Video Article

# The Assembly and Application of 'Shear Rings': A Novel Endothelial Model for Orbital, Unidirectional and Periodic Fluid Flow and Shear Stress

Luke A. White[1], Emily V. Stevenson[1], J. Winny Yun[1], Randa Eshaq[1], Norman R. Harris[1], David K. Mills[2], Alireza Minagar[3], Pierre-Olivier Couraud[4], J. Steven Alexander[1]

[1]Molecular and Cellular Physiology, Louisiana State University Health Sciences Center in Shreveport

[2]Biological Sciences, Louisiana Tech University

[3]Neurology, Louisiana State University Health Sciences Center in Shreveport

[4]Institut Cochin, Inserm U1016, Cnrs Umr8104, Université Paris Descartes

Correspondence to: J. Steven Alexander at jalexa@lsuhsc.edu

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## Abstract

Deviations from normal levels and patterns of vascular fluid shear play important roles in vascular physiology and pathophysiology by inducing adaptive as well as pathological changes in endothelial phenotype and gene expression. In particular, maladaptive effects of periodic, unidirectional flow induced shear stress can trigger a variety of effects on several vascular cell types, particularly endothelial cells. While by now endothelial cells from diverse anatomic origins have been cultured, in-depth analyses of their responses to fluid shear have been hampered by the relative complexity of shear models (*e.g.,* parallel plate flow chamber, cone and plate flow model). While these all represent excellent approaches, such models are technically complicated and suffer from drawbacks including relatively lengthy and complex setup time, low surface areas, requirements for pumps and pressurization often requiring sealants and gaskets, creating challenges to both maintenance of sterility and an inability to run multiple experiments. However, if higher throughput models of flow and shear were available, greater progress on vascular endothelial shear responses, particularly periodic shear research at the molecular level, might be more rapidly advanced. Here, we describe the construction and use of shear rings: a novel, simple-to-assemble, and inexpensive tissue culture model with a relatively large surface area that easily allows for a high number of experimental replicates in unidirectional, periodic shear stress studies on endothelial cells.

## Video Link

The video component of this article can be found at https://www.jove.com/video/54632/

## Introduction

Fluid shear stress has been shown to modulate endothelial gene programs[1-5] through activation of cis-regulatory elements[6], histone acetyltransferase activity[7] and shear stress response elements (SSRE)[8]. Shear stress influences endothelial contributions towards coagulation by modulating tissue factor[9] and tissue plasminogen activator (tPA)[10] expression. Shear stress also influences control of angiogenesis[11] and vessel remodeling by regulating PDGF-B synthesis and responsiveness[8]. The endothelial derived vasoactive mediators adrenomedullin, endothelin-1, urotensin II and relaxin are also regulated by shear[12]. Transcription of endothelial nitric oxide synthase production and nitric oxide production are both shear dependent[10]. Shear also controls endothelial ICAM-1 expression[13]. Flow-induced shear stress can therefore powerfully influence a large variety of endothelial responses. Importantly, vascular pulsations now also appear to play important roles in the pathophysiology of both normal vascular aging and forms of vascular dementia[14] and may even contribute to other neurodegenerative diseases, such as multiple sclerosis[15].

Venous and arterial endothelial cells are inherently exposed to diverse hemodynamic flow patterns *in vivo*, and many different endothelial cell phenotypes can be exhibited[16]. Depending on the magnitude and periodicity of flow, effects on endothelial cells may include inflammatory cell activation and apoptosis, which may reflect changes in gene or protein expression[17,18]. Studies on endothelial cell responses to shear phenomena therefore remain complicated by the difficulties in producing *in vitro* models that adequately produce such shear patterns.

Many different experimental protocols have been developed to apply fluid shear stress to endothelial cell monolayers. One of the most commonly used systems is the parallel plate flow chamber, which creates uniform laminar flow within the chamber[19-21]. A peristaltic pump is typically connected to create periodic flow, which can recapitulate flow characteristics typically found in many locations *in vivo*[22]. Another common set-up uses the 'cone and plate' model, where fluid shear stress is determined by the rotational speed of the cone[23]. Both systems, and other

 

arrangements similar to them, can be tedious to set up and require components that can be relatively expensive and inaccessible to many laboratories.

Another major limitation of these current models is the relatively low number of replicate studies that can be simultaneously performed, each with a relatively low surface area. This increases the time and complexity of such approaches. Therefore, an ideal model that induces unidirectional and periodic shear might be one where a high number of study replicates can be easily set up, each with a relatively large surface area. Furthermore, the aforementioned models require a fairly sophisticated setup, which may be cost-prohibitive for many users. A model that can produce fluid shear disturbances using basic laboratory materials might have several advantages.

A simple and highly economical method of applying unidirectional, periodic shear stress involves the placement of circular dishes on an orbital shaker[24]. This protocol is very simple and can be scaled up to achieve high numbers of study replicates, each with a relatively large surface area, as needed. However, cells located in the center of the dish are exposed to different flow patterns than cells along the periphery, yielding mixed cellular phenotypic responses in the same dish.

In this current report, we describe the construction and use of 'shear rings', our model for creating unidirectional and periodic shear stress. The design for the shear ring effectively limits 'mixed' cellular shear-induced phenotypes by restricting the flow pathway within a circular culture dish to the periphery through the placement of an inner ring. The construction and operation of the shear ring is simple and economical and can be easily scaled to accommodate a wide range of orbital shakers using widely available tissue culture supplies. This model can be applied in endothelial cell experiments to provide unidirectional and periodic flow patterns within physiological and pathophysiological levels.

## Protocol

# 1. Construction of 150 mm Diameter Shear Rings (Figure 1)

NOTE: Shear rings may be constructed to create many different dimensions by varying the outer and inner Petri dish sizes, resulting in devices with different total surface areas, cell yields and developed ranges of shear forces. This report describes a 150 mm dish combined with an inner 100 mm dish for a total surface area of 98 $cm^2$ (**Figure 2**).

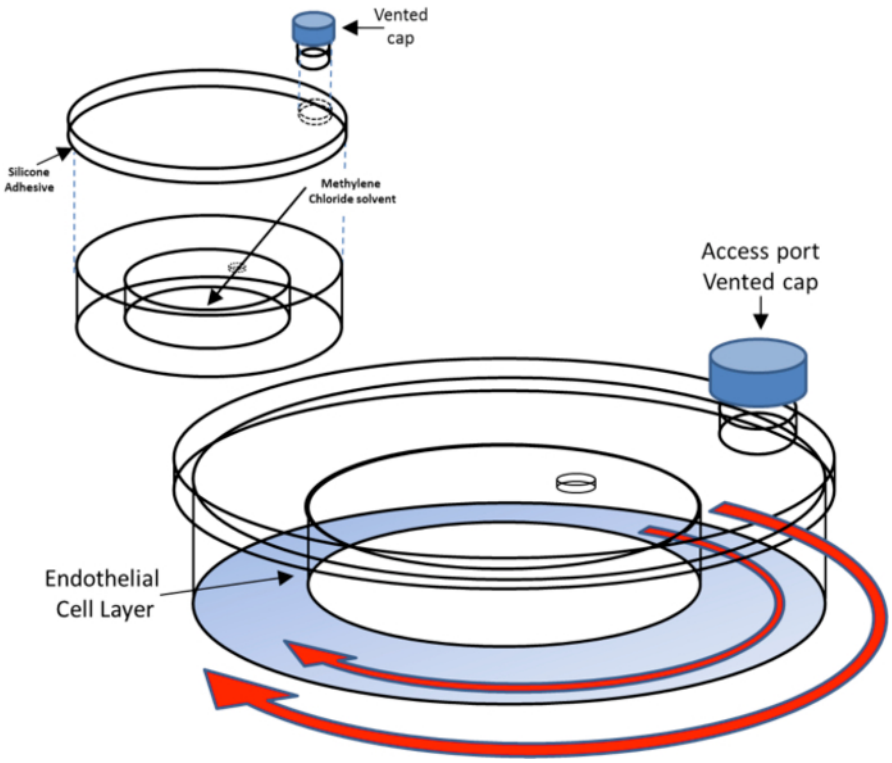


**Figure 1. Shear ring assembly.** The top portion of the figure shows a partially assembled shear ring. Inject 0.5 ml of methylene chloride through the 3 mm hole with a transfer pipet if a tight seal has not completely formed between the inner and outer dishes. The shear ring is sealed shut by applying a 1 mm thick bead of silicon rubber adhesive around the inner edge of the shear ring top. The bottom portion of the figure shows the assembled shear ring. The blue represents the area of plated endothelial cells. The outer and inner red arrows indicate the orbital motion of the shear ring and media inside the shear ring when placed on an orbital shaker. Please click here to view a larger version of this figure.

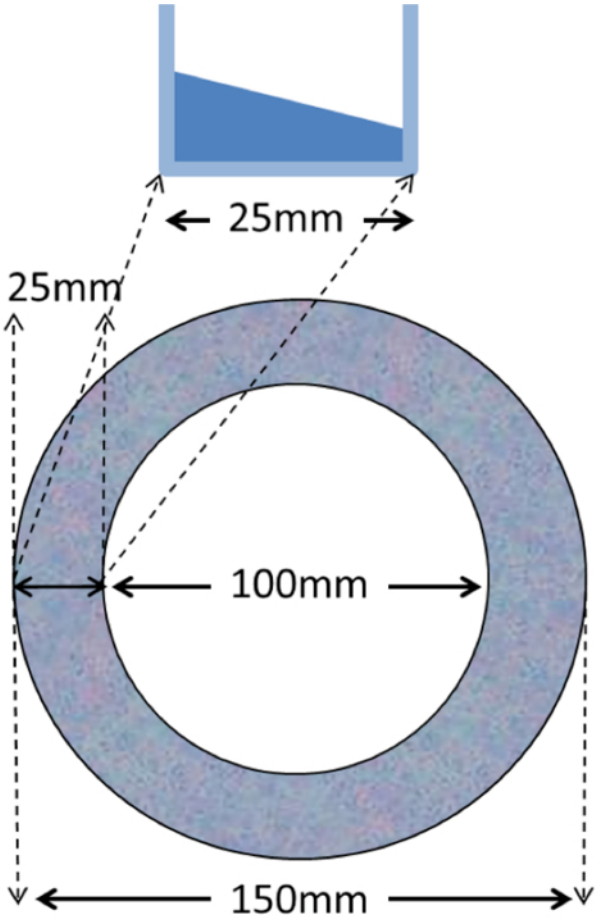


**Figure 2. Surface measurements for a 150 mm shear ring (not drawn to scale).** Top panel shows centrifugal shifting of fluid towards outer ring in response to orbital rotation. Please click here to view a larger version of this figure.

1. Create a shear ring construction template by generating a 150 mm outer edge profile in presentation software with a 100 mm internal diameter placed in the center of the 150 mm circle. Print the template on a sheet of A4 white paper.
2. Pipet 10 ml of methylene chloride (dichloromethane) into a 150 mm glass Petri dish. It is critical that the dish is glass and not polystyrene (or other vinyl plastic), as methylene chloride solubilizes most plastics and is used here to join plastic components.
   CAUTION: Use gloves for all construction with adequate hood ventilation. Methylene chloride is a contact irritant and is hepatotoxic.
3. Align a 150 mm plastic Petri dish onto the outer shear ring template.
4. Drill a 3 mm hole in the center of a 100 mm dish using a rotary tool. Remove any plastic shavings produced from the drilling.
5. While holding with a gloved hand, invert and dip the top edge of the 100 mm Petri dish from the previous step into the pool of methylene chloride for approximately 3 sec.
6. Transfer the "wetted" 100 mm dish edge-down onto the center of a 150 mm dish, carefully aligning the 100 mm dish onto the marked template. The methylene chloride will fuse the plastic, joining the 100 mm and 150 mm dishes. Gently rotate the 100 mm dish clockwise and counterclockwise a few times to ensure good adhesion to the 150 mm dish.
   NOTE: Take great care to ensure proper alignment of the inner dish to the center of the outer dish. Eccentric alignment may result in variations of the shear stress at different locations in the shear ring. Take care to not allow methylene chloride to accidentally drip onto the outer track portion of the 150 mm Petri dish during the placement of the 100 mm dish. This will melt the plastic on the bottom surface where cells will be growing, creating an uneven surface that may cause flow disturbances.
7. Once the methylene chloride has dried, flip the newly bound Petri dishes over and carefully inspect the points of contact to ensure that a tight seal has been formed between the two Petri dishes.
8. If a tight seal has not completely formed, inject 0.5 ml of methylene chloride through the 3 mm hole with a transfer pipet. Pick up the dish and gently rotate it to allow the methylene chloride to reach the edge.
   NOTE: If rotated too quickly, the methylene chloride can spill into the outer portion of the 150 mm dish, deforming the surface where cells will grow and ruining the shear ring. Leaking shear rings should be discarded.
9. Seal off the hole in the 100 mm dish by applying a 3 mm bead of silicon rubber sealant.
10. Using a rotary tool fitted with a flat cutting head, cut off the top 3 cm portion of a 15 ml conical polystyrene tissue culture tube, leaving at least 1 cm of the tube below the cap. Polish the edge of the cut tube until smooth and flat using the flat side of the cutting head. Remove any plastic shavings produced by grinding.
11. Trace the cut off 15 ml conical tube onto the lid of the 150 mm dish with a marker, approximately 0.5 cm away from the edge of the dish. Drill a hole inside the drawn circle, leaving a margin approximately 1-2 mm from the edge of the circle.
12. Place the cut off conical tube top over the drilled hole. Using a transfer pipet, apply approximately 250 µl of methylene chloride to the cut off edges of the conical tube to bind the conical tube to the 150 mm lid.

13. Seal the 150 mm lid onto the 150 mm dish by applying a 1 mm thick bead of silicon rubber sealant around the inner edge of the top Petri dish.

## 2. Sterilization of Shear Rings

1. Pipet approximately 10 ml of phosphate buffered saline into the newly formed shear ring through the 15 ml conical tube port. Swirl around to remove any debris inside the shear ring. Remove the phosphate buffered saline with a Pasteur pipet/vacuum aspirator.
2. Repeat the previous step until debris is removed.
3. To sterilize the shear ring, use a combination of a 70% ethanol rinse with UV irradiation.
    1. Unscrew the vented cap, pipet approximately 10 ml of 70% ethanol through the access port, and screw the cap back on. Rotate and flip over the shear ring multiple times, ensuring that the inside of the shear ring is thoroughly washed.
    2. Under a fume hood, aspirate out excess 70% ethanol. With a spray bottle, thoroughly spray the cap and access port with 70% ethanol.
    3. Place the shear ring and cap under UV radiation within the tissue culture hood until completely dry.
4. Once dry, screw the cap back on and store sterilized shear rings at room temperature until used in cell culture plating.

## 3. Shear Stress Studies

1. Plate endothelial cells in sterilized shear rings following the standard cell culture protocol typically using a 1:4 split ratio for transformed endothelial cell lines.
   NOTE: Rat retinal microvascular endothelial cells were obtained commercially. Human brain endothelial cells (hCMEC/D3) cells were provided as a generous gift from Dr. Pierre-Oliver Couraud (Inserm, France) and were cultured in complete endothelial basal medium (EBM).
2. Allow cultures to reach confluence prior to initiation of flow studies. Use 30 ml of tissue culture media (10% fetal calf serum, Dulbecco's modified Eagle's medium with 1% penicillin/streptomycin/amphotericin). Change cell culture medium every 3 days and maintain cells at 37 °C with 7.5% $CO_2$ and 92.5% room air.
3. Place the orbital shaker in the incubator.
   Note: Orbital shakers are typically heavy (>10 kg). Place the orbital shaker on the bottom shelf of the incubator to minimize shelf stress and vibration.
4. Place the experimental shear rings on the orbital shaker. Place static control group shear rings inside the same incubator.
5. Estimate the maximum shear stress within the shear ring with the equation $\tau_{max} = r\sqrt{\eta\rho(2\pi f)^3}$ where $r$ is the radius of rotation for the orbital shaker (in cm), $\eta$ is the viscosity of the medium (in poise), $\rho$ is the density of the medium (in g/ml), and $f$ is the frequency of rotation (in rotation/sec)[24].
6. Initiate the rotation setting on the orbital shaker to the desired rpm (*e.g.,* 90 rpm), leaving the shear rings on the shaker for the desired duration of shear stress application (*e.g.*, 72 hr).
   NOTE: Orbital shakers can produce heat, so the incubator temperature should be monitored initially to ensure 37 °C is maintained throughout the duration of the experiment. Alternatively, shear rings can be transferred into environmental chambers (*e.g.*, modular incubator chamber) and then placed within a rotating incubator.
7. After cell layers have been exposed to shear for the desired length of time, remove shear rings from the incubator. Pull off the shear ring lid and retrieve cells and/or media for examining desired endpoint analyses (*e.g.,* Western blotting, fluorescence activated cell sorting, *etc.*)[25].

## Representative Results

Here we present representative results from both hCMEC/D3 brain endothelial cell and rat retinal microvascular endothelial cell monolayers, cultured in shear rings.

After allowing hCMEC/D3 brain endothelial cell monolayers to grow to confluence in complete EBM, the shear rings were placed on an orbital shaker for 72 hours. Using the equation from step 3.5, the calculated maximal shear stress $\tau_{max}$ was approximately 2.8 dynes/cm$^2$ (with parameters $r$ = 0.95 cm, $\eta$ = 0.0101 poise, $\rho$ = 0.9973 g/ml[24], $f$ = 1.5 rotations/second). We have found that these endothelial cell monolayers sometimes exhibit alignment in parallel with the direction of the periodic flow (**Figure 3**), although this is not uniformly observed because cell layers usually have excellent adhesion to the shear ring surface throughout the study.

After allowing the rat retinal microvascular endothelial cell monolayers to grow to confluence in complete rat endothelial cell medium, including EGF/VEGF growth factor supplements, the shear rings were placed on an orbital shaker for 72 hours. Using the equation from step 3.5, the calculated maximal shear stress $\tau_{max}$ was approximately 12 dynes/cm$^2$ (with parameters $r$ = 0.95 cm, $\eta$ = 0.0101 poise, $\rho$ = 0.9973 g/ml[24], $f$ = 4 rotations/second). **Figure 4** shows that compared to the loading control of β-actin, there was a large and significant loss of platelet endothelial cell adhesion molecule (PECAM-1/CD31) from the endothelial cell surface.

PECAM-1 is an integral membrane protein which is a member of the immunoglobulin (Ig)-superfamily that contains an immunoreceptor tyrosine-dependent inhibitory motif or 'ITIM'[26]. PECAM-1 is not only expressed on endothelial cells but is also found on hematopoietic cells. PECAM-1 plays significant roles in endothelial cell-cell adhesion, leukocyte junctional transmigration, cell signaling, and importantly, mechano-transduction of shear stress. PECAM-1's role in sensing shear stress is critical for the functions of vascular endothelial cells and homeostasis[17]. When endothelial monolayers are exposed to shear stress, PECAM-1 responds directly to the mechanical force being exerted on it by altering its tyrosine phosphorylation, and subsequent activation of the ERK1/2 signaling cascade[27]. Furthermore, the PECAM-1-eNOS complex association is interrupted by disturbances in shear stress[28]. Therefore, PECAM-1 enables vascular endothelial cells to sense changes in fluid shear stress forces which can lead to reactive dilation of the vessel wall[29].

These data support this model showing that endothelial cells respond to exposure to periodic, unidirectional fluid shear by down-regulating an important junctional and adhesive determinant, which mediates intercellular contact as well as transvascular cell exchange.

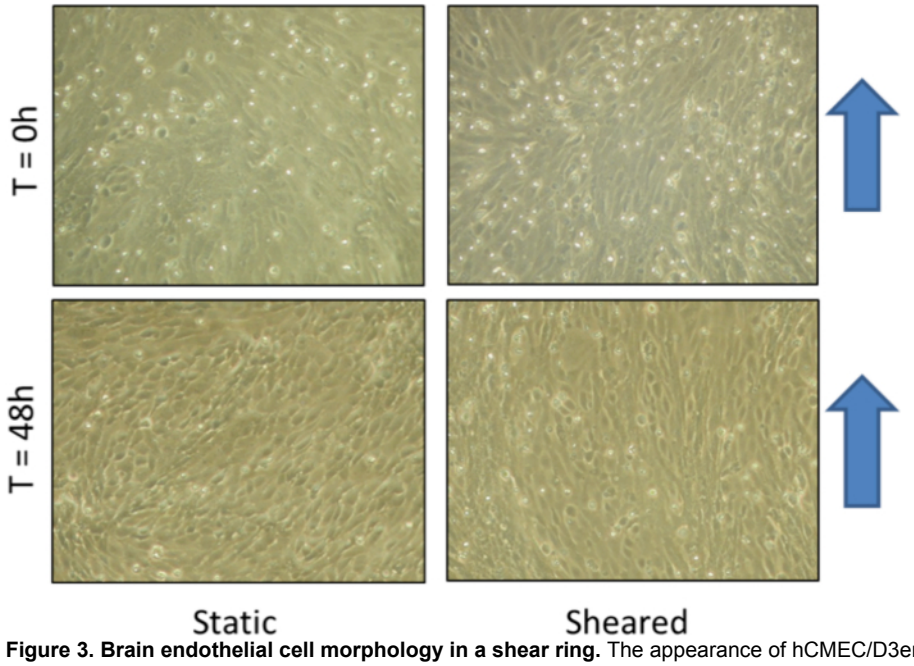


**Figure 3. Brain endothelial cell morphology in a shear ring.** The appearance of hCMEC/D3endothelial cell monolayers in shear rings following 48 hours of periodic fluid shear or static exposure. Alignment of cultures is not always observed parallel to flow direction (shown by arrow). Please click here to view a larger version of this figure.

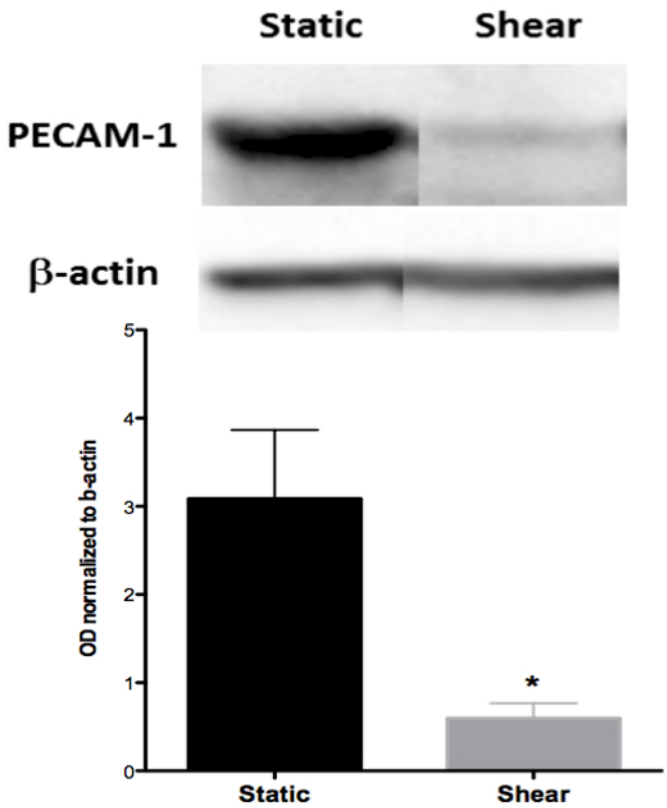


**Figure 4. Cell responses to periodic shear.** Rat retinal microvascular endothelial cell monolayers cultured in shear rings showed a reduction in PECAM1/CD31 relative to β-actin (n=3 each, students unpaired t-test, *-p<0.05, error bars refer to standard deviation), following 72 hours of periodic fluid shear in shear rings.

## Discussion

The construction of the shear ring system for exposing endothelial cells to shear is a simple approach to performing shear stress studies. Nevertheless, there are a few steps that are critical for obtaining superior shear rings and better results. A complete seal should be made between the inner and outer ring to prevent media from leaking which could create inconsistent shear stress among samples. If a complete seal is not made, a minimal amount of methylene chloride should be added to the edge between the inner and outer dish with a transfer pipet through the hole in the inner ring. Gently rotating the ring should allow for the methylene chloride to form a complete seal. Also, plastic shavings inadvertently produced from cutting may be present on the shear ring track, so rinsing out the shear ring after construction should remove any debris that could adversely affect cell growth and give inconsistent shear stress application.

The shear rings described in the article are relatively large in size, leading to a high volume of output per sample. However, smaller versions could be constructed using smaller Petri dishes (*e.g.*, 100 mm Petri dish with a 60 mm insert). Constructing multiple smaller shear rings could allow for higher numbers of study replicates while still maintaining relatively large fluid volumes and surface areas per sample when compared to other methods.

A few issues have been noted when using shear rings. First, some orbital shakers produce excess amounts of heat, and some incubators fail to control this increase in temperature. This can be avoided by the appropriate selection of rotators and the use of non-water jacketed incubators, which exchange heat much more readily. Culture contamination is another potential concern when using shear rings, particularly following assembly in an open-air environment. Shear rings must be thoroughly sterilized before use.

Here we have described a simple method to expose large numbers of endothelial cells to fluid shear stress. Other methods of producing unidirectional and periodic shear are available but are also far more costly, complex, and time-consuming when considering the surface areas and numbers of exposed cells per experiment. One such method involves connecting a peristaltic pump to a parallel plate flow chamber, which can difficult to set up and relatively costly. While the shear ring is simple to construct and use, the application of shear stress is less precise. Orbital flow can generate complex flow currents, which may model some, but not all, physical states of flow found in normal vascular physiology. The intrinsic inability to precisely control flow in the shear ring can be seen as one current limitation to the model. Furthermore, the mathematical formula described in the protocol above may be used as a tool to estimate maximal fluid shear stress in the shear ring. However, additional and more sophisticated experimental and computational studies are needed to definitively characterize flow patterns in the shear ring. As the characterization of flow patterns and fluid shear stress is better understood in the shear ring, one should be able to better adjust experimental parameters (optimal size of shear ring, rpm of the orbital shaker, volume of media in the shear ring, *etc.*) to generate the desired periodic, unidirectional shear stress.

## Disclosures

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