Int J Clin Exp Med 2017;10(3):5068-5074
www.ijcem.com /ISSN:1940-5901/IJCEM0043644

Original Article

Effects of adjustable impinging flow on the vascular endothelial cell layer in a modified T chamber

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Received November 5, 2016; Accepted January 12, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: The study aims to design a vitro device to mimic the fluid enviroment at a arterial bifurcation to explore the endothelial physiology. Confluent human umbilical vein endothelial cells were exposed to an impinging flow in a T chamber system we designed. The flow rate was set at 250 or 500 ml/min. The pressure in the chamber was 100 mmHg. The morphology of endothelial cells (ECs) was examined after 3, 6 and 12 hours. At 250 ml/min, ECs remained confluent, with no signs of damage, no gaps, no rounding up of cells been detected in 6 hours, and cells decreased in the stagnation point, and partly moved and crowded in downstream after 12 hours. At 500 ml/min, no differences in the shape and distribution of ECs were detected in 6 hours. After 12 hours, cells were over confluent and crowded at the stagnation point, while gaps between cells were enlarged and the number of cells decreased in the region with a high wall shear stress (WSS, >40 dynes/cm^2) and wall shear stress gradient (WSSG, >300 dynes/cm^3). ECs were over confluent and crowded in downstream, and highly elongated and aligned as the direction of the impinging flow. The modified device, characterized by the adjustable velocity of the flow and pressure values in the chamber, can mimic different hemodynamics at bifurcation apices.

Keywords: Endothelial cells, hemodynamic conditions, improved vitro device, morphology of ECs

Introduction

Vascular endothelial cells (ECs), which form the inner lining of blood vessel wall with direct exposure to blood flow, serve important homeostatic functions in response to various chemical and mechanical stimuli [1]. By now, hemodynamics is widely accepted as a major regulator of pathological angiogenesis, including velocity of flow [2], wall shear stress (WSS) and wall shear stress gradient (WSSG) encountered by endothelial cells [3, 4]. A combination of high WSS and WSSG represents a dangerous hemodynamic condition that predisposes the apical vessel wall to aneurysm formation [3], while atherogenesis is associated with a hemodynamics that a low and reciprocating WSS, and a high spatial and temporal WSSG [4]. Low rate of blood flow is thought to participate in the forming of coronary atherosclerosis and unstable plaques [2]. Little researches concerned about the response of ECs to an impinging flow at the bifurcation of an artery, where intracranial aneurysms usually form [3]. Szymanski et al. [5] designed a T chamber to mimic the bifurcation of an artery in vitro, however, the velocity of flow and pressure in his design was maintained, and cannot be adjusted after the flow was established. Thus, we designed a modified T chamber to simulate dynamically the hydrodynamics of endothelial cells at the apex of an arterial bifurcation in vitro, in which the pressure and velocity can be adjusted continuously and a real-time observation of the values of the pressure and velocity can be achieved.

Materials and methods

Chamber system design

The chamber system was produced by the industrial department of Jiangxi Agriculture University. The chamber system consists of a T chamber, a pump, a reservoir and pipes con-

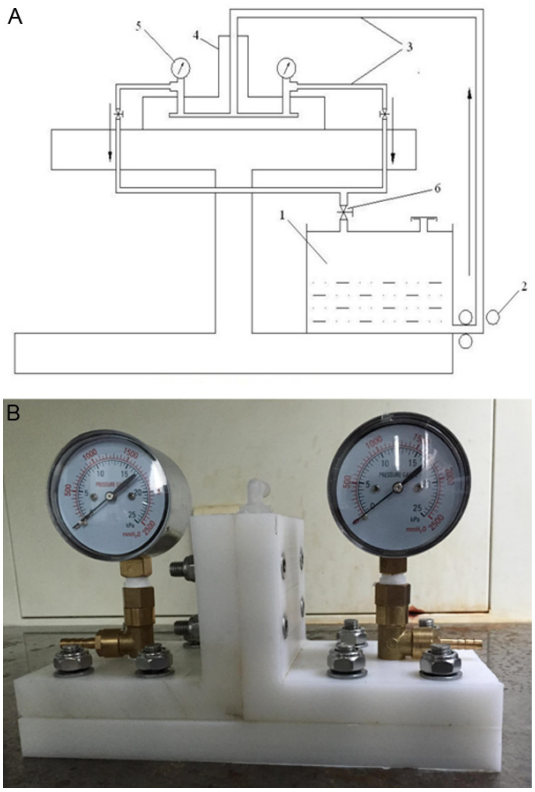

Figure 1. A. The map of the T chamber system: the chamber system, 1 reservoir, 2 pump, 3 tube, 4 the T-shape chamber, 5 pressure gauges, 6 flow constrictors; B. The T chamber.

necting the system (**Figure 1A** and **1B**). All rights of the system were reserved in state intellectual property office (SIPO) of the People's Republic of China (the reserved number: ZL 2014 2 0709792.5). The T chamber, with an impinging flow at the center of the chamber and then splitting in opposite directions along the chamber, contains a 22 mm × 50 mm rectangle area to accommodate a microscope coverslip for ECs culture. We set two pressure gauges at the outflow tracts of the chamber to detect the pressure in the chamber. The chamber was manufactured by machining the geometry from polycarbonate blocks, and stainless steel hardware were used to bolt the chamber together. We set three adjustable flow constrictors in the outlet tracts to adjust the pressure in the chamber.

Computational fluid dynamic (CFD) simulations

Hoi et al. [4] once described the validation of CFD. We used commercial software Fluent 6.0 to simulate. For the fluid simulations we assumed that: (1) a Newtonian fluid (μ=0.0034 Pa·s); (2) a incompressible, laminar flow (density =1020 kg/m^3); (3) the flow ran in a rigid-wall model, with traction-free boundary conditions at both outlets. The formula to calculate average shear stress is force per unit area, and is shown as follow:

$$\tau = \frac{F}{A}$$

where τ= the shear stress; F= the force applied; A= the cross-sectional area of material with area parallel to the applied force vector [6].

According to Szymanski et al. [5], three regions were set to define different conditions of hemodynamics in the modified T chamber. Region I: a stagnation point and a low to normal WSS relative to the baseline level in straight vessels. Region II: a high positive WSSG and high WSS. Region III: a recovery region characterized by a negative to zero WSSG and a normal WSS.

Cell culture

Human umbilical vein endothelial cells (HUVEC), between passages 4 and 14, were seeded in a sterile 22 mm × 50 mm glass coverslip. ECs were grown in 1640 Medium (Gibco, USA), within 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA). ECs were cultured in a humidified incubator at 37°C and 5% CO_2 and fed every 2 days. Fibronectin was used to fasten the HUVEC with the coverslip. Flow media consists of 90% 1640 medium within 100 U/mL penicillin and 100 mg/mL streptomycin, 10% FBS and 80 mg/mL Dextran 70 (GE Healthcare, USA). Wechezak et al. [7] elucidated that Dextran 70 serves as increasing the viscosity of the flow medium to 0.0034 Pa·s at 37°C. In our research, HUVEC were not incubated with any irreversible inhibitors of cellular proliferation, such as Mitomycin-C (MMC) [8], as Szymanski et al. [5].

Experimental protocol

All of the components of the T chamber system were sterilized by steam autoclave except pressure gauges, which were sterilized by been wrapped in alcohol (75%) for 12 hours. The entire system was assembled in a clean tissue hood using sterile methods. A coverslip of ECs

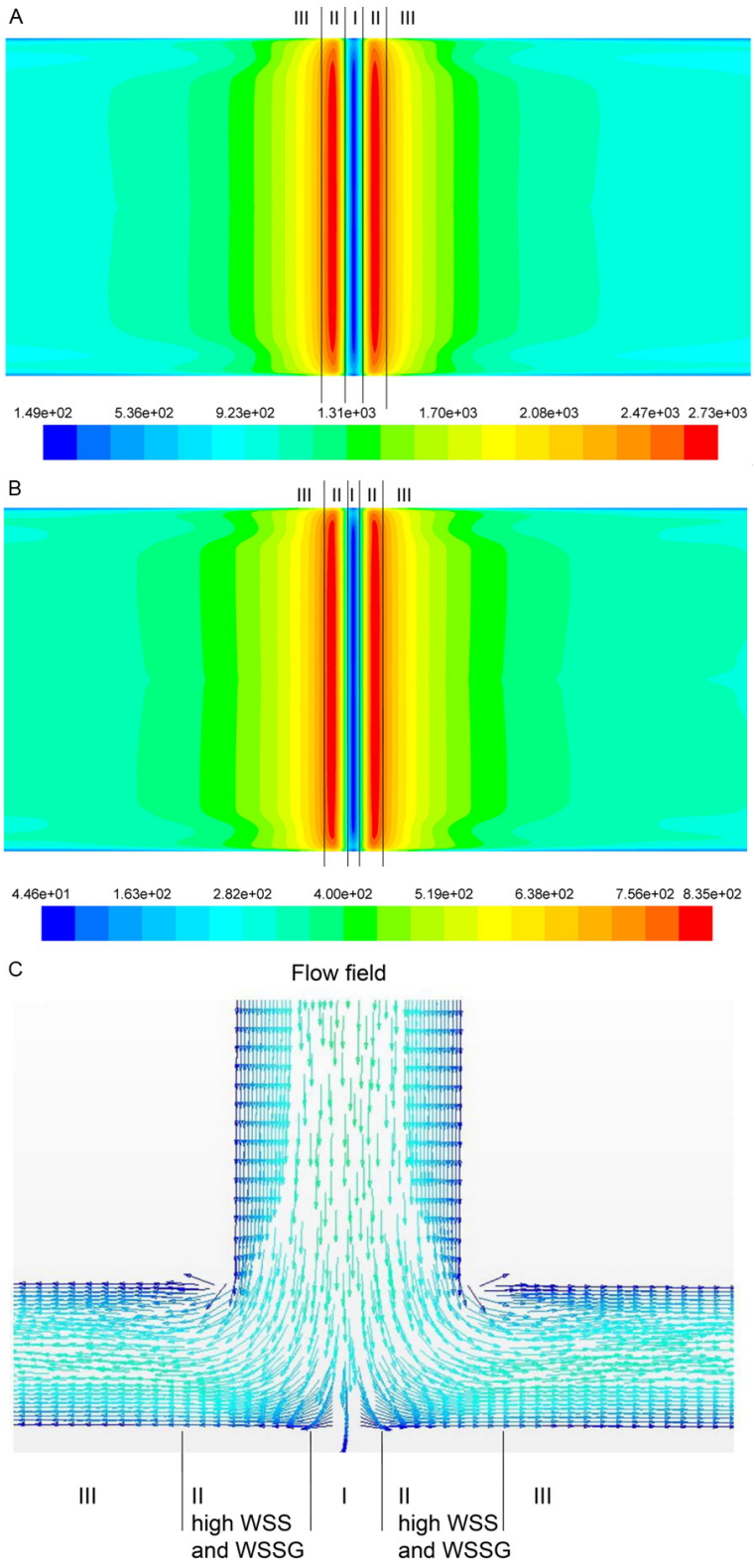

Figure 2. A. CFD simulation of WSS and WSSG of the impinging flow in the flow rate of 250 ml/min; B. CFD simulation of WSS and WSSG of the impinging flow in the flow rate of 500 ml/min; C. CFD stimulation for 2D geometry at the rate of 250 ml/min; Three flow zone, velocity magnitude and direction in the T chamber were shown in this image.

was then placed in the coverslip holder of the T chamber. The main body of the chamber was secured together using stainless steel bolts. All the components of the T chamber system were connected by pipes.

To avoid the cell monolayer from drying out, the flow pump was immediately started once the flow loop was connected. The flow rates were set up by the pump. In our search we set two levels of flow speed: 500 ml/min and 250 ml/min. The three flow constrictors located on the outlets of the T chamber were used to maintain the pressure in the chamber at a physiological level (100 mmHg), once the desired flow rate was established. Due to the two pressure gauges on the chamber and flow constrictors, a real-time detection of the pressure in the T chamber can be achieved, and the pressure can be adjusted once the values of the pressure were not 100. The entire T chamber system was placed in a humidified incubator at 37°C and 5% CO_2. Experiments were run for 3, 6, and 12 hours time periods. A coverslip with ECs was cultured under the same conditions in the incubator with flow media (no flow) and was used as a control. All experiments in any conditions were required to successfully run three times.

Morphological examination and cell density quantification

The characterization of the morphology, including shape, size, and orientation, of ECs on the coverslip was achieved by observing cells with an inverted phase contrast microscope (Olympus: IX71), taken at 100

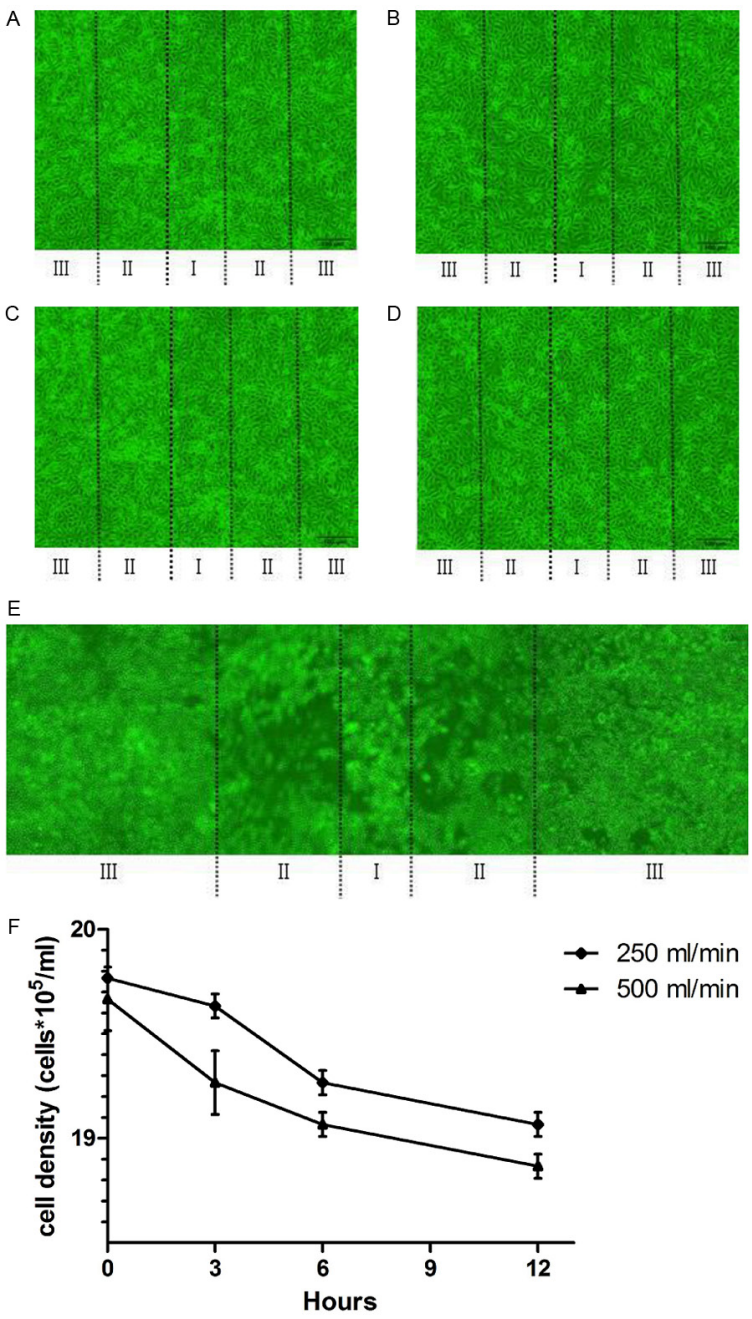

Figure 3. The morphology and distribution of ECs response to the impinging flow in different period and speed. A. 0 hours (control); B. 6 hours in the rate of 250 ml/min; C. 12 hours in the rate of 250 ml/min; D. 6 hours in the rate of 500 ml/min; E. 12 hours in the rate of 500 ml/ml; F. Cell density quantification of HUVEC on the coverslip.

× magnification. Digital images were taken using an inverted microscope (Olympus: IX71) and IP Lab software. Cells on the coverslip was dissociated using tryposin (Sigma). Cell density quantification on the coverslip was achieved by using a standard medical blood count chamber [9].

Statistical tests for association between the cell density on the coverslip and the period that ECs been subjected in the impinging flow were performed using ANOVA, and data was analyzed by the SPSS software (version 19.0, IBM Institution). A P value <0.05 was considered to be statistically significant.

Results

Flow data

Two distinct flow patterns were found in the modified chamber at 500 ml/min and 250 ml/min, and each corresponding to specific WSS and WSSG distributions along the EC layer. The pressure in the chamber was maintained at 100 mmHg by adjustable flow constrictors just located at the outflow tracts of the chamber, and the pressure in the outlet tracts can be detected through the pressure gauges on the chamber.

The results of CFD stimulation at the rate of 250 ml/min were shown as **Figure 2A** (250 ml/min), **Figure 2B** (500 ml/min) and **Figure 2C**. According to CFD simulations, three regions in the T chamber were defined as: impingement region (Region I): a low to normal WSS and a positive WSSG; acceleration region (Region II): high WSS and high, positive WSSG and recovery region (Region III), decelerating flow with WSS gradually decreased to a normal level as the edge of Region I. The magnitude of WSSG overall was less than in the acceleration region, with a negative WSSG. When the WSS returned to a normal level, seen in the straight part of outlet tracts, the WSSG reached zero. For the rate of 250 ml/min, the flow from inlet tract impinged on the bifurcation, creating a

central stagnation point (WSS=0 Pa, where 1 Pa=10 dynes/cm^2), and accelerated away from the bifurcation into branches. WSS reached 2 Pa at the boundary of this region. The rapid increasing of WSS over a short distance produced a large spatial and positive WSSG (250 dynes/cm^3). Flow in this region continued to accelerate until maximum (about 7 Pa), which defines the distal edge of region II. The WSSG in region II persisted at elevated levels (500 dynes/cm^3).

When the velocity of flow increased to 500 ml/min, WSS reached 8.3 Pa at the boundary of region I with a high and positive WSSG (900 dynes/cm^3). In region II, the maximum WSS reached 20.8 Pa, with a high and positive WSSG (1000 dynes/cm^3). The region III characterized with a negative to zero WSSG and normal level of WSS.

The morphology, alignment and distribution of ECs

ECs, which was subjected to the impinging flow, remained confluent after 12 hours at the rate of 250 ml/min, and no signs of damage, no gaps and no rounding up of cells were detected in 6 hours (**Figure 3B**), compare with 0 hour (control, **Figure 3A**). After 12 hours, cells decreased in the stagnation point (region I) and moved and crowded in region III, while areas with sparse cells were detected and observed (**Figure 3C**). When the speed rate increased to 500 ml/min, no significantly differences were detected in 6 hours (**Figure 3D**). After 12 hours, a spatially varying density was exhibited on ECs subjected to the stronger impinging flow. Cells in Region I were remained confluent but crowded at the stagnation point, and the shape of cells in region I was polygonal and enlarged, while the alignment of these cells was nonpolarized. In Region II, gaps between cells were enlarged and the number of cells in this region decreased. ECs in Region III (**Figure 3E**) were over confluent and crowded downstream in both branches of the impingement chamber, but were highly elongated and aligned as the direction of flow. Quantification of HUVEC density after 3, 6 and 12 hours, at the rate of 250 and 500 ml/min, were shown as **Figure 3F**. A part of cells lost from the coverslip, but the decreased number of cells showed no statistical differences ($P>0.05$), no matter in 250 or 500 ml/min.

Discussion

Our device is able to mimic the hemodynamic condition in the bifurcation of an artery

Hemodynamics is widely accepted as a crucial factor in inducing vascular diseases. Various of researches in vivo and vitro suggests that WSS and WSSG caused by different type of flow are correlated with pathogenesis of vascular disease [1, 10], for instance, intracranial aneurysms [3] and atherogenesis [4]. In vivo, the method that surgically created new branch points in carotid vascular of dogs has been used to analyze the complex hemodynamics at the apex of an arterial bifurcation [3, 11]. While in former vitro research, Szymanski et al [5] designed a T chamber to gain knowledge of the response of ECs to impinging flow at the apex of bifurcations, where intracranial aneurysms usually form, and stated that cells were accumulated at high WSSG.

The results of our study support the statement from Szymanski et al [5]. When subjected to a low speed impinging flow, the shape of endothelial cells showed no observed differences after 12 hours compared with 0 hours. However, a part of cells moved to downstream in both branches of the chamber following the direction of the impinging flow after 12 hours. When the speed increased to 500 ml/min, no differences of morphology and alignment were observed after 6 hours. After 12 hours, a part of cells were crowded at stagnation point and others moved to and crowded in the downstream. In region II, characterized by high WSS and high positive WSSG, cells were sparse. Concerning the results of cell density quantification, little part of ECs on the coverslip ($P>0.05$) were flown away by the impinging flow, which suggests that changes in the morphology, alignment and distribution of ECs were induced by the impinging flow. In our research, we did not use MMC to inhibit the proliferation of cells, though Szymanski stated that no differences in their research were observed whether MMC was used or not. What is more, the proliferation of ECs may be a possible response to the impinging flow, and the treatment, MMC, would interfere with the observa-

tion and results. However, a further research is required.

The advantages and conveniences of our modified T chamber system

As described by Szymanski et al [5], authors did not mention the speed controlling and whether flow rate was adjustable, moreover, pressure clamps were used to set the specific pressure value in the chamber after the experiment flow was established, but authors showed no methods to adjust the velocity of flow and pressure in the chamber after the flow was established. For the velocity of blood flow can hardly be a same value in one day and such differences in the velocity of blood flow may induce some vascular diseases, hemodynamic conditions of endothelial cells at the apex of an arterial bifurcation cannot be stimulated completely in such devices. An improved vitro device to mimic fluid environment of the human vascular system is still wanted to explore endothelial physiology. In our research, a pump with a digital LED screen is used to adjust the velocity of the flow, and the flow rate is able to be observed through the LED screen. In our research, we set the speed at 250 and 500 ml/min, however, other specific speeds, such as 10, 125 and 400 ml/min, are also available to any other research.

We set up two pressure gauges at both branches of the T chamber to detect the pressure in the outlet tracts, which means a real-time observation of the pressure values in both branches is available. In outlet tracts outside the chamber, we set three adjustable constrictors to control the pressure (**Figure 1A**), so that, as the flow rate changed by the pump, we are able to control the pressure in the chamber, and different pressure values in the two branches of the chamber are also available as we wish. And once the value is changed for any reasons, we are able to adjust and maintain the value at the level we wish as soon as possible by using the three constrictors.

During our research, after the flow was established, we are able to adjust the flow rate to any level as we wished and maintain the pressure level as we require, without disturbing the established flow. These conveniences may contribute to mimic a more real hydrodynamic condition of endothelial cells at the apex of an arterial bifurcation in vitro, and decrease the rate of infection to ECs during experiments. Vascular diseases characterized by specific flow rates and pressure values in different period are able to be stimulated by our modified device.

A possible mechanism of changes of ECs induced by the impinging flow

The changes in the morphology, alignment and distribution of ECs may be partly related to the impairment of adherens junctions between cells induced by the impinging flow. Adherens junctions(MeSH Unique ID in Pubmed: D022-005) are composed of specialized areas of the plasma membrane where bundles of the actin cytoskeleton attach to the membrane through the transmembrane linkers, Cadherin, which in turn attach through their extracellular domains to cadherins in the neighboring cell membranes. Intercellular adherens junction formation is mediated by VE-Cadherin, whose functions are regulated by P120 catenin (P120 ctn).

In vitro research, Sakatoma et al. [12] showed that 85% of ECs were aligned to the direction of flow and the changes of P120 ctn and VE-Cadherin in distribution and expression, after exposure to shear stress for 24 hours in a parallel plate flow chamber system. And Li et al. [13] firstly showed that ECs changed from a fusiform to column shape, and gaps between ECs enlarged, in response to sustained high blood flow in rabbits. Our research suggests that the response of ECs to the impinging flow showed a similar result in morphology and distribution of ECs as Sakatoma et al. [12] and Li et al. [13]. So that, we make the conclusion that the changes of ECs in morphology and distribution, in response to the impinging flow, is induced by the impairment of adherens junctions between ECs. However, a further research is required to clarify the specific molecular mechanism in the response of ECs to the impinging flow in our vitro device, for instance, the expression of P120 catenin, VE-Cadherin et al.

Limitations

One limitation of our research was that the period of flow running was 12 hours at most, which was 72 hours in Szymanski et al. [5]. We had tried to enhance the period to 18 and 24 hours, and little endothelial cells were detected

through the microscope, however, the infection of cells was also not detected. The reason in our opinion was that we use a different type of experimental cells in our study, HUVEC, but Bovine Aortic Endothelial Cells (BAECs) in Szymanski et al. [5]. In our research, BAECs were not available to us, so that we selected HUVEC instead. A further research based on BAECs is required.

Conclusion

The modified T chamber system we designed can mimic different hemodynamic conditions at bifurcation apices of an artery, and is convenience and practical. Hemodynamics, characterized by high WSS and high positive WSSG, is a crucial factor in inducing the endothelial cells injury.

Acknowledgements

Since starting neurological training at the first affiliated hospital of Nanchang University in Nanchang, Professor Mei-hua Li, M.D. and Ph.D., has supported the author's interests in intracranial aneurysm. I am grateful to his support and help, and to everyone in the first affiliated hospital of Nanchang university. This research was granted by National Natural Science Foundation of China (NSFC): 81360188 and 30960397.

Disclosure of conflict of interest

None

Abbreviations

ECs, endothlial cells; WSS, wall shear stress; WSSG, wall shear stress gradient.

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