

# Different Effects of Orbital Shear Stress on Vascular Endothelial Cells: Comparison with the Results of In Vivo Study with Rats

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**Purpose:** An attempt was made to characterize the orbital shear stress by comparing the effects of orbital shear stress on vascular endothelial cells (ECs) with the results of animal experiments.

**Materials and Methods:** In the laboratory study, cultured ECs were distinguished by center and periphery then exposed to orbital shear stress using an orbital shaker. In the animal study, arteriovenous (AV) fistulas were made at the right femoral arteries of Sprague-Dawley rats to increase the effect of the laminar flow. The condition of the stenosis was given on the left femoral arteries. The protein expression of inducible nitric oxide synthase (iNOS) and Akt phosphorylation were observed and compared.

**Results:** Under orbital shear stress, ECs showed an increase in iNOS protein expression and phosphorylation of Akt but most of the protein expressions derived from the periphery. When compared to the animal study, the increased expression of iNOS protein and phosphorylation of Akt were observed in the sample of AV fistula conditions and the iNOS protein expression was decreased in the stenosis conditions.

**Conclusion:** Orbital shear stress did not show the characteristics of a pure turbulent shear force. By comparing the observation with the morphological changes of vascular ECs and site-specific protein expression on the results of animal experiments, uniform directional lamina shear stress forces were expressed at the periphery.

**Key Words:** Orbital shear stress, Endothelial cells, Akt phosphorylation, Nitric oxide synthase

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## INTRODUCTION

Shear stress is a resistant force from the object that internally moves towards an external force that attempts to deform. The blood flow in the blood vessels causes

the shear stress on the endothelial cells, and the size and type of this force are known to determine the primary characteristics of endothelial cells [1-3]. The normal laminar flow of the blood vessels generates the laminar shear stress, which has atheroprotective actions [3-5]. On the other

hand, turbulent flow generated by abnormal hemodynamic changes is known to cause various vascular pathologic changes such as neointimal hyperplasia and arteriosclerosis. Previous studies have focused on studying the response of vascular endothelial cells due to changes in the type and size of these forces [2,4,6-8].

Most of the previous studies are about the effect of the laminar flow on the vascular endothelial cells and many already have been organized about the universal reaction of endothelial cells that occurs normally due to laminar shear stress. However, research about the effects of the turbulent flow is insignificant compared to the laminar flow. Furthermore, different results have been observed on past studies in order to investigate the effects of the turbulent flow by applying the orbital shear stress generated by the orbital shaker. Hence, the actual reaction of vascular endothelial cells due to the turbulent flow is still not clear [2,9-11].

The orbital shear stress effects on vascular endothelial cells through laboratory research was reported by Dardik et al. [12] in 2005 and the differences of shear stress on the center and periphery were thought to be associated with the opposing results of previous research. Considering the study of Dardik et al. [12] and other research, we assumed that an orbital shear stress, generated by the spiral flow of the culture medium, characteristically differs from the actual turbulent flow occur in blood vessels. By comparing the changes in actual blood vessels through the animal study, we wanted to find out the reason why the different results derived from previous studies. In addition, we tried to design an appropriate experimental model to observe the reactions of endothelial cells by using the orbital shear stress in the future.

## MATERIALS AND METHODS

### 1) Culture of endothelial cells for in vitro

Vascular endothelial cells of bovine aorta were obtained by the scraping of the intimal surface of the aorta obtained from the local slaughter house. Endothelial cells were cultured in Dulbecco's modified Eagle's medium containing antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 250 ng/mL; GIBCO BRL Life Technologies, Gaithersburg, MD, USA), and 10% fetal bovine serum (FBS) and on 37°C in 5% CO<sub>2</sub>. Endothelial cells were identified as being of a typical cobblestone appearance and possibly verified by specified immunohistochemical staining with anti-factor VIII. Endothelial cells were cultured until they form as one complete cell layer on six 35 mm-diameter wells that are coated with type 1

collagen. Endothelial cells were stabilized for more than 24 hours after changing with serum-free culture medium. Then the orbital shear stress were applied after adding 10% FBS. Endothelial cells which used in the experiment were subcultured three to five times. In each experiment for the enzyme protein expression, endothelial cells were subcultured the same number of times. In order to limit the portion of seeding only on the periphery or the center, we used a silicon gasket. The area of whole well, periphery and center portion was estimated at 9.07, 7.06 and 2.01 cm<sup>2</sup> respectively. The silicon gasket was removed just before the orbital shear stress application.

### 2) Shear stress application

Orbital shear stress was applied by using an orbital shaker (Orbital Shaker SH30; FINEPCR Co., Seoul, Korea). The strength of the shear stress exerted in the entire well is soluble via the formula as follows [12,13]; the case of 210 rpm of rotational frequency of the orbital shaker can give the shear stress of 11.5 dyne/cm<sup>2</sup>. Orbital shear stress was applied for five days to observe the morphological change of endothelial cells adequately. For comparison, we have observed the endothelial cells which have subcultured same number of time with a static condition and the same period as the control group (0 dyne/cm<sup>2</sup>).

$$\tau_{\max} = a \sqrt{\eta \rho (2\pi f)^3}$$

a is an orbital radius of the rotation of the orbital shaker (0.95 cm),  $\eta$  is the medium (0.0101 poise),  $\rho$  is the density of the culture medium (0.9973 g/mL), f is the frequency of the rotation (rotation/sec).

### 3) Animal study

#### ① Model of animal study

Ten male Sprague-Dawley rats of between 240 gm from 310 gm were housed in the appropriate environment with a standard feed (Super Feed Co., Ltd., Wonju, Korea) in the animal laboratory of The Catholic University of Korea. Induction and inhalation anesthesia was acquired by using Isoflurane. The animals were not anti-coagulated during operation. Surgery was performed with an operating microscope that had 20 magnifications to ensure the surgical field. The femoral arteries were exposed after separating of the muscle and the subcutaneous fat layer by 5-6 cm of vertical incision on both sides of the groin. On the right side of the rats, 2-3 mm sized incisions were made each in the femoral artery and vein that runs along it. Then the arteriovenous (AV) fistula was completed by side to side anastomosis with the continuous suture technique using a

10-0 prolene (Microsurgery Instrument Inc., Bellaire, TX, USA). On the left side, the arterial stenosis condition of the exposed femoral artery was induced by partial ligation with No.2 silk. Before closing the operation field, distal blood flows were checked with an ultrasonic flow probe (0.5VB Probe; Transonic System Inc., Ithaca, NY, USA). An average operation time for each rat was estimated one and half hour totally including 15 minutes of vascular stenosis procedure and about 55 minutes of AV fistula procedure (Fig. 1).

#### ② Blood flow and sample collection

At least a 6 week observation period was taken after the surgery. The previous surgical site was inspected by re-incision under an Isoflurane inhalation anesthesia. Careful dissection was made to prevent the injury of each femoral artery. Arterial flow rates were measured at distal to the left stenosis and proximal to the right AV fistula site by ultrasonic flow probe (0.5VB Probe). Length of 2 cm sample tissues was harvested from 1 cm proximal to the site of AV fistula on the right, and 1cm distal to the site of stenosis on the left. Tissues from the abdominal aorta where 1 cm above the bifurcation site of the iliac arteries were harvested for the comparison control group. The tissues were then fixed into two parts for histological and molecular biological study after being irrigated with saline.

#### 4) Observation and comparison of endothelial cell morphology

##### ① Endothelial cell staining and culture in vitro

In order to observe the morphological changes in the endothelial cells, the endothelial cells were fixed with 3.7% of formaldehyde for 10 minutes, stained with 0.125% of crystal violet for 2 minutes and then observed under an optical microscope.

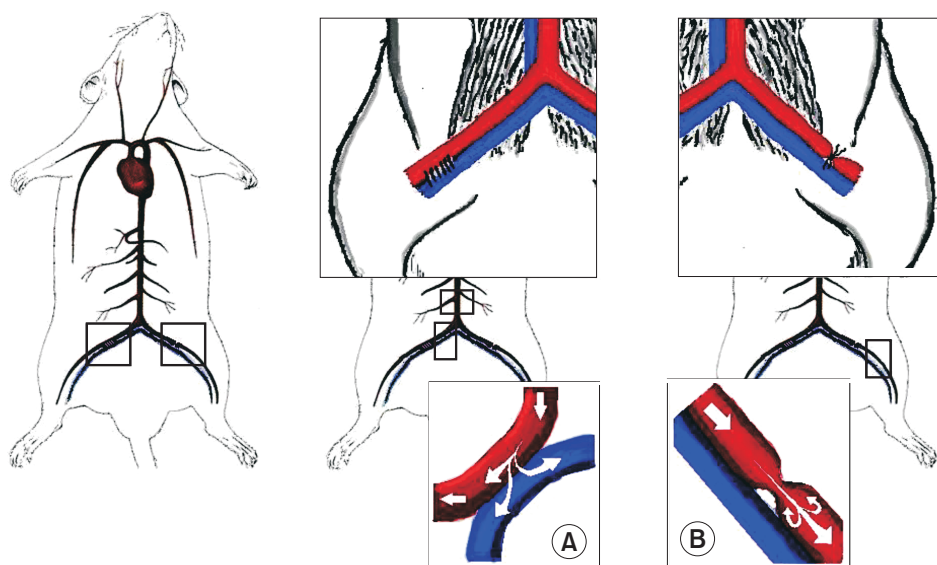
##### ② Scanning electron microscopy

In the animal studies, in order to observe the morphological change of the endothelial cells, the harvested tissues from the rats were fixed primarily for 12 hours with 2.5% of glutaraldehyde and 4% of paraformaldehyde in 0.1 M phosphate-buffered saline then fixed secondary by using 1% of osmium tetroxide for an hour. The fixed tissue went through the dehydration process gradually over a period of 12 hours from 50% to 100% by using acetone. The morphological changes of the cells were observed in the electron microscope (JSM-5410LV; JEOL, Tokyo, Japan) after the application of gold using the sputter coater.

##### 5) Western blot analysis

##### ① Cultured endothelial cells in vitro

In order to observe the degree of nitric oxide synthase (iNOS) protein expression and the Akt phosphorylation, hemodynamic force loaded cells were lysed with buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% TritonX-100, 1.5 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 gm/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and quantified by the Bradford assay [14]. The extracts were mixed with Laemmli sample buffer and denatured for 5 minutes at 95°C, then separated by with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking the non-specific binding of the antibody for 1 hour with tris-buffer saline (TTBS) containing 0.1% Tween-20 and 5% nonfat dry milk, specimens were treated with either anti-iNOS antibody (Chemicon International Inc., Temecula, CA, USA), or anti-phospho-Akt antibody (Ser473; Cell Signaling,



**Fig. 1.** Diagram of rat model. (A) Femoro-femoral arteriovenous fistula was performed by side to side anastomosis to increase laminar shear force. (B) Black silk ligation was done on the other side of femoral artery to make turbulent flow. Patent, but sluggish distal flow was checked by palpation. Assumed blood flow is demonstrated at the bottom of the figure (boxes: specimen harvesting area).

Beverly, MA, USA). After washing 3 times for 15 minutes in TTBS, a second reaction was performed with 1:1,000 anti-rabbit immunoglobulin G (Cell Signaling) for an hour. The specimens were exposed to x-ray film (Amersham Pharmacia Biotech) using the chemiluminescent substrate (ECL kit; Amersham Pharmacia Biotech) after washing three times with TTBS.

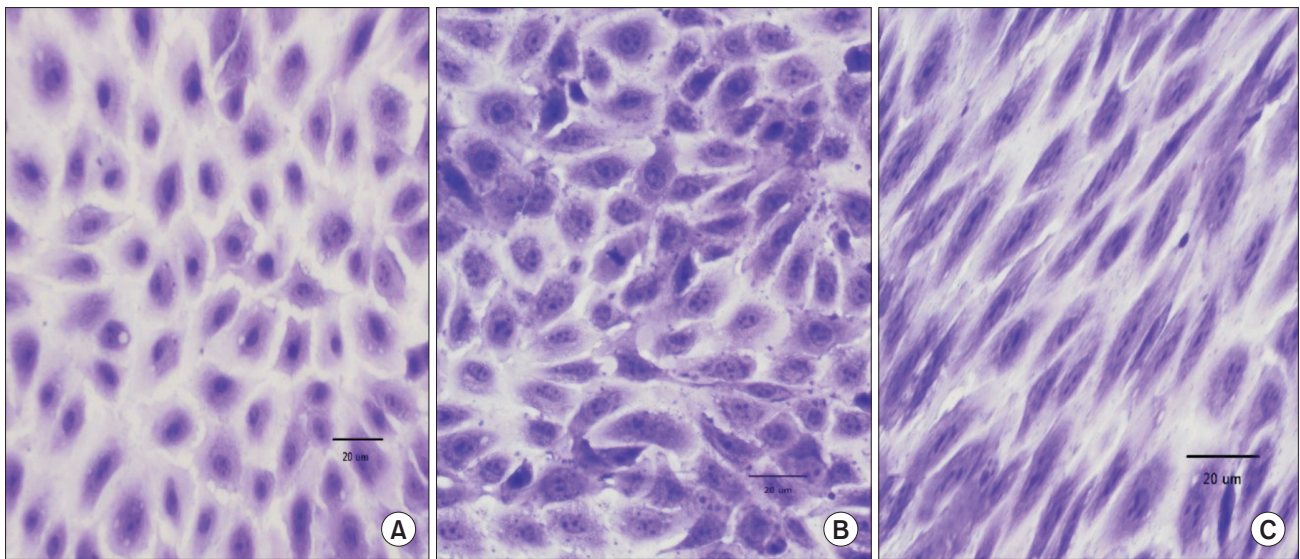
② Specimen of animal study

The tissues from the rats were pulverized with the addition of 2 mL buffer solution (8.0 M urea, 40 mM Trizma base [Sigma-Aldrich, St. Louis, MO, USA], 4.0% CHAPS, pH 11.0, 10 mM herpes, 60 mM KCL, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100 [Sigma-Aldrich], 1 mM PMSF,

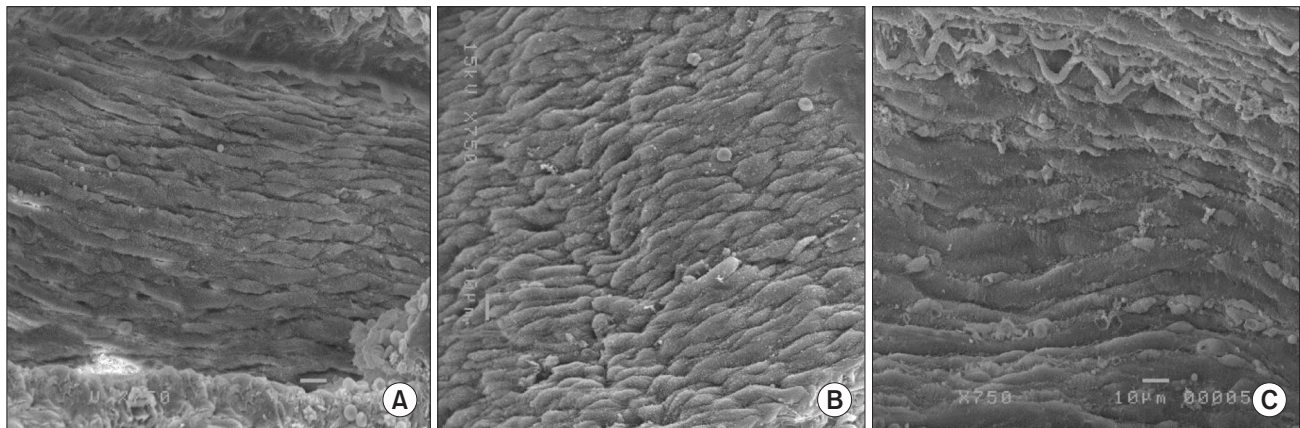
0.1% aprotinin) to get protein effluent. Supernatant was obtained by centrifugation of the effluent for 15 min at 4°C, 3,000 rpm. The protein extracted from the sample (20 µg) were quantified by Bradford assay and processed in the same way to go through with Western blotting as in vitro.

6) Statistical analysis

The expression level of iNOS protein and Akt protein kinase was calculated by analyzing the blackening of the film using ImageMaster VDS 2.0 image analyzer (Amersham Pharmacia Biotech) and compared with the percentage (%) for the control group. All measurements of experiment were presented as mean±standard error. Data were analyzed



**Fig. 2.** Morphology of cultured endothelial cells (ECs) in vitro after 5 days. (A) Control ECs (static condition). (B) ECs exposed to orbital shear stress (center of the well). (C) ECs exposed to orbital shear stress (periphery of the well). The edge of the culture well is to the right of the panel. (A-C) Stained with crystal violet (×400).



**Fig. 3.** Scanning electron micrographs of luminal surface of specimens. (A) Control (abdominal aorta). (B) Arteriovenous fistula side specimen (right femoral artery); endothelial cells (ECs) were regularly spaced and more elongated. (C) Stenosis side specimen (left femoral artery); polygonal ECs (A-C: ×750).

with the Student's t-test, post hoc test and ANOVA test and then judged as statistically significant when the P-value was less than 0.05.

## RESULTS

### 1) Morphology

#### ① Cultured endothelial cells in vitro

The endothelial cells of the peripheral portion were elongated and aligned with the direction of shear stress, as shown in a normal laminar shear force. The endothelial cells of the central portion, similar with the endothelial cells in static condition, were round and randomly aligned (Fig. 2).

#### ② Animal study

In comparison with the stenosis condition, the endothelial cells in the AV fistula condition were in a tightly lined-up pattern and elongated with the direction of the blood flow (Fig. 3).

### 2) Phosphorylation of Akt

#### ① Cultured endothelial cells in vitro

Endothelial cells under an orbital shear stress showed an increased phosphorylation of Akt when compared to a static condition ( $100 \pm 4.9$  vs.  $248.6 \pm 23.7$ ,  $P < 0.05$ ). The Akt phosphorylation of the peripheral endothelial cells increased significantly ( $377.2 \pm 33.8$ ,  $P < 0.05$ ) when compared to the control group. In contrast, there was no significant change in the endothelial cells of the central portion ( $127.3 \pm 22.7$ ) (Fig. 4).

#### ② Animal study

Significant increase in Akt phosphorylation was shown under the AV fistula condition. In contrast, there was minimal increase in the stenosis condition ( $459.3 \pm 17.6$  vs.  $151.5 \pm 26.6$ ,  $P < 0.05$ ) (Fig. 4).

### 3) iNOS protein expression

#### ① Cultured endothelial cells in vitro

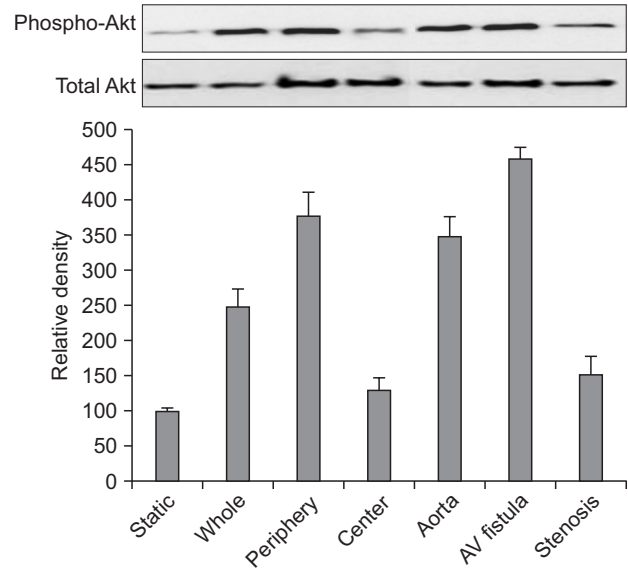
The expression of iNOS protein of the peripheral endothelial cells increased significantly when compared to the static condition ( $100 \pm 7.2$  vs.  $232.1 \pm 25.6$ ,  $P < 0.05$ ). There was significant difference in iNOS protein expression between endothelial cells in the periphery and the center ( $232.1 \pm 25.6$  vs.  $169.7 \pm 16.3$ ,  $P < 0.05$ ) (Fig. 5).

#### ② Animal study

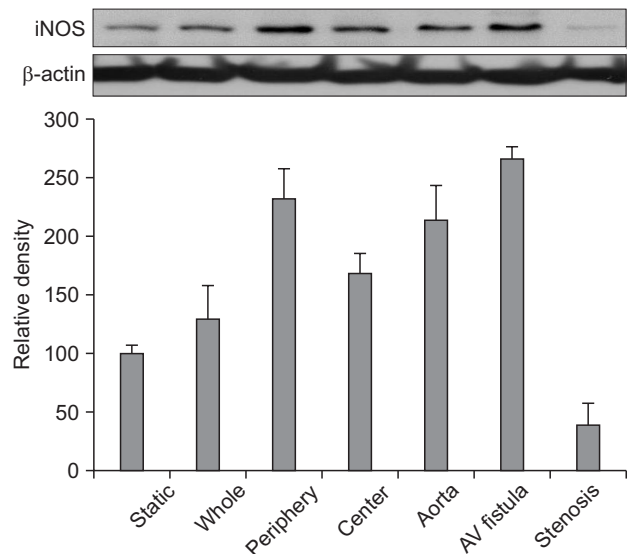
An increase in iNOS protein expression was shown under the AV fistula conditions. In contrast, there was few protein expression in the stenosis ( $265.6 \pm 11.1$  vs.  $39.7 \pm 18.1$ ,  $P < 0.05$ ) (Fig. 5).

### 4) Flow meter

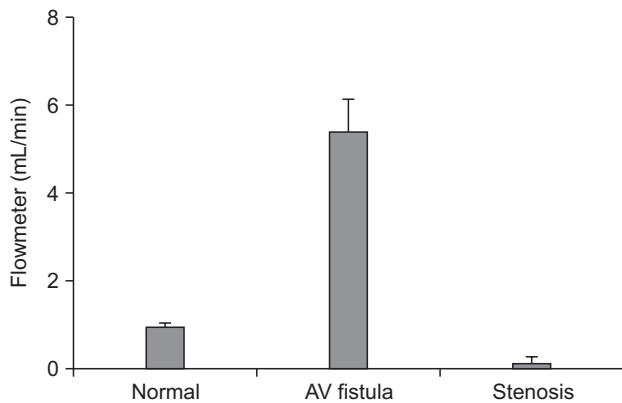
The blood flow of both femoral arteries before surgery



**Fig. 4.** Akt phosphorylation with orbital shear stress and in rat model. Bar graph represents the mean relative ratio of phosphorylated to total Akt. A representative Western blot is shown. The relative difference in Akt phosphorylation between periphery and center cells exposed to orbital shear stress is significant ( $P = 0.0002$ , post hoc). In rat model, arteriovenous (AV) fistula side specimen demonstrate significantly increased Akt phosphorylation compared with stenosis side specimen ( $P < 0.0001$ , post hoc).



**Fig. 5.** Nitric oxide synthase (iNOS) protein expression with orbital shear stress and in rat model. Bar graph represents the mean relative ratio of iNOS to  $\beta$ -actin. A representative Western blot is shown. The relative difference in iNOS expression between control and periphery cells exposed to orbital shear stress is significant ( $P = 0.0008$ , post hoc). In rat model, arteriovenous (AV) fistula side specimen demonstrate significantly increased iNOS expression compared with stenosis side specimen ( $P < 0.0001$ , post hoc).



**Fig. 6.** Bar graphs for flow rates. Blood flow on the proximal portion of right arteriovenous (AV) fistula site counted as  $5.41 \pm 0.699$  mL/min, and left stenosis site was  $0.38 \pm 0.361$  mL/min.

was  $1.0 \pm 0.029$  mL/min. After applying the condition for 6 weeks, the blood flow on the proximal portion of the right AV fistula site counted as  $5.41 \pm 0.699$  mL/min. On other hand, the flow of the distal portion of the left stenosis site was  $0.38 \pm 0.361$  mL/min (Fig. 6).

## DISCUSSION

As a result of observing the morphology of cells applied with orbital shear stress, we could confirm a significant difference between the cells of the central and the peripheral portion of the well. The direction of elongated cells at the periphery is relatively uniform with constant angle with the wall of culture well, which was determined to coincide with the spiral-shaped flow of the culture medium. On the other hand, cells in the central portion appeared as polygon-shaped cobble stones similar with the exposure of pure turbulent shear stress [3,15-17].

The morphology of cells in the periphery is very similar to the AV fistula condition on the animal study. And when compared with the results of iNOS protein expression and Akt phosphorylation in the animal study, the response of peripheral cultured cells appear to be similar. Therefore, we derived the belief that at least a portion of the downstream intracellular signal transduction occurs in the periphery of the well, similar with endothelial cells which were exposed by laminar force. In addition, the result of the protein expression and morphology of the cells in the center of the well are similar to the condition of the stenosis in the animal study.

Many researchers have studied about the hemodynamic response of endothelial cells through blood flow for nearly 30 years with many different methods such as lab, animal study etc [3,18-22]. In order to see the effect of the laminar

flow, pre-existing studies used a parallel flow chamber for applying the laminar shear stress. Also, the orbital shaker and cone-and-plate device, which applies a rotating fluid field by using a cone shape plate, were commonly used to observe the effect of the turbulent flow [12,15,23-25]. For observing the effect of the turbulent flow, except for some of the early studies, the method using an orbital shaker rather than the cone-and-plate device has been gradually increasing. The cone-and-plate device was impossible to apply the same experimental conditions into many plates at the same time without having more equipment [12,19-21]. On the other hand, an orbital shaker applies the shear stress by only using culture media and it is free from expensive costs and space limitations with the complex devices, such as a circulatory system or fluid reservoir.

When comparing the endothelial cells under orbital shear stress to the laminar shear force using a parallel flow chamber, an increase in cell proliferation and apoptosis have been clearly observed [9,12,23,25]. These results derived the belief that an orbital shear stress is not the laminar shear force and it shows the effects of an incomplete or disturbed laminar flow or rather a turbulence flow. Therefore, many researchers have applied an orbital shear stress for the effects of the turbulence flow. However, studies associated with orbital shear stress have brought about different results [9-12,26]. Dardik et al. [12] confirmed that there were differences in magnitude of force on the center and periphery among the wells in the orbital shaker and assumed these differences are the reason for the contradictory results of other studies.

In the study, we reconfirmed the presence of the force differences among the center and periphery of the wells and in addition to the differences we also found that the constant direction of the force is present in the shear stress of the periphery. We designed an animal experimental model for specifying the forces of the center and periphery if we can compare the results with the changes of the actual blood flow in vivo. When the AV fistula formed, due to the decrease of the blood flow resistance on the distal portion, the blood flow in the proximal artery of the AV fistula gradually increased. The flow increases up to 5 times if the size of AV fistula is 1.5 times larger than the inner diameter of the inflow artery and the flow increases up to 8 times when the diameter becomes 3 times [22,27]. We made a condition of the AV fistula and stenosis on the other side in the animal study to observe the effects of the laminar shear stress and turbulence flow in vivo. Partial ligation was made to produce the turbulent flow [28]. And we made sure the blood flow distal to the stenosis kept running by using an ultrasonic flow probe (0.5VB Probe). The difference of the flow rate was about 14 times, and 4 times of the shear

stress difference was calculated by the modified Hagen-Poiseuille equation ( $\tau = 4\eta Q/\pi r^3$ ;  $\eta$  is blood viscosity,  $Q$  is flow rate in milliliters per second, and  $r$  is radius in centimeters) after 6 weeks of applied conditions. Serine/threonine protein kinase Akt, known as protein kinase B or Ras active enzyme, plays a number of important roles in substrate adhesion and integrin mediated signaling pathway. Also, it inhibits the apoptosis by secretion of growth factors [29-31]. Under normal laminar shear force, phosphoinositide-3 kinase (PI3K) was activated by the phosphorylation of a lipid phosphatidylinositol 4, 5 biphosphate which results in inducing the phosphorylation and activation of Akt [32,33]. The activated Akt activates NOS to induce the secretion of nitrogen monoxide and stimulates the secretion of transcript proteins and active enzymes which control the angiogenesis, and cell protection. Downstream intracellular signal transduction which represents the phosphorylation of Akt in cell gets conducted by the mechanical receptors of the cell surface and gets stimulated at the moment the endothelial cells are subjected to mechanical hemodynamic forces and the inhibition of apoptosis and cell growth inhibition occurs as a result to maintain the stability of the inner membrane of the blood vessel [29-33].

In this study, Akt phosphorylation among a whole well increased [9,11,12,34]. However, the phosphorylation of Akt increased mostly in the periphery and any activation on the center was minimal. This result suggests that the size or types of the force on the center and the periphery might be different even within the well. Also, this result can be comparable to the results which phosphorylation of Akt increases under the condition of AV fistula and minimal

changes are shown on the stenosis condition in the animal study. Along with increase of Akt phosphorylation, iNOS protein expression on a periphery well and the AV fistula condition increases also. These results strongly suggest that the activation of iNOS is induced by the phosphorylation of Akt and also there is a quite similar to downstream of intracellular signal transduction on peripheral portion of well like when exposed on laminar shear force [29-31].

## CONCLUSION

Through this study, we confirmed that the orbital shear stress generated by the orbital shaker is not a pure turbulence shear force. Therefore, simply applying the orbital shear force generated by orbital shaker should be avoided. On the other hand, it is applicable for a model by applying the different conditions of shear stress using the cells under the same conditions by separating the center and periphery. The center and periphery can each be utilized as part of a self-control group with the same variables excluding shear force. Because cells on the center are similar with the atherogenic activated and dysfunctional endothelial cells [3,15-17], the center of the well can be used for a study of the turbulent shear force effect which also could be producible of plenty of wells with the same conditions at one time.

## ACKNOWLEDGEMENTS

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