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Review article

Understanding mechanobiology in cultured endothelium: A review of the orbital shaker method

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HIGHLIGHTS

- Endothelial cells experience pro- or anti-atherogenic flow in swirling culture wells.
- Flow characteristics in different parts of the well can be estimated or computed.
- Inflammatory, homeostatic and other cell properties depend on location in the well.

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ABSTRACT

A striking feature of atherosclerosis is its highly non-uniform distribution within the arterial tree. This has been attributed to variation in the haemodynamic wall shear stress (WSS) experienced by endothelial cells, but the WSS characteristics that are important and the mechanisms by which they lead to disease remain subjects of intensive investigation despite decades of research. *In vivo* evidence suggests that multidirectional WSS is highly atherogenic. This possibility is increasingly being studied by culturing endothelial cells in wells that are swirled on an orbital shaker. The method is simple and cost effective, has high throughput and permits chronic exposure, but interpretation of the results can be difficult because the fluid mechanics are complex; hitherto, their description has largely been restricted to the engineering literature. Here we review the findings of such studies, which indicate that putatively atherogenic flow characteristics occur at the centre of the well whilst atheroprotective ones occur towards the edge, and we describe simple mathematical methods for choosing experimental variables that avoid resonance, wave breaking and uncovering of the cells. We additionally summarise a large number of studies showing that endothelium cultured at the centre of the well expresses more pro-inflammatory and fewer homeostatic genes, has higher permeability, proliferation, apoptosis and senescence, and shows more endothelial-to-mesenchymal transition than endothelium at the edge. This simple method, when correctly interpreted, has the potential to greatly increase our understanding of the homeostatic and pathogenic mechanobiology of endothelial cells and may help identify new therapeutic targets in vascular disease.

1. Introduction

Vascular endothelial cells regulate vessel tone, vascular permeability, inflammation and thrombogenesis. Endothelial dysfunction is associated with the early stages of atherosclerosis. Studying endothelial properties is therefore important for understanding vascular physiology and in the fight against cardiovascular disease.

Endothelial cells are exposed *in vivo* to frictional drag exerted by the flowing blood; the force per unit area, given by the product of viscosity and the near-wall velocity gradient, is known as haemodynamic wall shear stress (WSS). Endothelial cells are able to sense and respond to this stress through complex mechanosignalling pathways [1]. WSS varies from site to site depending on vessel geometry; straight unbranched arteries are exposed to pulsatile but uniaxial flow and relatively high time-averaged WSS (TAWSS) whereas areas of branching, bifurcation and high curvature are associated with multidirectional flow and either low magnitude or extreme TAWSS [2]. Studies using animal vessels have increased our understanding of endothelial mechanobiology [3–6] but there is also a need for good *in vitro* models.

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2. *In vitro* methods for applying uniform flow

A range of methods for studying the influence of WSS on endothelium *in vitro* have been described including, for example, culturing endothelial cells on microcarrier beads packed into columns [7] or on the inner wall of the capillary-like tubes in hollow-fibre bioreactors [8]. Advances have also been made in the development of 3D tissue models, where endothelial cells can be co-cultured in bio-engineered cylindrical constructs resembling whole vessels [9], or in microvascular networks of cylinders [10], and exposed to flow. However, the most widely published methods are the parallel-plate flow chamber and the cone-and-plate viscometer [11,12]. In these latter methods, the apparatus can be perfused at different steady flow rates or with time-varying flow, including reversing flow [13,14]. In both cases, however, the cells are exposed to flow along one axis and all cells are exposed to approximately the same flow.

3. *In vitro* methods for applying non-uniform flow

The restriction to spatially homogeneous flows is a serious one because numerous experiments are required to collect data over the range of WSS magnitude and pulsatility to which endothelial cells are exposed *in vivo*. Furthermore, spatial gradients in WSS have themselves been postulated as triggers of atherosclerosis [15]. To overcome this limitation, the geometry of parallel plate flow chambers has been altered in order to provide spatial variation in shear. Most commonly, taper is introduced to provide a gradient of shear stress magnitude along the chamber [16,17]. The addition of a backward-facing step upstream of the region of interest has also been used; it produces flow separation and recirculation, both in cone-and-plate devices [18] and in parallel-plate flow chambers [19,20].

4. *In vitro* methods for applying multiaxial flow

Tapering of parallel plate flow chambers results in WSS variation only along the length of the chamber. Even with the backwards step, the flow can be considered predominantly two dimensional. Again, this is a significant limitation. Intimal thickening in the carotid artery bifurcation co-localises with high values of the Oscillatory Shear Index (OSI) [21], and this has given rise to the view that flow is atherogenic if it shows a significant reversal in direction during the cycle. However, the OSI – despite its name – captures not only WSS that changes direction along one axis but WSS that is at any angle to the mean WSS vector at the same location; reversing uniaxial flow and truly multidirectional flow are confounded. A new metric – the transverse WSS (transWSS) [2] – captures only the multiaxial flow by averaging over the cardiac cycle those components of the instantaneous WSS vectors that are perpendicular to the mean WSS vector. Reversing uniaxial flows have a transWSS of zero. There is a strong correlation between areas of high transWSS and atherosclerotic lesion location, suggesting that it is multiaxial flow which is atherogenic [22]. Hence devices that can expose cells to flow which cyclically changes axis may have particular value.

Ibidi® have created a y-shaped, bifurcated parallel-plate flow chamber with the aim of mimicking the effects of vascular branching [23]. This device could produce multiaxial flow if perfused at a cyclically varying flow rate. However, the very low Reynolds number means that the degree and extent of such flow is likely to be small.

The parallel plate flow chamber has also been modified by the addition of inlet and outlet ports on the sides of the chamber, allowing flow to be switched between conventional and orthogonal directions [24]. Switching the orientation of flow did alter the angle and consistency of cell alignment, but the rate of switching was orders of magnitude slower than the changes in direction expected during a single cardiac cycle *in vivo*.

Effects of transWSS have also been studied using a conventional parallel-plate flow chamber in which the cells are cultured on a circular cover slip that can be rotated. Following 24 h of uniaxial flow, rotation through 90° resulted in increased production of reactive oxygen species and activated NF-κB whilst rotation through 180° had no effect, suggesting that endothelial cells are sensitive to flow across their long axis [26] and supporting the postulated pro-atherogenic role of multiaxial flow [25,26]. However, the cyclical changes in direction that occur *in vivo* were not replicated.

5. Use of the orbital shaker method to understand endothelial mechanobiology

An ideal method for investigating effects of flow on endothelium would allow chronic, high-throughput exposure of cells to both multiaxial and uniaxial flows using standard laboratory equipment, and the multiaxial flow would have cyclical changes in direction with a period on the order of the cardiac cycle.

The orbital shaker or ‘swirling well’ method appears to fulfil these requirements. It involves culturing cells in standard plasticware on the horizontal platform of a shaker that produces a circular movement in the plane of the platform, thus inducing a wave of culture medium that circles around the well (Fig. 1).

The peak of the wave rotates at the angular velocity of the platform. The resulting movement of the fluid exposes cells cultured on the base

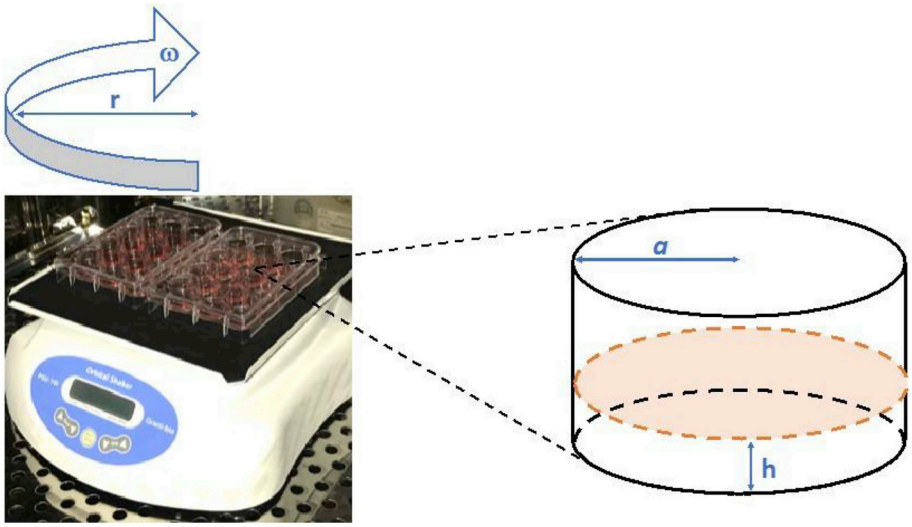

Fig. 1. The swirling well method.
An orbital shaker with 12-well culture plates on the shaker platform, and a schematic of one well. The arrow indicates the plane of rotation of the platform. *a* is the radius of the well, h is the height of the medium at rest, ω is the angular velocity of the platform and r is the orbital radius of the platform. Movement of the platform induces a wave that rotates within the well and consequently shears the cells cultured on the base.

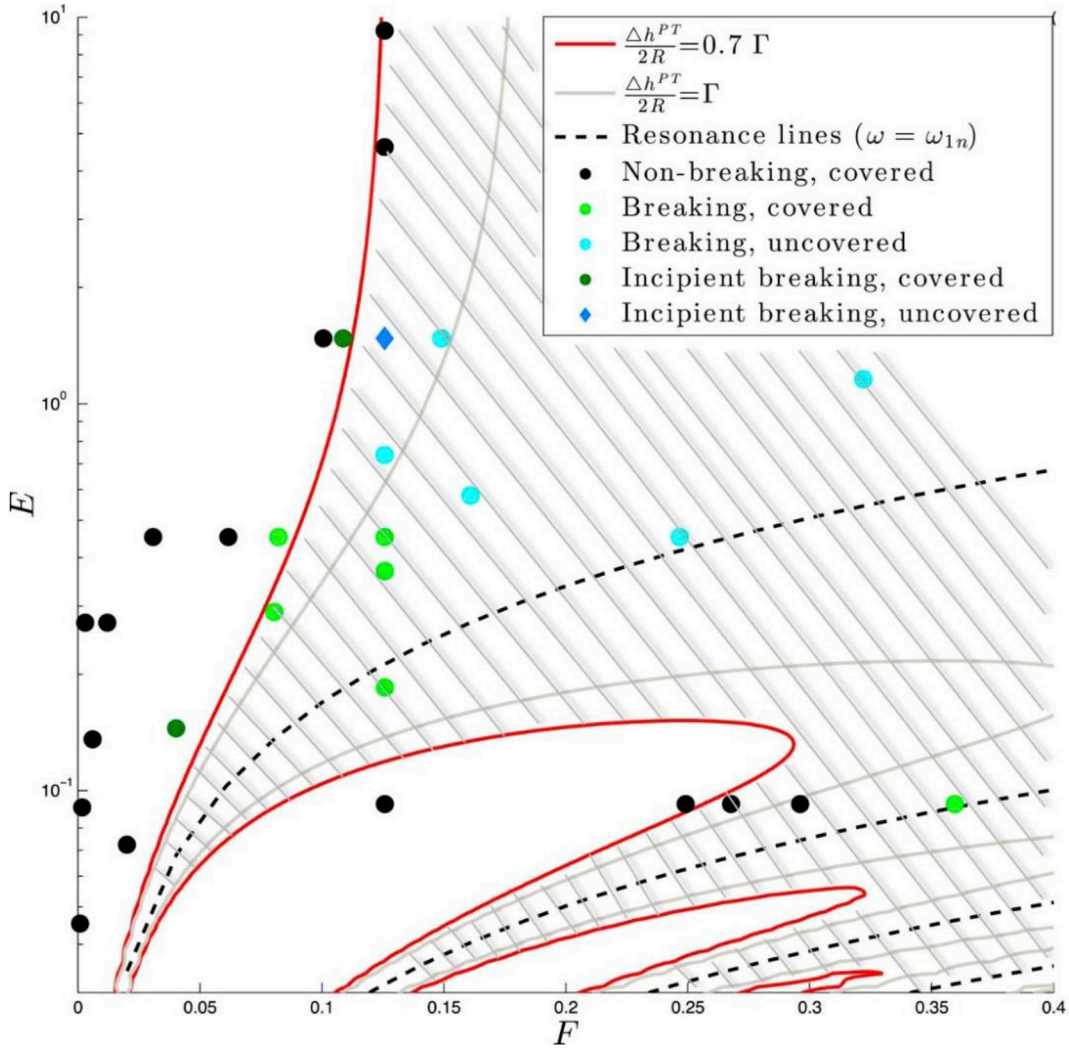

Fig. 2. Classification of the different wave-breaking regimes.
The red line is a contour for the breaking limit, resonance lines are represented by the dashed black lines and the grey striped area represents the wave-breaking region. Adapted from Alpresa et al. [34].

of the well to shear stress that is multiaxial at the centre of the well and uniaxial at its edge (see below). The apparatus can be placed in a conventional incubator for ten days or more.

Here we (i) review studies of the flow created using this apparatus, (ii) detail its effects on endothelial function and (iii) critically appraise the benefits and limitations of the method.

6. Characterisation of flow in swirling wells

Analytical, numerical and experimental methods have been used to estimate values of WSS in the well. Note that the term WSS is used for consistency with the stresses experienced by endothelium *in vivo*, but the parameter which has been estimated in all cases is the shear stress on the base of the well, where the cells grow, and not on the walls.

6.1. Analytical methods

Early studies used the extended solution of Stokes' second problem to estimate WSS in the well [27,28]. Stokes' second problem concerns a plate oscillating sinusoidally along one axis in the plane of the plate, with a liquid above it. The extended problem is where out-of-phase sinusoidal oscillations occur along two such axes at right angles to each other, giving circular motion. The problem is unbounded and assumes that the fluid is unperturbed in the far field. As a result of these assumptions, the calculated WSS, given by

$$\tau_w = a\sqrt{\rho\mu\omega^3}$$

where

τ_w = shear stress
a = radius of rotation
ρ = density
μ = dynamic viscosity
ω = angular velocity,

is uniform throughout the well, unaffected by its side walls and independent of the starting height of the fluid. All three of these properties are incorrect [29–31] and the calculated WSS magnitude can be in error by an order of magnitude [31–33].

An improved approach has recently been presented by Alpresa et al. [31,34]. Termed the potential theory-Stokes (PT-Stokes) model, it divides the fluid into two layers. The upper layer is modelled using potential flow [31] (i.e. the fluid is assumed to be frictionless and individual elements in it do not rotate). Velocity vectors within it are independent of depth, unaffected by the side walls, and determined by forcing from the orbital motion. Comparisons with numerical

simulations (see below) show that this part of the model describes the free-surface height well and the velocity field within the upper layer reasonably well, except when the orbital frequency is close to a natural frequency and resonance occurs.

The lower, boundary layer is dominated by viscous forces. At its upper surface, velocity vectors are equal to those in the inviscid layer, but they decrease in magnitude to zero at the base of the well, and also change direction in a spiral fashion with depth as a result of Coriolis-type effects.

The PT-Stokes model generally agrees much better with numerical solutions than does the extended solution to Stokes' second problem. The direction of WSS vectors is less well predicted than the magnitude, particularly towards the edges of the well, because the boundary conditions at the wall and secondary flows within the inviscid layer are not considered. Nevertheless, it provides a rapid method for obtaining approximate solutions except in cases with resonance or very high forcing.

Because the potential theory accurately models the free-surface height, it can be used to determine whether a particular experimental set-up will give non-breaking waves, breaking waves, breaking waves with the bottom of the well uncovered, or resonance [34]. For endothelial experiments, the first condition is preferred. To achieve this, flow in the well can be modified by altering the height of the medium at rest, the angular velocity and orbital radius of the platform, or the radius of the well. Additives to the culture medium could also be used, to alter its density and viscosity.

These variables can be reduced to a set of four dimensionless parameters – dimensionless forcing (***F***, the ratio of centripetal acceleration to gravitational acceleration), eccentricity (***E***, the ratio of orbital radius to well radius), shallowness of the fluid layer ($\boldsymbol{\Gamma}$, the ratio of fluid depth at rest to well radius) and Reynolds number (***Re***, the ratio of linear velocity times well radius to kinematic viscosity). For conventional culture medium and $\boldsymbol{\Gamma} = 0.18$ ($\approx$2 mm medium depth in a 12-well plate and $\approx$3 mm in a 6-well plate), Fig. 2 shows which combinations of ***E*** and ***F*** are required to avoid resonance, wave breaking and uncovering of the base.

A MATLAB implementation of the PT-Stokes model is available online at.

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Non-breaking waves with full coverage of the bottom of the well will be achieved if

$$\Delta h/(2R) < 0.7\Gamma$$

where Δh is the amplitude of the wave (obtainable from the MATLAB implementation) and R is the radius of the well (see Box 1).

6.2. Numerical methods

Higher accuracy can be obtained by computational fluid dynamics (CFD), in which numerical techniques are used to solve the Navier-Stokes equations governing flow. WSS can then be calculated from velocity gradients near the base. Such methods are routinely employed to obtain WSS in arteries, but the swirling well system has the additional complexity of a free surface with varying height. It is necessary to track the surface and to ensure that the volume of liquid is conserved. Surface tension is generally ignored but the effect of gravity (which acts to level the surface) must be included. A forcing function, representing the motion of the well, is also required.

Starting with the work of Berson et al. [35], numerical models of the orbital shaker system [30–32,34,36–46] have shown that WSS magnitude fluctuates with time, more so towards the edge of the well than at its centre, and that TAWSS is also greater towards the edge. Additionally, the direction of the instantaneous WSS vector rotates evenly through 360° at the centre of the well during each orbit whereas at the edge, vectors tend only to switch between forward and backward orientations along a line that is approximately parallel to the wall. Hence there are low magnitude, multidirectional shear stresses at the centre and high magnitude, uniaxial shear stresses at the edge. This behaviour has been captured by polar plots of instantaneous WSS vectors [37,47,48], by a combination of the OSI and transWSS metrics, or by a metric termed the Directional OSI [DOSI] [37,49].

A set of 33 flow simulations, corresponding to cases from across the literature, has been conducted to validate the potential theory and PT-Stokes models described above [31,34].

6.3. Experimental methods

Dardik et al. [29] used optical Doppler velocimetry to obtain velocities near the centre and edge of the well. WSS magnitudes increased with angular frequency and were higher at the edge than the centre. A very complete study by Salek et al. [30] combined optical Doppler velocimetry, Particle Image Velocimetry (PIV) and video recording, comparing the measured flows and free surface height with CFD simulations. Thomas et al. [33] also compared PIV and CFD.

In general, the agreement between experimental and numerical methods has been excellent. Thomas et al. reported that the velocity vector components differed by 5% between PIV and CFD, and the velocity magnitudes by 2.5% [33]. Additionally, there is good qualitative agreement between CFD simulations and flow visualisation [42]. However, the experimental methods have limitations. Optical Doppler velocimetry cannot, as currently implemented, obtain the direction of WSS vectors. PIV can obtain vector direction as well as magnitude but the laser sheet used to illuminate the particles is typically nearly as thick as the depth of the medium and the technique is difficult to use near the wall. Hence although direct measurement has provided good validation of numerical simulations, it is unlikely to replace CFD as the method of choice for obtaining WSS characteristics, unless more practicable methods are developed, perhaps based on tracking fluorescent microspheres or using mechanosensitive fluorescent molecular rotors [50].

6.4. Conclusion

The orbital shaker method provides a rich flow environment. WSS metrics including TAWSS, OSI, transWSS and DOSI vary with radial distance from the centre to the edge of the well. In general, WSS characteristics regarded as proatherogenic (low TAWSS and DOSI, high OSI and transWSS) are more pronounced at the centre of the well and

Box 1
Choosing experimental variables to give a specific flow profile.

- The shear stress that the cells experience depends on the orbital radius and angular velocity of the platform, the height of the medium at rest and the radius of the culture well.
- Identifying suitable values of these variables requires calculation of the dimensionless eccentricity (***E***, the ratio of orbital radius to well radius), forcing (***F***, the ratio of centripetal acceleration to gravitational acceleration) and shallowness (Γ, the ratio of fluid depth at rest to well radius).
- For a typical Γ value of 0.18, Fig. 2 shows which values of ***E*** and ***F*** are required in order to avoid resonance, wave breaking or uncovering of the cells.
- Pairs of (***E***,***F***) near the wave-breaking threshold give the most diverse WSS patterns in a single well.

those regarded as atheroprotective (high TAWSS and DOSI, low OSI and transWSS) are more pronounced at the edge of the well, making the system a valuable one in which to investigate cellular events that might lead to disease.

However, the nature of the WSS characteristics that promote or retard disease remains controversial and it is therefore also necessary to distinguish effects of the different metrics. That can be achieved by comparing their radial profiles with the radial profile of the cellular property of interest, and/or by changing the physical variables that influence flow patterns. For example, in 12-well plates having a static medium depth of 2 mm, rotated at 150 rpm with an orbital radius of 5 mm, TAWSS decreases from the centre to a radial distance of 7.8 mm before rapidly increasing as it approaches the edge, whereas in the larger 6-well plates with the same medium depth and orbital parameters, TAWSS is uniform until a radial distance of 10 mm before the rapid increase [47,48]. This type of diversity can be used to separate the effects of different metrics.

7. Investigation of responses to shear stress *vs.* static conditions using the orbital shaker

Many early studies used the orbital shaker simply as a method for inducing flow over cells, with no regard to variation of flow within the well; cells cultured under static conditions were used as the control. The findings from these studies, along with the flow regimes used, are summarised in Table 1 [27,28,50–56].

This paradigm was adapted to assess effects of flow on endothelial permeability by culturing confluent monolayers on Transwell® filter inserts suspended within the wells. A 1-h exposure to orbital flow significantly increased permeability to albumin when compared to static conditions [39], supporting previous studies [58], whereas a 5-day exposure significantly reduced it through an NO-dependent mechanism [39].

8. Investigation of responses to different flow characteristics using the orbital shaker

Radial variation in WSS profiles additionally allows the effects of putatively pro- and anti-atherogenic flows to be investigated when the method is used in conjunction with spatially resolved readouts of cell behaviour. Here we summarise studies that compared cells cultured at the centre and the edge of the well. Note that the terminology used to describe the flow characteristics has varied. For example, some authors classify the centre as a low WSS region without reference to its multi-directional component. Here, for simplicity, we use the terms “centre” and “edge” to describe the two flow regimes.

8.1. Cellular morphology and biomechanics

Endothelial cells at the edge of the wells exhibit elongation and alignment whereas cells in the centre are unaligned [36,37,49,59]. Cells at the centre of the well are also more compliant than those at the edge [58].

8.2. Proliferation, apoptosis and senescence

Cells at the centre of the well exhibit increased rates of proliferation [36,37] and apoptosis [6,36] relative to the edge. Mechanistic studies have shown that the expression of PERP, a positive regulator of apoptosis, is increased in the centre [6]. Cells at the centre also exhibit increased levels of senescence and thus accelerated endothelial ageing [38]. The increased senescence depended on enhanced expression and activation of p53. Sirt1, which deacetylates and inhibits p53, was reduced at the centre of the wells and Sirt1 activation reduced the senescence [38].

8.3. Atheroprotective gene expression

Expression of KLF4 [40] and eNOS [38,40] is higher at the edge than at the centre. eNOS phosphorylation is also higher [25]. Contradictory results have been obtained for COX-2, however: Filipovic et al. [40] found higher expression at the edge than the centre but Potter et al. [59] did not. Filipovic et al. used HUVEC and exposed them to flow for 24 h, whereas Potter et al. used porcine aortic endothelial cells and exposed them for seven days, so this contradiction may reflect the different cell origin or flow duration.

8.4. Pro-inflammatory signalling and endothelial dysfunction

Expression of ICAM-1 [36,49,60], VCAM-1 [60], E-selectin [38,49] and IL-6 [49] are significantly higher in the centre than at the edge, although there is disagreement about whether co-stimulation with TNF-α is [43] or is not [18,49] required in order to see the difference in ICAM-1. The activity of the NF-κB subunits RelA and p50, which can

Table 1
Use of the orbital shaker method to investigate responses to flow compared to static conditions in endothelial and vascular smooth muscle cells.

Reference	Cell type	Effects of shear compared to static culture	rpm	Reported shear stress (dyne/cm^2)	Computation of shear stress	Duration of flow
Ley 1989 [28]	HUVEC	↑granulocyte adhesion to EC	50	0.44	Mathematical solution	0.5h
			100	1.25		
			150	2.29		
Tsao 1995 [56]	BAEC	↑ production of NO ↓ adhesion of monocytes	120	None	None	4h
Pearce 1996 [57]	HUVEC	↑Cpla$_2$ activity	50	1.04	Mathematical solution	5–60min
		↑Arachidonic acid production	100	2.92		
		↑ERK-2 activity	150	5.31		
			200	8.3		
Kraiss 2000 [27]	HUVEC	↑ phosphorylation and activation of pp70^{S6k} ↑ Bcl3 protein levels	200	12	Mathematical solution	0.5–1h
Kraiss 2003 [512]	HUVEC	↓ translation of E-selectin	200	12	Mathematical solution	4–24h
Yun 2002 [51]	MicroVEC	↓MT1-MMP expression	270	14	Mathematical solution	1–8h
Haga 2003 [53]	BASMC	↑ VSMC proliferation ↑ Akt activity	270	14	Mathematical solution	1–5 days
Asada 2005 [54]	BASMC	↑ VSMC proliferation ↑ERK1/2 activity	210	11.5	Mathematical solution	1–10 days
Walshe [55]	HUVEC	↑TGFβ-ALK5-Smad2/KLF2		10	From refs	24–72h

BAEC (bovine aortic endothelial cells), BASMC (bovine aortic smooth muscle cells), Bcl-3 (B cell lymphoma-3), cPLA2 (cytosolic phospholipase-A2), ERK (extracellular signal-related kinase), HUVEC (human umbilical vein endothelial cells), KLF2 (Kruppel-like factor-2), MT1-MMP (membrane type 1-matrix metalloproteinase), microVEC (microvascular endothelial cells), TGFβ (transforming growth factor-β), VSMC (vascular smooth muscle cells). 1 dyne/cm^2 = 0.1 Pa.

Box 2
Advantages of the orbital shaker method for shearing endothelial cells *in vitro*.

- The method is straightforward and economical to set up and run.
- It gives high-throughput since many multi-well plates can be placed on one platform.
- The use of standard plates means that cells can be sheared for extended periods (ten days or more).
- Cells in the centre of the well are exposed to multi-directional flow, which cannot readily be produced with other methods.
- Effects of putatively pro- or anti-atherogenic shear profiles can be compared within the same well, removing confounding variables such as cell donor and passage number.
- The range of stresses applied within a well can easily be varied (see Box 1).
- Small molecule inhibitors can be used, and secreted products examined, due to the small volume of fluid in the well.
- The method can be adapted to study effects of co-culture.

drive expression of inflammatory genes, was also higher at the centre [61]. Furthermore, adhesion of THP-1 monocytes to endothelial cells was greater at the centre than at the edge of pre-swirled wells, although in these experiments the expression of ICAM-1 and VCAM-1 was not different between regions [48]. Collectively, these data suggest there is chronic inflammatory activation of endothelial cells in the centre but not at the edge of the wells.

8.5. Endothelial-mesenchymal transition

Recently the orbital shaker has been used to investigate effects of different flows on endothelial-to-mesenchymal transition (End-MT) and the signalling pathways that regulate it [62]. Cells at the centre exhibited increased expression of mesenchymal lineage genes (N-cadherin and α-SMA) and End-MT transcription factors (Slug and Snail) [60,63]. Silencing of GATA-4 and TWIST-1 reduced the induction of Snail in cells at the centre but did not alter the expression of other mesenchymal markers, suggesting that flow-induced End-MT is regulated by multiple signalling pathways [60]. Furthermore, flow-induced End-MT may be incomplete since the expression of VE-cadherin, indicative of endothelial cell lineage [49,53], was similar at the centre and edge [60,63].

8.6. Permeability

Transendothelial transport of macromolecules has also been investigated in the swirling well [47]. Transport was measured using a modification of the method of Dubrovskyi et al. [64], who grew endothelial cells on biotinylated gelatin and used FITC-labelled avidin (69 kDa) as a tracer; the FITC-avidin is added to the medium and binds to the biotin immediately on crossing the endothelium, thus remaining under – and hence identifying – the structure through which it travelled. This principle was extended to larger tracers by also using R-phycoerythrin-labelled avidin (300 kDa) and quantum dot-labelled avidin (radius 20 nm). The dominant transport pathways for the tracers of increasing size were, respectively, junctions between neighbouring pairs of cells, tricellular junctions, and a transcellular route. Paracellular transport was elevated in the centre of the well and reduced at the edge, but there was no difference in transcellular transport [47].

9. Advantages and limitations of the swirling well method

Studying endothelial mechanobiology *in vivo* has limitations: the mechanical environment is hard to characterise (for example, it is difficult to estimate or eliminate effects of altered strain), endothelial properties may be influenced by other cell types, the use of toxic or expensive reagents is difficult, studies cannot easily be conducted in human arteries, and cost is high while throughput is low.

All methods for applying flow to endothelium *in vitro* overcome the problem of concomitantly altered mechanical strain, permit the use of human cells and of endothelial cells in isolation from other cell types, and allow toxic reagents to be employed. However, the swirling well method additionally has the ability to impose both multidirectional and uniaxial flow. Furthermore, exposing the cells to both shear profiles in a single well removes confounding variables such as passage number and cell donor.

Another significant advantage of the orbital shaker method is the ability to shear cells for extended periods. Cone-and-plate viscometers and parallel-plate flow chambers are rarely used beyond 48h, but cells can be maintained on the orbital shaker for ten days or more. Furthermore, the cost of the shaker is low and no consumables are required other than standard cell culture plates and medium, while the use of multi-well plates and the fact that they can be stacked on the platform creates a high-throughput system.

The use of culture wells also allows addition of expensive inhibitors to interrogate signalling pathways; the large fluid reservoirs required for parallel plate flow chambers can make this impracticable. Similarly, the large volume of effluent makes it difficult to investigate the role of soluble mediators or secreted microparticles that play a role in endothelial mechanosignalling [65]. Transwell® plates can also be used with the orbital shaker to investigate the role of co-culture on mechanosignalling [66]; this under-exploited potential is worthy of future study (see Box 2).

The use of standard plasticware does bring with it some of the potential disadvantages of conventional tissue culture techniques [67]. For example, the regulation of endothelial cell properties is not only governed by imposed mechanical forces but also influenced by the stiffness of the substrate to which the endothelial cells are adhered [68]. Many have improved on the inherently rigid substrate by culturing cells on a thin layer of more compliant material such as functionalised polyacrylamide hydrogels [69] or polydimethylsiloxane (PDMS) [70] to imitate the *in vivo* environment. It is plausible that these techniques could be incorporated into the model by culturing cells on a biomimetic surface within the wells, although no studies have combined the methods to our knowledge. It may also be possible to develop the use of biomimetic substrates with co-culture models, further enhancing the physiological relevance of the orbital shaker model.

Despite the numerous advantages of the orbital shaker, the difficulty of separating effects of different flow metrics can be challenging. In addition to the methods already described, it is possible that varying the geometry of the wells may improve separation; this is an area of ongoing study. A further complication (and an often unrecognised problem for other *in vitro* methods) is that cells exposed to a certain shear profile in one location may release mediators into the medium that alter the behaviour of cells at another location, experiencing a different shear profile, thus obscuring or corrupting the true relation between shear and cell properties [54]. To turn this potential disadvantage into a benefit, surface coating methods have been developed for growing cells in only some areas of the well; these methods allow the demonstration and identification of soluble mediators. Data obtained in such studies are consistent with the release of a mediator from cells at the edge of the well that has anti-inflammatory effects on activated endothelium; the mediator may be of importance in atherogenesis [54].

10. Conclusion

The orbital shaker method is a simple technique for exposing cells to different types of flow occurring in arteries *in vivo*. The flow created in the centre of the well has characteristics that are regarded as pro-atherogenic and promotes inflammatory activation, proliferation, apoptosis, senescence, End-MT and elevated permeability, all of which are associated with early endothelial dysfunction and the development of disease. Conversely, flow at the edge has characteristics, and induces endothelial properties, that are regarded as protective. The method provides a convenient, cost-effective, reliable and robust model for investigating endothelial mechanosignalling and understanding the focal nature of atherosclerosis.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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