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ORIGINAL ARTICLE

The Bonn Criteria: Minimal Experimental Parameter Reporting for Clinostat and Random Positioning Machine Experiments with Cells and Tissues

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Abstract Published reports on studies in clinostats and random positioning machines frequently do not include adequate operational data on physical parameters of the culture device or cell culture conditions. This failure to report minimum physical and chemical data on how experiments are performed makes it impossible to determine specific hardware utilization or to calculate forces delivered. This makes experimental comparisons difficult and isolation of critical methodological differences between investigational results impossible. A minimum set of parameters to be reported in clinostat or random positioning machines is proposed to be known as the Bonn criteria. For random positioning machine experiments, the minimum experimental parameters to be reported should include angular velocity of rotation, highest angular acceleration, operating mode (random, centrifuge, or clinostat in rpm or freely programmable mode). For both clinostat and random positioning machines, experimental reporting should include the properties of the culture vessel, culture media and carrier beads. These should also include dimensions and rotation speed of vessel, chemical consistency including density and viscosity of media, size, density, and porosity of beads, size, density, and porosity of cells, whether cells are motile or non-motile, density of beads with cells attached, as well as time of rotation, nature of controls, operating temperature, and gas content.

Keywords Suspension culture · Shear · Terminal velocity · Clinorotation · Cell culture · Clinostat · Random positioning machine · Rotating wall · Vessel

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Introduction

From white cells to hydridomas, and primary hepatocytes to bacteria, scientists all over the world put cells in suspension culture every day (Hammond and Hammond 2001; Helmrich and Barnes 1998; Klaus et al. 2004). This intense cell biology interest in suspension culture has been driven by observations that, unlike plate culture during which most cells dedifferentiate, suspension culture maintains some of the differentiated features of many cell types (Hammond and Hammond 2001; Helmrich and Barnes 1998; Klaus et al. 2004).

A diverse array of suspension culture devices aim to keep cells buoyant in the fluid phase of a culture vessel during cell growth and propagation (Helmrich and Barnes 1998). Beads are commonly added to provide an adhesive surface for adhesion dependent cell types (Hammond and Hammond 2001; Helmrich and Barnes 1998). The cellular effects of suspension culture

are heavily dependent on the set of forces delivered to the cells. To make the desired biological effects freely reproducible, the experimental parameters must be defined, known, and reported to assure repeatable controlled conditions.

For microgravity related studies, rotating wall vessels and clinostats are popular, as they provide suspension culture at sufficiently low shear levels that fragile mammalian cells can be studied (Briegleb 1983; Klaus et al. 1998; Tsao et al. 1994; Wolf and Schwartz 1992). These vessels replicate some of the physical culture conditions of space flight in that they provide suspension culture with minimal wall impacts, co-localization of cells of differing size and density, optimally reduced fluid shear and turbulence, and provide three-dimensional spatial freedom with gas exchange by diffusion (Hammond and Hammond 2001; Klaus et al. 2004).

The random positioning machine is a more recent development which greatly enhances and expands the ability to vector average the effect of gravity in multiple dimensions (van Loon 2007; Borst and van Loon 2008). Two independently moving frames allow a central biological sample to be moved in a complex manner which averages the gravity vector. Variable instrument settings allow different angular velocities of rotation and operating modes (random, centrifuge, clinostat, or freely programmable) to apply different approaches to gravity vector averaging (van Loon 2007; Borst and van Loon 2008). The non-clinostat modes of the random positioning device allow low shear suspension culture with a different set of forces than traditional clinorotation.

A popular way to ensure simple interpretation of the results of an experiment unambiguously and potentially to reproduce the experiment is to incorporate the use of data sets. For instance, MIAME is an acronym which describes the Minimum Information About a Microarray Experiment that is needed for interpretation and reproduction of experiments (Brazma et al. 2001). For microarray data the MIAME initiative has led to standard data formats, journals which will only accept MIAME compliant data, and public data repositories which are facile to use to freely available tools (see http://www.mged.org/Workgroups/MIAME/miame.html).

The utility of published reports on studies in random positioning machines and clinostats, which includes rotating wall vessels, would be greatly strengthened by the reporting of a minimum set of parameters which we propose. As the symposium formulating this new systematic reporting was held in Bonn, Germany, we propose the parameter set to be known as the Bonn criteria.

Methods and Results

Determinants of Terminal Velocity and Shear in Rotating-Wall Vessels & Clinostats

A cell or cell aggregate in suspension culture using clinorotation accelerate through the fluid until it reaches a terminal velocity (V_s), at which the pull of gravity is balanced by equal and opposite hydrodynamic forces including shear, centrifugal, and Coriolis forces. This is important as the shear on a particle is proportional to the terminal velocity of the cell aggregate, which is in turn determined by Eq. 1 (Schwarz and Wolf 1991; Tsao et al. 1994; Wolf and Schwartz 1991)

$$V_s = \left[2gr^2(\rho_\rho - \rho_f)\right]/(9\mu\rho_f) \quad (1)$$

where V_s is the terminal velocity, g is gravity, r is the radius of the particle, ρ_ρ is the density of the culture particles, ρ_f is the density of culture medium (fluid), $\rho_\rho - \rho_f$ is the difference in density between culture particles and medium, and μ is the viscosity of cell culture medium.

Hence, to calculate terminal velocity of a particle during clinorotation and secondarily calculate shear it is critical to know the major direct determinants of the terminal velocity of a cell aggregate in suspension culture: gravity and the radius of the particle squared. There are direct effects of cell and fluid density and inverse effects of viscosity and density of the culture medium. Note that the rotation speed of the vessel does not affect the terminal velocity of the cells in suspension; rotation speed is not in the equation to calculate terminal velocity (Hammond and Hammond 2001).

Although the classic Navier Stokes equation does not have density in the demoniator, some authorities have argued that this should be included for many applications in the rotating wall vessel and other forms of clinorotation (Schwartz and Wolf 1991; Tsao et al. 1994; Wolf and Schwartz 1991), as density and viscosity are not independent. No matter where one stands in this debate, having the parameters available allows comparison of the mechanical conditions applied.

Shear Stress Shear stress (τ_{max}) is a function of the terminal velocity as shown by the following (Andereck et al. 1986; Gao et al. 1997; Wolf and Schwartz 1991):

$$\tau_{max} = 3\mu V_s/2r \quad (2)$$

where μ, V_s, and r are defined as in Eq. 1.

Equation 1 applies to creeping flow around a solid sphere (Birch and Arathoon 1990), which adequately

describes the movement of a particle during clinorotation. Note that similar to the case with terminal velocity, rotation speed of the vessel does not affect shear stress (Hammond and Hammond 2001).

Discussion and Recommendations

Terminal velocity and shear have been shown to play an important, and perhaps central, role in determining the effects of clinorotation on a variety of biological effects on cells in culture (Hammond and Hammond 2001; Kaysen et al. 1999; Unsworth and Lelkes 1998; Jessup et al. 1997; Zhau et al. 1997). The equations above identify all the parameters which are necessary to know to calculate terminal velocity and shear during simple clinorotation culture.

It is noteworthy that rotation speed has no direct effect on either terminal velocity or shear during simple clinorotation culture other than a tiny centrifugal force effect (Gao et al. 1997; Hammond and Hammond 2004). The rotation speed does effect whether terminal velocity can be reached. The sum of forces includes a Coriolus force, which determines that the cells or aggregates move through a clinorotator in tiny corkscrews, and the diameter of the corkscrews is also influenced by the rotation speed (Gao et al. 1997; Hammond and Hammond 2001). Some more complex clinorotation devices have a coaxial oxygenator which can be rotated at a different speed to the outer wall to reintroduce shear (Andereck et al. 1986; Spaulding et al. 1993). In this case, other parameters are needed to calculate shear, specifically the difference in rotation speed of the inner and outer walls (Goodwin et al. 1993) and the ratio of the inner and outer wall radii. Hence, when tabulating suggested minimal criteria for reporting clinorotation experiments (see Table 1), we have included rotation speed and vessel diameter to cover these more complex situations.

Table 1 Minimum parameter set for both clinostat and random positioning devices experimental reporting

▶Dimensions of vessel (cm)
▶Rotation speed of vessel (rpm)
▶Density of media (mass per unit volume)
▶Viscosity of media (Pascal-second, or centepoise)
▶Size, density and porosity of cells
▶Inoculum density of cells
▶Density and porosity of beads (if present)
▶Whether cells are motile or non-motile
▶Density of beads with cells attached (mass per unit volume)
▶Operating temperature (degrees Fahrenheit or Celsius)
▶Gas content (including type and presence of any oxygenator)
▶Time of rotation (minutes)
▶Nature of controls

Table 2 Minimum experimental parameters for random positioning device experimental reporting in addition to the parameters in Table 1

▶Angular velocity of rotation (rads/sec)
▶Highest angular acceleration (rad/s^2)
▶Operating mode
•Random rad/sec
•Centrifuge rpm
•Clinostat • freely programmable mode (with set parameters stated)

The importance of documenting mechanical culture conditions is underscored by several lines of evidence that shear stress selectively effects diverse cellular functions, and that cumulative cell sedimentation is the dominant effect on clinorotation cultures.

In our own lab, we examined whether the expression of renal cell protein markers is dependent on initial mechanical culture conditions (Cowger et al. 2002). During rotating wall vessel cultivation of human renal cells, size and density of glass-coated microcarrier beads were changed to modulate initial shear, and renal-specific proteins were assayed after 2 days. Analysis of vitamin D receptor demonstrated peak expression at intermediate shears, with 30% reduction outside this range. Activity of cathepsin C showed the inverse pattern, lowest at midshear, with twofold increases at either extreme. Dipeptidyl-peptidase IV had no shear dependence, suggesting that the other results are specific, not universal, changes in membrane trafficking or protein synthesis. On addition of dextran, which changes medium density and viscosity but not shear, vitamin D receptor assay showed no differences from controls. Neither cell cycle, apoptosis/necrosis indexes, nor lactate dehydrogenase release varied between experiments, confirming that the changes are primary, not secondary to cell cycling or membrane damage. This study provides direct evidence that mechanical culture conditions modulate protein expression in suspension culture.

Benoit and Klaus (2005) report a novel approach for evaluating the effects of reduced cell sedimentation through use of *Escherichia coli* cultures genetically modified to be neutrally buoyant. Since clinorotation would not (or would only minimally) affect cell distribution of this already near-colloidal cell system, it was hypothesized that the effects on final population density would be eliminated relative to a static control.

Gas-vesicle-producing *E. coli* cultures were grown under clinostat and static conditions and the culture densities at 60 h were compared. As a control, *E. coli* that do not produce gas vesicles, but were otherwise identical to the experimental strain, were also grown under clinostat and static conditions. As hypothesized, no significant difference was observed in cell populations at 60 h between the clinorotated and static gas-vesicle-producing *E. coli* cultures, while the cells that did not produce gas vesicles showed a mean increase in population density of 10E5 ($P = 0.001$). These results further suggest that the lack of cumulative cell sedimentation is the dominant effect of space flight on non-stirred, in vitro *E. coli* cultures.

Although we are not currently aware of a similar analysis of the determinants of hemodynamic forces in the random positioning machine, there are some guidelines. First, the same physiological and material properties which are active during clinorotation are likely to play a role during random positioning machine culture (van Loon 2007; Borst and van Loon 2008). What remains to be determined is the exact relationship between the parameters and their quantitative importance. Second, the variable instrument settings are critical to reproduction of an experiment (van Loon 2007; Borst and van Loon 2008).

Therefore, we propose that the minimal reportable criteria for random positioning machine experiments should be the same as the criteria for clinorotation experiments (Table 1) with the addition of the variable instrument settings, specifically angular velocity of rotation and operating mode (Table 2).

For some investigators, determination of densities and viscosity will be burdensome. In this case, sufficient details of the media, cells and other reagents should be included for others to make the determinations when they will be of utility.

We propose a minimum set of parameters to be reported for clinorotation or random positioning machine experiments. As this proposal was made at the European Low Gravity Research Association meetings in Bonn, we propose that the standards be known as the Bonn criteria.

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