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Mathematical modeling predicts physical parameters which are pertinently affecting the 2D- and 3D- culture microenvironments

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Abstract
The 2-D disc and 3-D RWV cultures showed quite a big discrepancy in a diversity of biological responses. The reasons are still poorly understood. We hypothesize that such discrepancy could arise from the genotypic variance and the microenvironment interventions involving the physical, chemical, and biological parameters. Theoretical elucidation showed 3D-cultures exhibit larger total surface area (>78.54×10^{-6} cm^2), larger effective surface area available for mass transport (>52.36×10^{-6} cm^2 per cell), and larger contact surface area for anchorage (>26.18×10^{-6} cm^2). The 3-D and not the 2-D culture always faces a rotating force 3.107×10^{-9} N/cell and a weaker gravitational force, ≤ 6.77×10^{-7}N/cell. As contrast, the 2-D cultured cells uniquely encounter a static hydraulic pressure 37.51 N/cm^2. Kinetic analysis indicated 2-D culture mostly is accompanied with diffusion control over the substrate, contrasting with the kinetic control of 3-D cultures. For note, in principle severe cytotoxicity or mutagenicity only can occur at higher level toxicant under a kinetic control regime.

Keywords: Microenvironment; 3-D culture; rotational force; gravitational force; static hydraulic force; effective surface area.

Abbreviations: A: the substrate chemical; B: the hypothesized overall metabolic intermediate; D: the diffusion coefficient; $\vec{F}/V = -\vec{\nabla}\Pi$; $\vec{F}_d$: the drag force the drag force associated with fluid resistance in floating medium; $\vec{F}_g$: the gravitational force; $\vec{F}_{hd}$: the total static hydraulic force exerted on the cell; $\vec{F}_x$: the force vector in the direction of axis x; $\vec{F}_y$: the force vector in the direction of axis y; $\vec{F}_z$: the force vector in the direction of axis z; $\sum\vec{F_S}$: the force vector sum; $\vec{F}_{rot}$: the rotational force; $\vec{P}_{hd}$: the static hydraulic pressure exerted on the cell surface; $\mathcal{R}_{app}$: the apparent intracellular reaction rate; S: the surface area contributing to mass transport; S_{TSA}: = total surface area of a single cell; S_{ESA} = the effective surface area of a single cell available for mass transport; S_{ECSA}= the effective contact surface area of a single cell to the solid disc surface; *V*: the volume of a cluster of cells in the medium; V_c: the specific volume of a single cell; X: the distance from the particle (cell) centre mass to the rotational axis of 3-D RWV; *g*: gravity acceleration constant; f_{conv}: the fraction conversion; $f_{conv,max}$: the maximum fraction conversion; $f_{m,bm}$: the diffusion flux from the bulk to the microenvironment surface; k_{metab}: the overall metabolic rate coefficient for digestion of substrate chemical 'A'; *m*: the mass of a cell; q: the conversion factor; r: the radius of microenvironment surrounding the cell; r_c: the radius of a cell; Π: the scalar function that describes the pressure at all locations in space; η: the viscosity of the medium; π: the circumference ratio; θ : the effectiveness factor; ρ_w: the SI unit of the medium density; υ: the rotational speed; ω_{cyc}: the rotational speed in cycles per second; ω_{rad}: the angular speed in radians per second.

1. Introduction

Conventional adherent tissue culture involves growing cells on solid flat surfaces as two-dimensional (2-D) monolayers. Although such practices are routine and suitable for transformed or immortalized cell lines, de-differentiation and loss of specialized functions

occurs when primary cells are removed from their host tissue and grown as 2-D monolayers. This is generally believed to be a result of the dissociation of primary cells from their native three-dimensional (3-D) structure *in vivo* to their 2-D propagation on flat impermeable substrates *in vitro* [1]. As such, there is a continuing need to develop tissue culture systems which can either promote re-differentiation of laboratory cell lines or prevent primary cell lines from de-differentiating. The NASA-engineered rotating wall vessel (RWV) tissue culture bioreactor system, manufactured by Synthecon (Houston, TX), addresses this need [1] (Fig. 1).

A wide variety of observations on cells in space, admittedly made under constraining and unnatural conditions in many cases, have led to experimental results that were surprising or unexpected.

The RWV is a horizontally rotating cylindrical culture vessel, which offers an optimized suspension cell culture. RWV has many advantages: largely reducing shear and turbulence generated by conventional stirred bioreactors, minimizing mechanical cell damage. In RWV cells are cultured in “suspended animation” where they are continuously free-falling, promoting the assembly of 3-D cellular aggregates which allows for more efficient cell-to-cell interactions and exchange of growth factors [1].

Gathered data have indicated that the 3-D cultured cells behave differently from the 2-D cultured (The Scientist-Magazine of the Life Sciences. The Spin on Rotary Culture [2, 3] (Fig. 1).

Rhee demonstrated that nonrandom genetic and phenotypic changes in prostate epithelial cells can occur through an event that resembles “adaptive mutation” such as has been described in bacteria subjected to nutritional starvation. The occurrence of such permanent changes may be highly contact-dependent, and appears to be driven by specific microenvironmental factors surrounding tumor cell epithelium grown as 3D-prostate organoids (Fig. 1) [4]. Cancer-stromal interaction results in the co-evolution of both the cancer cells and the surrounding host stromal cells. As a consequence of this interaction, cancer cells acquire increased potential and stromal cells became more inductive [5]. Tumor microenvironment promotes cancer progression, metastasis, and therapeutic resistance [6]. The reason(s) eliciting different biological outcomes by different microenvironments is still unclear. To interpret such discrepancy, we performed both theoretical and the experimental approaches. A tremendous parameters including the physical, the chemical, and the biological that probably can discriminate the differential chemico-biological interactions were evaluated and presented.

2. Materials and methods

2.1. Theoreticals

Any single cell (synonymously referred here as a tiny mass particle) in a culture system may encounter a certain microenvironments (Fig. 2, Fig. 3A, Fig. 3B). This microenvironment can be created by a diversity of different stresses exerted by the physical, chemical, and biological factors depending on the cultivation condition. Herein the microenvironments created by the 2-D disk culture and the 3-D rotary cell culture system (RCCS), i.e. the rotary wall vessel (RWV) system will be compared. In principal, the stability function of a microenvironment ($\check{S}_{ME}$) can be mathematically formulated as

$$\check{S}_{ME} = \xi(\text{Physical})\bullet \Phi(\text{Chemical})\bullet\Psi(\text{Biological}) \quad \ldots\ldots 1$$

Exclusive of the pH values, the incubation temperature, and the ionic strength, that are usually held constant during incubation of cells, the major physical variables in the term ξ(Physical) involves the total surface area, the effective surface area available for mass transport, and the effective contact surface area of a cell for attachment onto the disc surface, the force vector sum, the static hydraulic pressure, and the gravitational force. The second term Φ(Chemical) mainly deals with the chemical kinetics and the mass transport associated with the externally supplied nutrients and medicine. While the third term Ψ(Biological) considers the cell viability, growth, and proliferation, and further the overall toxicity as a consequence of chemical-cell interactions. Eq. 1 can be approached from the following sub-functions.

2.1.1 The Function ξ(Physical)

The function *ξ(Physical)* in reality is contributed by the following phenomena.

2.1.1.1. The surface area vs. the effective surface area

Assume a cell to be spherical in shape having a radius r, its total surface area (S_{TSA}) is

$$S_{TSA} = 4\pi r^2 \quad \ldots\ldots 2$$

Considering a single cell to be cultured in a common 2-D culture, most of the cells will lose one dimension when become adhered onto the disc bottom (Fig. 3A). Consequently, the effective surface area (S_{ESA}) of a cell available for the mass transport will be reduced, supposedly to 2/3 of S_{TSA}, which is

$$S_{ESA} = (2/3)S_{TSA} \quad \ldots\ldots 3$$

Substituting Eq. 2 into Eq. 3 leads to

$$S_{ESA} = (8/3)\pi r^2 \quad \ldots\ldots 4$$

2.1.1.2. The static force vector sum vs. the centrifugal force

The force field accessible by a cell in 2-D culture is simply the vector sum:

$$\sum \overrightarrow{Fs} = \overrightarrow{Fx} + \overrightarrow{Fy} + \overrightarrow{Fz} \quad \ldots\ldots 5$$

According to Newton’s law, the vector sum $\sum \overrightarrow{Fs} = 0$ for a 2-D culture (Fig. 3A). However, in the RSCC 3-D culture (Fig. 1, Fig. 3B), there exists constantly rotating force $\overrightarrow{F_{rot}}$, therefore $\sum \overrightarrow{Fs} \neq 0$, and

$$\sum \overrightarrow{Fs} = \overrightarrow{F_{rot}} = m\,\vec{\omega}_{rad}^{\,2}\,X \quad \ldots\ldots 6$$

The inter-conversion of angular speed to rotational speed can be accomplished by

$$\omega_{rad} = 2\pi\,\omega_{cyc} \quad \ldots\ldots 7$$

where ω_{rad} is angular speed in radians per second; and ω_{cyc} is rotational speed in cycles per second.

This rotating force in the 3-D culture creates a multifunctional damping change in hydraulic pressure surrounding this cell (Fig. 1, Fig. 3B). Consequently, this cell will consistently feel a "Floating motion", which definitely can not be perceived in the 2-D culture (Fig. 3A).

2.1.1.3. The static hydraulic pressure exerted on a cell in 2-D culture

The static hydraulic pressure is expressed as

$\vec{P}_{hd} = \vec{F}_{hd} / S_{ESA}$ 8

where $\vec{F}_{hd}$ is the total static hydraulic force exerted on the cell, $\vec{P}_{hd}$ is the static hydraulic pressure exerted on the cell surface, and S_{ESA} is the effective cell surface area exposed to the medium fluid (Fig. 3A).

$\vec{F}_{hd}$ = (9.8 N/kg) × ($h\rho_w \times 10^{-3}$ kg) 9

Where h is the height of medium liquid surface level in perpendicular to the cell surface, and in 2-D-culture with plates the value of h can be approximated to be 2 mm (0.2 cm). ρ_w is the density of the medium fluid (here supposed ρ_w =1.002 gcm^{-3}). Thus, substitution of these parameters to Eq. 8 leads to

$\vec{P}_{hd}$ =(9.8 N/kg) × ($0.2 \times 1.002 \times 10^{-3}$ kg)/S_{ESA} 10

In contrast to conventional static cell culture systems (e.g. flat culture flasks and dishes), cells grown in the RWV are cultured in "suspended animation" where they are continuously free-falling and overtime would not encounter a net static hydraulic pressure.

2.1.1.4. Gravitational force

The gravitational force exerted on a single cell having a mass *m* in the 2-D culture is simply

$\vec{F}_g = mg$ 11

which is entirely different from the 3-D RWV system (Fig. 2, Fig. 3B). When a cell having a specific volume of V_c is suspended in a medium with a density of ρ_w, the net gravitational force exerted on this cell becomes [7]

$\vec{F}_g = mg - V_c\rho_w mg = (1 - V_c\rho_w)mg$ 12

In the floating state, the particle will reach a limiting speed υ when this force is equal to the frictional force $\vec{F}_f$, which is equal to the frictional coefficient μ multiplied by the speed υ [7]:

$\vec{F}_f = \mu\upsilon = (1 - V_c\rho_w)mg$ 13

Expectedly, a particle like a cell having mass m, when suspended in a rotational medium in 3-D culture will 'feel' a weaker gravity force than a cell in 2D- culture, which is actually the magnitude $(1 - V_c\rho_w)mg$

If Stokes' law applies to the particle (cell), $\vec{F}_f$ is given as

$6\pi r_c \eta \upsilon = (1 - V_c\rho_w)mg$ 14

Where π is the circumference ratio, r_c is the radius of the cell, η is the viscosity of the medium suspending the cell, υ is the rotational speed. The limiting speed to maintain a particle in a balanced floating state without sedimentation would require [7]

$\upsilon = (1 - V_c\rho)\, mg / 6\pi r\eta$ 15

2.1.1.5. Continuum mechanics of the aggregate of a cluster of particles

Newton's laws and Newtonian mechanics in general were first developed to describe how forces affect idealized point particles rather than three-dimensional objects. However, in real life, matter has extended structure and forces that act on one part of an object might affect other parts of an object. For instance, a tiny piece of tissue (or a cell sheet under culture) holding together the cells in an object is able to flow, contract, expand, or otherwise change shape, the theories of continuum mechanics describe the way forces affect the materials including the solid and rigid, the pseudoplastic, and the viscous, provided that the relevant parameters characteristically feasible for each specific material are appropriately incorporated in Eq. 12. Macroscopically, when the drag force (F_d) associated with fluid resistance becomes equal in magnitude to the force of gravity on a falling object (F_g), the object reaches a state of dynamical equilibrium at terminal velocity (Fig. 2). For example, for the case of RWV, the cells in extended fluids differences in pressure result in forces being directed along the pressure gradients as follows [8]:

$\vec{F} / V = -\vec{\nabla}\Pi$ 16

where *V* is the volume of the cluster of cells in the fluid and Π is the scalar function that describes the pressure at all locations in space. Pressure gradients and differentials result in the buoyant force for fluids suspended in gravitational fields and the lift associated with the floating medium. A specific instance of such a force that is associated with dynamic pressure is fluid resistance: a body force that resists the motion of an object through a fluid due to viscosity. For so-called "Stokes' drag" the force (or the centrifugal field of force) is approximately proportional to the rotational angular velocity ω_{rad} but opposite in direction [8]:

$\vec{F}_d = - qm\vec{\omega}_{rad}^2 X$ 17

where: q is a constant that depends on the properties of the fluid and the dimensions of the object (usually the cross-sectional area). $\vec{\omega}_{rad}$ is the rotational angular velocity of the object (Fig. 2), and X is the distance from the center of rotation. Eq. 17 is actually an extension of Eq. 13. If the dimension of particle is small enough and the floating medium is water, q =1.0, i.e.

$\vec{F}_d = - m\vec{\omega}_{rad}^2 X$ 18

Substitution of Eq. 18 into Eq. 15 gives the centrifugal or rotational velocity

$\upsilon = (1-V_c\rho)\, m\, \vec{\omega}_{rad}^{2}\, X / 6\pi r\eta$ .....………………….....……19

2.1.2. The Function Φ(Chemical)

The function *Φ*(*Chemical*) in fact depends on the variation of following phenomena.

2.1.2.1. Kinetic Analysis

Taking the oxygen and CO_2 supplies to be constant, both the 2-D- and the 3-D- cultures can be treated as "pseudobatch" in view of kinetics. Most of the biochemical reactions initially are only simple linear sequential pathways. The intermediates (herein denoted as 'B') produced then diverge to net work metabolic pathways. To simplify the argument, the classical first order consecutive linear reactions will be considered:

$$A \xrightarrow{k1} B \xrightarrow{K2} C \quad \text{...20}$$

where B represents the key metabolic intermediate. The consumption rate of substrate chemical 'A' is:

$-dA/dt = k_1 A$ ……..……………………………………..21

The overall metabolic rate is simplified as

$\mathcal{R}_{metab} = dB/dt = k_1A - k_2B$ ……………….…………......22

C is determined by material balance.
In terms of time t since

$A/A_0 = \exp(-k_1 t)$ ……………………………….….………...23
and

$\mathcal{R}_{metab} = dB/dt = k_1A_o \exp(-k_1t) - k_2B$ ………………………24

This linear, first-order differential equation is of the form

$dy/dx + Py = Q$ …………………………….....…………25
the general solution being [9]

$$y = \exp(\int Pdx)[\int Q\exp(\int Pdx) + const] \quad \text{………26}$$

Therefore for $B = B_o$ at $t = 0$, the fractional conversion is

$f_{conv} = B/A_o$
$= (k_1/(k_2-k_1)[\exp(-k_1t) - \exp(-k_2t)] + B_o/A_o \exp(-k_2t)$…..27

The time at which the maximum in B occurs is obtained by differentiation of Eq. 27; for $B_o = 0$

$t_{max} = \ln(k_2/k_1)/(k_2 - k_1)$ ………………………….……28

while the maximum fractional conversion is

$f_{conv,max} = B_{max}/A_o$
$= (k_1/k_2)^{k2/(k2-k1)}$………...29

Predictably, different initial concentration of A_o can elicit different values of maximum fractional conversion or B_{max}. Obviously, the subsequent biochemical steps (i.e. C in Eq. 20 and others to follow) can be greatly affected as well. Worth noting, should the intracellular concentration of A and B_{max} are extremely high, e.g. far exceeding the mutagenic dose, the cell phenotype or genotype can be significantly altered [4].

2.1.2.2. Mass Transport

2.1.2.2.1. The Mass Transport in 2-D Cultures

In an isothermal reaction system, the reaction rate decreases with advancement of reaction. The phenomenon, called gel effect, is due to a change in properties of the reaction medium and to the fact that one of the steps in the chain becomes diffusion controlled [9].

Supposing that the interaction between a medicinal component A and the cell can be considered as a 'pseudohomogeneous step', this type of reaction can become diffusion-controlled at least in an elementary fashion when not reacting rigorously. In a 2-D culture, the cell (B), assumed to be spherical in size, is surrounded by molecules 'A' segregated by a microenvironment having a radius r from the bulk fluid with a concentration of A_o (Fig. 3A). The diffusion flux from the bulk to the microenvironment surface is

$f_{m,bm} = DS \cdot d[A]/dr$ …………………….………….........30

where $f_{m,bm}$ is the diffusion flux, D is the diffusivity, S is the surface area of the spherical microenvironment. Integration with the boundary conditions between A_o and A_{cex}, and r and r_c (Fig. 3A) neglecting the radius of molecule A leads to

$$f_{m,bm}\int_{r}^{rc} dr = DS \cdot \int_{A_o}^{A_{cex}} dA \quad \text{.......31}$$
or

$f_{m,bm}(r - r_c) = 4\pi r^2 D(A_o - A_{cex})$..32

and the concentration of 'A' at surface of extracellular membrane:

$A_{cex} = [4\pi r^2 DA_o - f_{m,bm}(r-r_c)]/4\pi r^2 D$ 33

Assume the intracellular metabolic rate of 'A' in cell 'B' obeying the second order reaction, we have:
$\mathcal{R}_{app} = k_{metab}[A][B]$ ……………………………………...34

here $\mathcal{R}_{app}$ is the apparent second order reaction rate, k_{metab} is the second order metabolic kinetic coefficient. When this intracellular reaction is undergoing very fast, the concentration of molecules 'A' at the surface of 'B' (cell) becomes depleted, i.e. a condition $A_{cex} << A_o$ is reached. Under such a condition, a diffusion flux sets in to replenish the molecule 'A' that is consumed in the reaction. Hence the flux must be equal to the reaction rate:
$4\pi r_c^2 DA_o = k_{metab}[A][B]$ ……….....……........…………....35

In reality, the cell is adhered to the disc bottom in the 2-D culture (Fig. 3A, Fig. 4A), the effective surface area will be greatly reduced, supposedly to 2/3 of the total area (S_{TSA}), the transport equation is further modified as
$8/3\pi r_c^2 DA_o = k_{metab}[A][B]$ …………………………........36

Recognizing that the single cell 'B' is fixed at the center of the spherical microenvironment having a radius r, and the reaction being fast enough to deplete A around the cell, the limiting reaction rate becomes

$8/3\pi r_c^2 DA_o = k'_{metab}[A]$ 37

where k'_{metab} is the pseudo-first order metabolic kinetic coefficient.
Assume $\mathcal{R}$ to be the actual rate of reaction taking place when 'A' gets inside the cell 'B':

$\mathcal{R} = k'_{metab}[A_{cex}]$..38

Comparing with the condition without the microenvironment resistance, the rate would have attained a maximum rate, i.e.

$\mathcal{R}'_{max} = k'_{metab}[A]_o$ 39

The ratio of the actual to the maximum rate is generally termed "the effectiveness factor θ " which measures the diffusion limitation:

$\theta = \mathcal{R}/\mathcal{R}'_{max} = [A_{cex}]/[A]_o$..40

Eliminating $[A_{cex}]$ and $[A]_o$ between Eqs. 38-40 we get:

$\theta = 1/[1+\{k'_{metab} /(8/3)D\pi r_c \}$ 41

By definition, the value of θ ranges from 0.0 to 1.0. The actual reaction rate hence is

$\mathcal{R} = \theta\, k'_{metab}[A]_o$..42

3. Results and Discussion

The data obtained either from the theoretical calculation are listed in Tables 1-3. Table 1 gives the parameters dealing with the function physical, ξ(Physical); Table 2, with the function chemical, Φ(Chemical); and Table 3, with the function biological, Ψ(Biological).

3.1. The total surface area vs. the effective surface area

The total surface area, the effective surface area, and the effective contact surface area were 78.54×10^{-6} cm^2, 52.36×10^{-6} cm^2, and 26.18×10^{-6} cm^2, respectively (Table 1). Every living organism is made up of tiny cells. The average human cell is about 50 micrometers or 0.05 mm (5×10^{-3}cm; or r = 2.5×10^{-3}cm) [13]. Assume a cell to be spherical in shape, its total surface area (S_{TSA}) is

$S_{TSA} = 4\pi r^2 = 78.54\times10^{-6}cm^2$..43

The attachment to the disc bottom would lose some surface area. Assume the effective cell surface area (S_{ESA}) available for mass transport to be reduced to approximately 2/3 of S_{TSA}, which is.

$S_{ESA} = (2/3)S_{TSA} = 52.36\times10^{-6}cm^2$ 44

Consequently, we have the effective contact surface area (S_{ECSA}, for cell contact to the solid disc surface) is 26.18×10^{-6}cm^2 (Table 1). Cells from many different tissues sense the stiffness and spatial patterning of their microenvironment to modulate their shape and cortical stiffness [14]. Dish surface adhesivity determines the cell shape [15]. Cell cortical stiffness increases as a function of both substrate stiffness and spread area. As can be seen, the effective surface area for contact with the solid disc is larger in the 3-D culture (26.18×10^{-6}cm^2 in the 2-D vs. >26.18×10^{-6}cm^2 in the 3-D cultures) due to the larger cellular size in the latter (Table 1). The cells in 3-D culture may commit higher membrane-stretch and cellular spread.
Cell surface contact and cell shape give rise to distinctly different regulatory responses [15]. Dish surface adhesivity determines the cell shape. DNA and rRNA synthesis and mRNA production are profoundly, but protein biosynthesis is not, affected by cell shape [15]. As mentioned, contact of a limited portion of the plasma membrane with the solid culture dish surface is a sufficient signal by itself [15].
For soft substrates, the influence of substrate stiffness on cell cortical stiffness is more prominent than that of cell shape, since increasing adherent area does not lead to cell stiffening [14]. On the other hand, for cells constrained to a small area, cell shape effects are more dominant than substrate stiffness, since increasing substrate stiffness no longer affects cell stiffness [14]. Thus cell size and substrate stiffness can interact in a complex fashion to either enhance or antagonize each other's effect on cell morphology and mechanics [14].

3.2. The force vector sum

The force vector sum for 2-D culture at a static state simply is $\sum \overrightarrow{Fs} = \overrightarrow{Fx} + \overrightarrow{Fy} + \overrightarrow{Fz} = 0$ (Table 1). While in the 3-D RWV, the cell is constantly encountering a rotational force $m\omega_{rad}^2 X$ (Eq. 6), the 3-D culture operated at 45 rpm yields an angular velocity

$\omega_{rad} = 2\pi(45/60) = 1.5\ rad\ s^{-1}$ 45

Suppose a cell in the RWV transiently may be localized at a point with 2 cm apart from the central axis of rotation, the rotational force is

$\overrightarrow{F_{rot}} = m(1.5\ rad\ s^{-1})^2(2\times10^{-2})$ 46

Given the cell density ρ_{cell} =1.055g/cm^3 and the average human cell size ≈50 micrometers or 0.05 mm (5×10^{-3}cm; or r = 2.5×10^{-3}cm) [13], we have the mass per cell

$m = (4/3)\ \pi\ (2.5\times10^{-3}cm)^3 \cdot 1.055g/cm^3$
$= 6.905\times10^{-8}$ kg/cell 47

Substitution of Eq. 47 into Eq.46 yields the rotational force encountered per cell (Table 1):

$\overrightarrow{F_{rot}} = (6.905\times10^{-8}\ kg/cell)\ (1.5\ rad\ s^{-1})^2(2\times10^{-2})$
$= 3.107\times10^{-9}$ N/cell 48

Recent studies suggest that physical forces influence signal transduction, gene expression, secretory function, cell differentiation and proliferation [16]. Transmitters released from acinar cells may modulate the secretory activity of salivary tissue, and interact with classical regulatory

pathways [16]. A study to overview membrane stretch-activated cellular events evidenced from a variety of tissues suggests that mechanical forces may alter the properties of acinar cells leading to cytoskeletal reorganisation, changes in ion fluxes, modulation of secretory activity and subsequent release of transmitters such as ATP [16].

3.3. The static hydraulic pressure exerted on the 2-D- and the 3-D cultures

In 2-D culture, the cells are usually submerged in a medium having a height h (assume h = 0.2 cm; in the direction of axis z over the cell) (Fig. 3A) and a density ρ (assume to be 1.002 g/cm^3). Presuming that the hydraulic principle is applicable to cells, the cells would 'feel' a static hydraulic force:

$$\overrightarrow{F}_{hd} = (9.8\ \text{N/kg}) \times (0.2\times\ 1.002\ \times 10^{-3}\ \text{kg}) = 1.964\times 10^{-3}\ \text{N} \quad (49)$$

And a static hydraulic pressure

$$\overrightarrow{P}_{hd} = \overrightarrow{F}_{hd}/S_{ESA} = 1.964\times 10^{-3}\ \text{N}/52.36\times 10^{-6}\text{cm}^2 = 37.51\ \text{N/cm}^2 \quad (50)$$

Thus a single cell in the 2-D culture system will 'feel' a total static hydraulic force approximately 1.964×10^{-3} N (per cell) or a static hydraulic pressure 37.51 N/cm^2 (per cell) (Fig. 3A; Table 1). Obviously, such a phenomenon is unperceivable in the 3-D RWV culture (Fig. 3B).

3.4. The gravitational force

As mentioned in the above, a cell having a mass *m* in 2-D culture will 'feel' a gravitational force (Fig. 2):

$$\overrightarrow{F}_g = mg = (6.905\times 10^{-8}\ \text{kg/cell})\ (9.8\ \text{ms}^{-2}) = 6.77\times 10^{-7}\ \text{N/cell} \quad (51)$$

In contrast, this same cell if cultured in the 3-D RCCS will 'feel' less gravitational force (Eq. 12):

$$\overrightarrow{F}_g = (6.77\times 10^{-7}\text{N/cell})[1-[(4/3)\pi(2.5\times 10^{-3}\text{cm})^3 \cdot 1.002\text{g/cm}^3] \quad (52)$$

$$= (6.77\times 10^{-7}\text{N/cell})[1-6.558\times 10^{-8}] \le 6.77\times 10^{-7}\text{N/cell} \quad (53)$$

Apparently, the gravitational force faced by a cell having a volume V_c = 6.545×10^{-8} cm^3 in 3-D culture suspended in a medium having a density ρ_w = 1.002 g/cm^3 merely is slightly weaker than the gravitational force normally faced by a cell in 2-D culture, i.e. ≤ 6.77×10^{-7}N/cell (Eq. 53) (Table 1). This cell will be suspended and rotated in RWV and will be more affected by the influence of just a tiny rotating force 3.107×10^{-9} N/cell (Eq. 48) (Table 1).

Gravity directly and indirectly affects the smallest functional units (e.g a single cell) where the primary receptor, positioned inside and outside of such a unit (cell), interacts with gravity [17].

Gravity seems to be positively correlated with cytochrome oxidase activity in the magnocellular nucleus of developing fish brain. In the inner ear the energy metabolism is decreased under microgravity concerning utricle but not saccule. Hypergravity has no effect on cytochrome oxidase activity in sensory inner ear epithelia [18].

Reception of physical environmental signals caused by alteration of the gravitation field leads to the shift of morpho-physiological cell characteristics. The gravity influence on a cell may be direct or non direct, its extent varying in dependence of the cell model applied [19]. Gravity affects morphogenesis processes, such as locomotion, adhesion, intercellular contacts, etc. At the same time, all the processes named are under control of cell integral systems of the signal transduction. Minor disturbances in this system coming from the environment, due to amplification, may provide significant modulations of the signals [19].

Several experiments in space show that mitogenic T cell activation is lost at null g (i.e. 0 g). Immunocytochemistry indicates that such effect is associated with changes of the cytoskeleton. The lack of expression of the interleukin-2 receptor is one of the major causes of the loss of activity [20]. In addition, the acceleration stimulus induces changes in the level of second messenger, adenosine 3',5'-cyclic monophosphate (cAMP) [21].

3.5. The diffusion control vs. the kinetic control reaction regimes

To obtain the maximum effectiveness factor, i.e. θ_{max}=1.0, the condition in Eq. 42 requires $(8/3)D\pi r_c >> k'_{metab}$. Eq. 42 states that when the reaction becomes diffusion-controlled (Fig. 4A), the condition $k'_{metab} >> (8/3)D\pi r_c$ must be fulfilled (Table 2). On the contrary, in the absence of microenvironment transport resistance, the condition $k'_{metab} << (8/3)D\pi r_c$ must be satisfied, which actually is a kinetic control regime (Fig. 4B, Table 2), evidently implicating a large discrepancy of microenvironmental circumstance between the 2-D and the 3-D cultures.

Previously we have demonstrated that phytochemicals exhibit antitumor bioactivity by suppressing cell growth and proliferation, and occasionally, cell shape can be distorted with colony segregated, e.g. the human urinary bladder cancer cell line, RT4 (a well-differentiated papillary tumor of the bladder having wt *p53* and *Rb* gene) when treated with the ethanolic extract of *Antrodia camphorata* [12]. Worth mention, the outcome of chemico-biological interaction depends not only on the microenvironment, but also on the cell genotypes.

Furthermore, as seen in tables 1-3, the two culture systems (2-D and 3-D) may differ from each other in many aspects: physically, chemically and biologically, eliciting tremendous discrepancies between the 2-D and the 3-D cultures. The 3-D RWV cultures yield larger cells with larger total- and effective surface area to facilitate the mass transport. The force vector sum in 3-D culture gives constantly rotating motion but don't 'feel' any static hydraulic pressure. Instead, the 2-D culture would encounter approximately a total static hydraulic force 1.964×10^{-3} N/cell, or a static hydraulic pressure 37.51 N/cm^2. Conversely, the 3-D culture will face less amount of gravitational force, which is ≤ 6.77×10^{-7}N/cell, comparing to 6.77×10^{-7}N/cell of 2-D cultures (Table 1). In view of chemical kinetics, given the 2-D and 3-D culture systems a same level of substrate chemical 'A' in the bulk fluid (A_o), the intracellular level of 'A' could reach approximately A_o in the 3-D culture due to the absence of microenvironment resistance (Fig, 4C and 4D), but could be relatively low in

the 2-D cultures due to the presence of microenvironment resistance (Fig. 4A and 4B) (Table 2). Most of the occasions, the cells in 2-D cultures will survive dependent on "diffusion control" only (Fig. 4A and 4C). Nonetheless, at a status when the accumulation of substrate chemical 'A' is in far excess over the capacity normally operating, cell mutagenicity or cytotoxicity ultimately may occur. At this point, the transport regime obeys "kinetic control", despite of 2-D or 3-D cultures (Fig. 4B and 4D) (Table 2). Conferring the biological response, the 3-D cultures could always show much easier cellular aggregation, more efficient cell-to-cell interactions and exchange of growth factors, and greatly promoted cell re-differentiation [1]. Interestingly, researchers recently have demonstrated organoid formation, co-evolution, cancer progression, metastasis, and therapeutic resistance are only perceivable in the 3-D cultures [4-6] (Table 3).

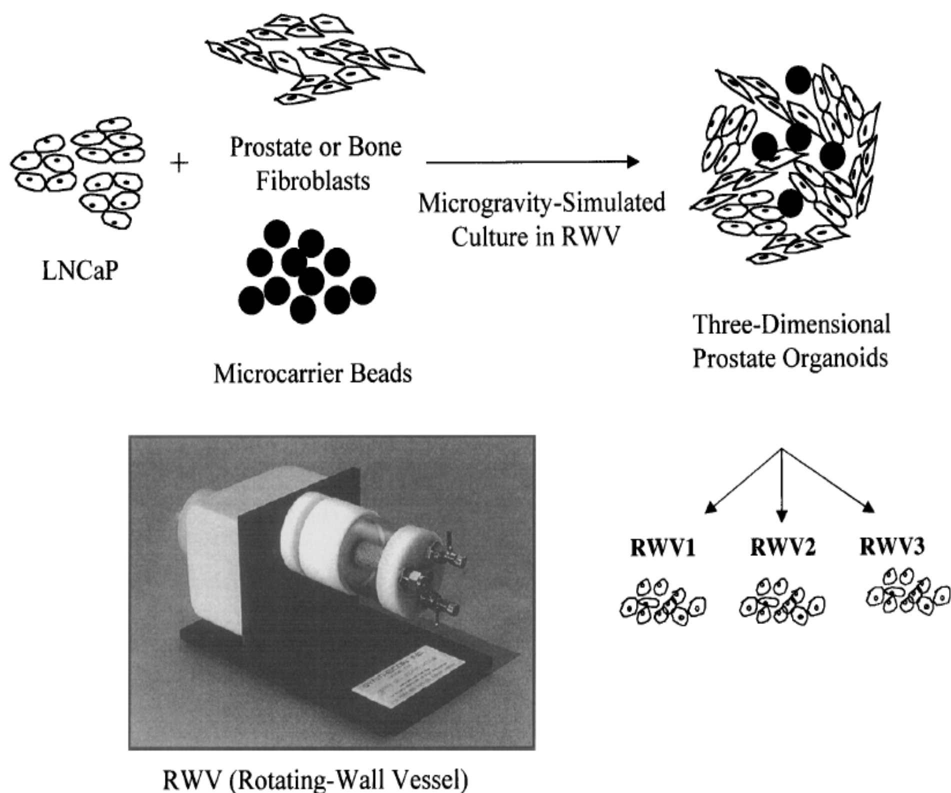

Fig 1: A scheme for producing RWV-1, -2, and -3 cells from the 3D prostate organoids cultured under simulated microgravity conditions with either microcarrier beads alone (RWV-1), or with prostate (RWV-2), or bone (RWV-3) fibroblasts. (depicted from Rhee et al., 2001).

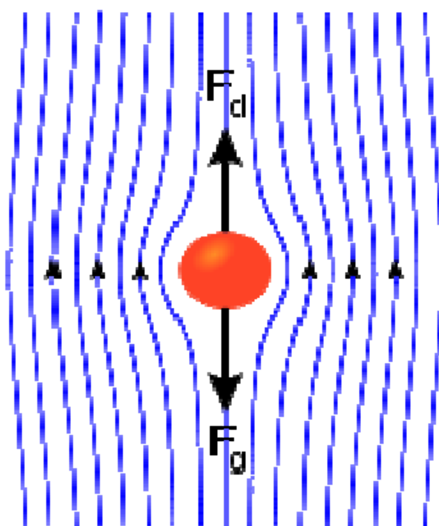

Fig 2: The motion of a particle in the rotating fluid balanced by the gravity force (F_g) and the Stokes' drag force (F_d). (depicted from Wikiapedia The Encyclopedia).

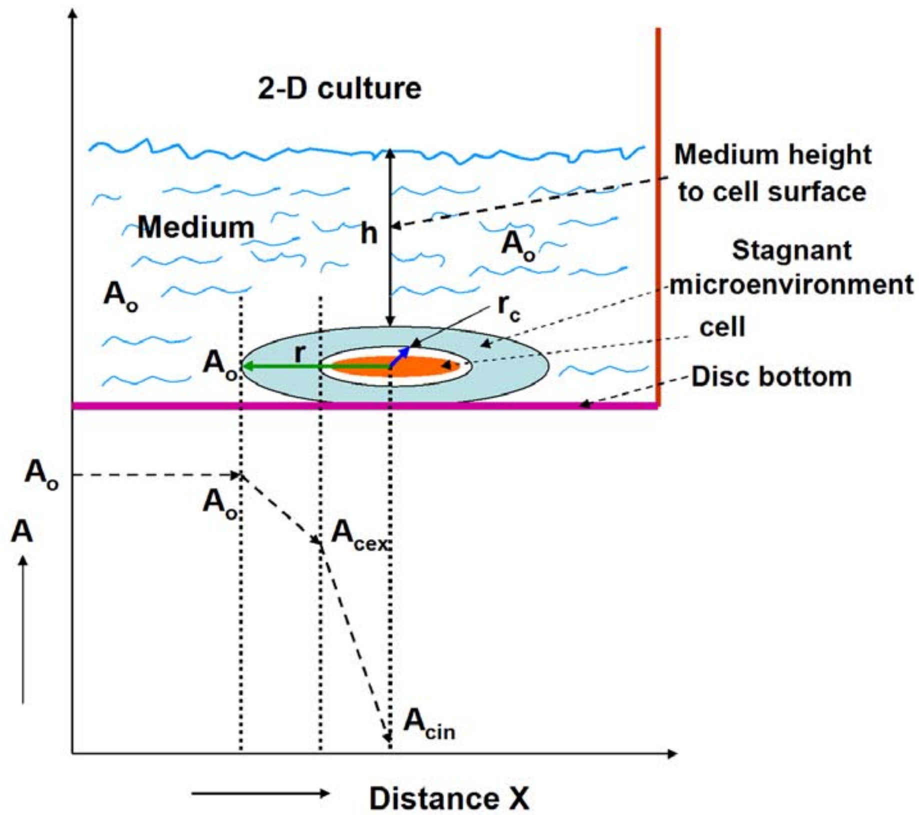

Fig 3A.

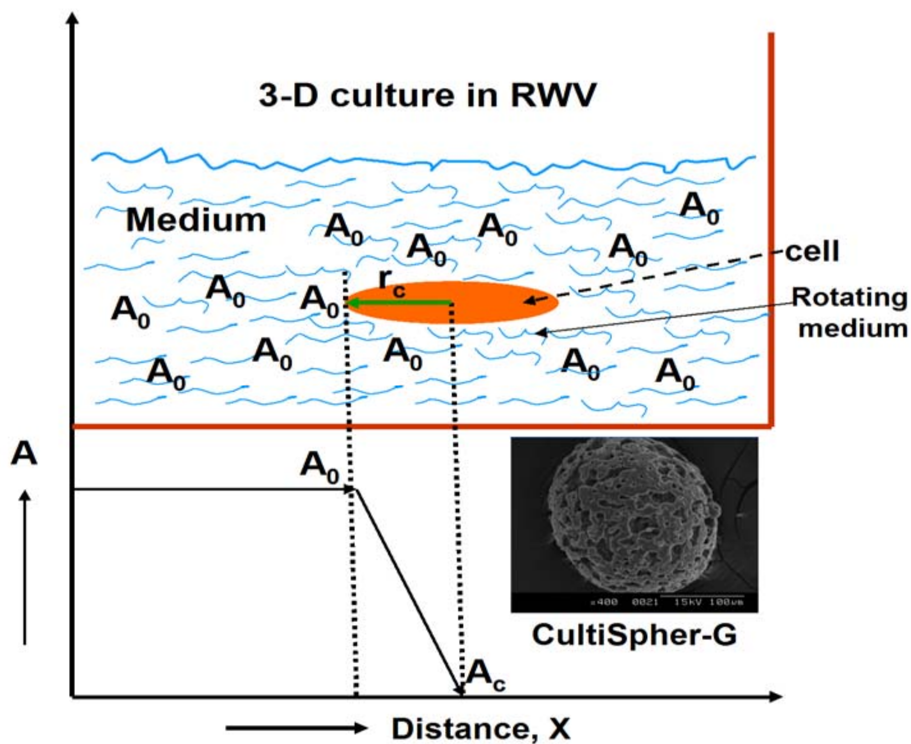

Fig 3B.

Fig 3: The diagrammatic model showing the mass transport phenomenon in a single cell. A) in 2-D culture model, and B) in 3-D model. The insert 'CultiSpher-G' at the lower right hand corner of Fig. 3B is the carrier particle onto which the cells became attached.

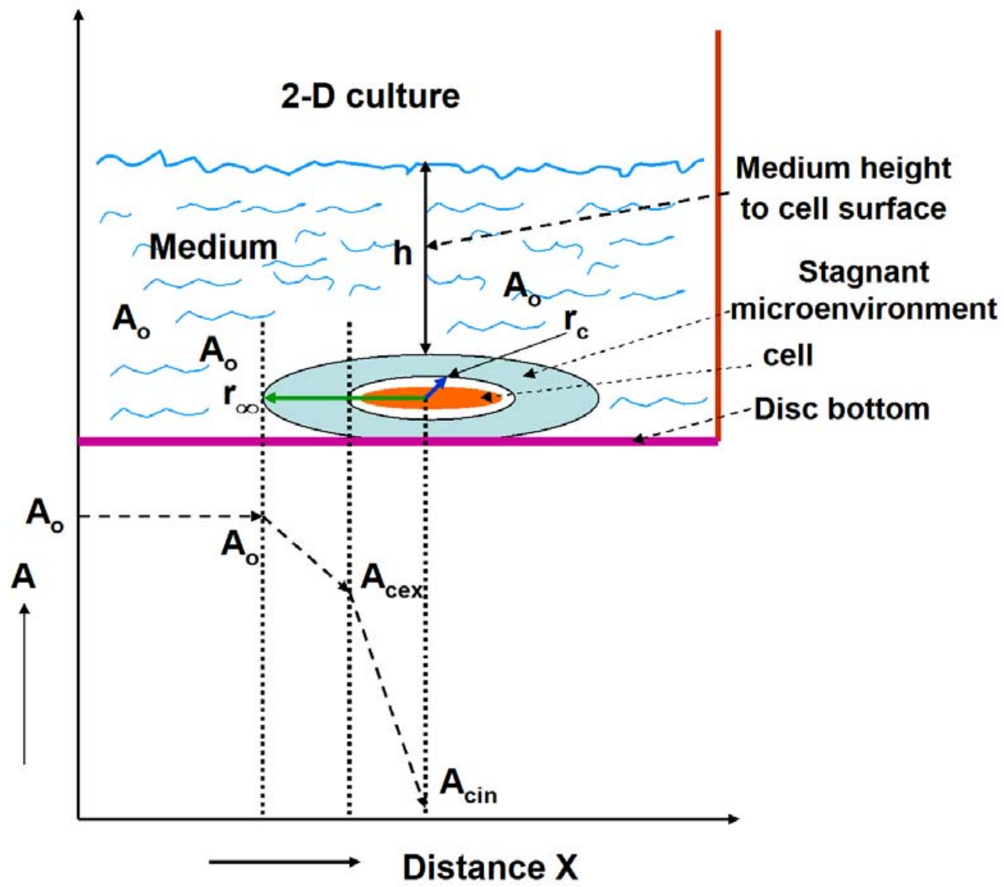

Fig 4A: 2-D diffusion control

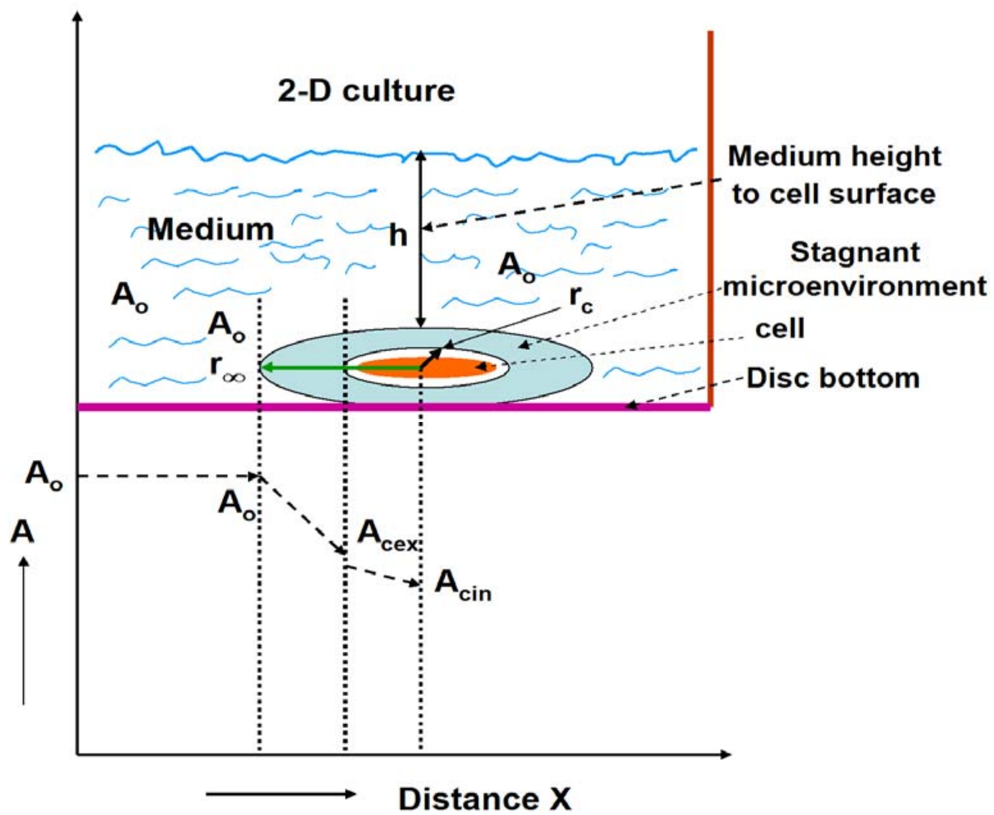

Fig 4B: 2-D kinetic control

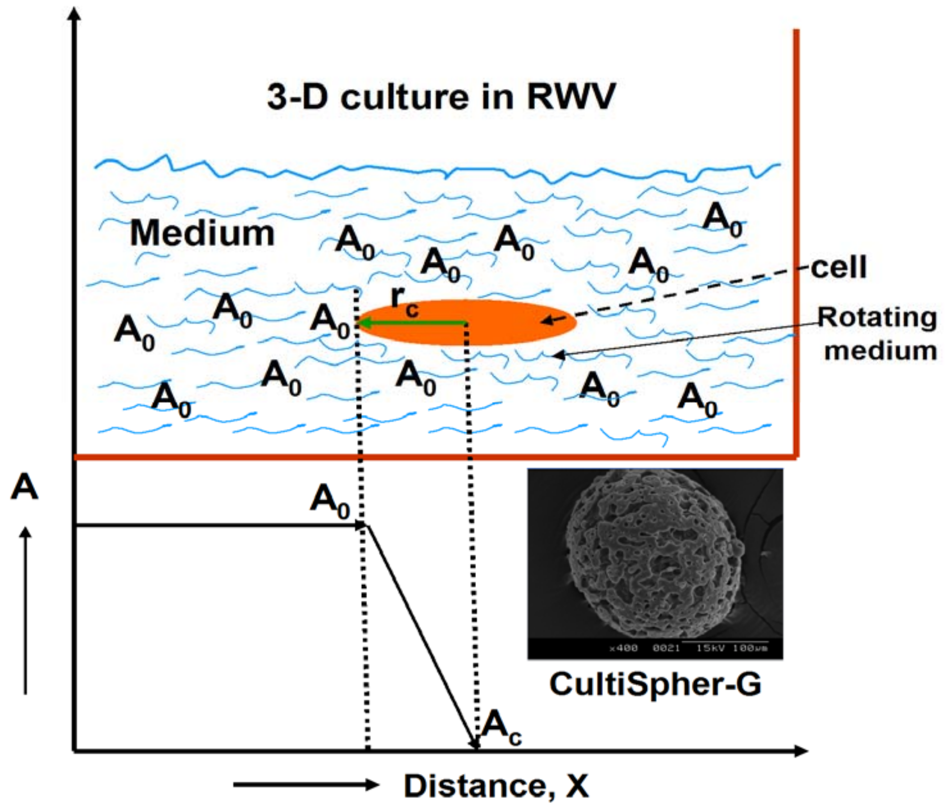

Fig 4C: 3-D diffusion control

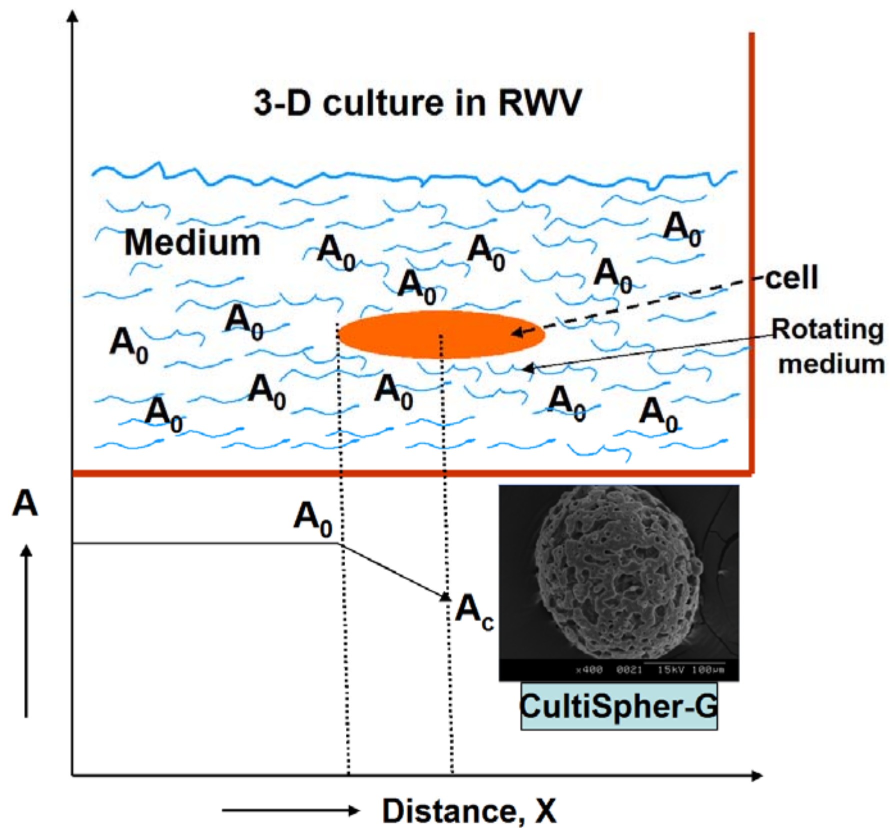

Fig 4D: 3-D kinetic control

Fig 4: The diffusion control and the kinetic control reaction patters occurring in different microenvironments elicited by 2-D and 3-D cultures. Diffusion control (A), and kinetic control in 2-D cultures (B). Diffusion control (C), and kinetic control (D) in 3-D cultures.

Table 1: Comparison of the microenvironments elicited by different culture conditions— Function Physical

Micro-environmental parameters	Culture system		Reference /data source
	2-D-disc	3-D-RWV	
ξ(Physical)			
total surface area/cell, S_{TSA}	$78.54\times10^{-6}cm^2$	$>78.54\times10^{-6}cm^2$	Eq. 43 in this paper
effective surface area/cell for transport, S_{ESA}	$52.36\times10^{-6}cm^2$	$>52.36\times10^{-6}cm^2$	Eq. 44 in this paper
effective surface contact area, S_{ESCA}	$26.18\times10^{-6}cm^2$	$>26.18\times10^{-6}cm^2$	Calculated from Eq. 44 in this paper
force vector sum, $\sum \vec{F}_s$	0	$\neq 0$	Section 3.2. in this paper
rotating force, $\vec{F}_{rot}$	0	3.107×10^{-9} N/cell	Eq. 48 in this paper
static hydraulic force, $\vec{F}_{hd}$	1.964×10^{-3} N (per cell)	≈ 0	Eq. 49 in this paper
static hydraulic pressure, $\vec{P}_{hd}$	37.51 N/cm^2	≈ 0	Eq. 50 in this paper
gravitational force, $\vec{F}_g$	6.77×10^{-7}N/cell	$\leq 6.77\times10^{-7}$N/cell	Eqs. 12 and 53 in this paper
physical forces	influence signal transduction, gene expression, secretory function, cell differentiation and proliferation	influence signal transduction, gene expression, secretory function, cell differentiation and proliferation	Zelles *et al*., 1999.

Table 2: Comparison of the microenvironments elicited by different culture conditions—Function Chemical

Micro-environmental parameters	Culture system		Reference /data source
	2-D-disc	3-D-RWV	
Φ(Chemical)			
microenvironment transport resistance	+++	none	Fig. 5A-5D
diffusion-control	$k'_{metab}>>(8/3)D\pi r_c$	$k'_{metab}>>(8/3)D\pi r_c$	Eq. 41 of this paper; Fig. 5A & 5C
kinetic control	$k'_{metab}<<(8/3)D\pi r_c$	$k'_{metab}<<(8/3)D\pi r_c$	Eq. 41 of this paper; Fig. 5B & 5D
intracellular substrate chemical level	low, $A_{cex} << A_o$	high, $\approx A_o$	Fig. 5A-5D
operation behavior under normal conditions	usually diffusion control: $k'_{metab}>>(8/3)D\pi r_c$	usually kinetic control: $k'_{metab}>>(8/3)D\pi r_c$	Fig. 5A-5D
reaction regime when mutagenicity or cytotoxicity occurs	Kinetic control; $k'_{metab}<<(8/3)D\pi r_c$	Kinetic control; $k'_{metab}<<(8/3)D\pi r_c$	Fig. 5B and 5D

Table 3: Comparison of the microenvironments elicited by different culture conditions—Function Biological

Micro-environmental parameters	Culture system		Reference /data source
	2-D-disc	3-D-RWV	
Ψ(Biological)			
cellular aggregation	difficult	much easier	Synthecon Inc., January 05, 2010
cell-to-cell interactions	much less efficient	more efficient	Synthecon Inc., January 05, 2010
exchange of growth factors.	much less efficient	more efficient	Synthecon Inc., January 05, 2010
cell re-differentiation,	much less effective	greatly promoted	Synthecon Inc., January 05, 2010
condition for inducing mutagenicity or cytotoxicity	high level accumulation of chemical substrate by kinetic control regime	high level accumulation of chemical substrate by kinetic control regime	This paper. Fig. 5B and 5D
viability affected by ferulic acid (2 mM)	viability 54.79%	viability 0%	This paper: Fig. 6
organoid formation	unlikely	Yes	Rhee *et al*., 2001
co-evolution	unlikely	Yes	Wang *et al*., 2005

(Continued)

Table 3: Comparison of the microenvironments elicited by different culture conditions—Function Biological

Micro-environmental parameters	Culture system		Reference /data source
	2-D-disc	3-D-RWV	
Ψ(Biological)			
cancer progression, metastasis, and therapeutic resistance	none-promoting	promoting	Sung and Johnstone, 2007.
cell shape	determined by disc surface adhesivity		Ben-Zéev *et al*., 1980
Cytochrome oxidase	?	positively correlated with gravity	Paulus *et al*., 1996
Inner ear energy metabolism	?	decreased under microgravity	Paulus *et al*., 1996
morpho-physiological shift: locomotion; adhesion; intercellular contacts; signal modulation	?	affected by gravity	Tairbekov,1996
signal transduction; gene expression; secretory functional cell differentiation; proliferation; cytoskeletal reorganisation	affected by physical forces	?	Zelles *et al*., 1999
changes in ion flux	affected by physical forces	?	Zelles *et al*., 1999
ATP release	affected by physical forces	?	Zelles *et al*., 1999
mitogenic T cell activation	?	lost at 0 g	Schwarzenberg *et al*., 1999
IL-2 receptor	?	lacking expression at 0 g	Schwarzenberg *et al*., 1999
cAMP	?	Acceleration induces change of cAMP	Block *et al*., 1995

4. Conclusions

Huge discrepancy in biological responses actually exhibits between the 2-D disc and 3-D RWV cultures. The cells in 3-D RWV culture exhibit larger total surface area (>$78.54\times10^{-6}cm^2$), larger effective surface area for transport (>$52.36\times10^{-6}cm^2$), and larger effective surface contact area (>$26.18\times10^{-6}cm^2$), facilitating the mass transport. The force vector sum exerts a net rotating force 3.107×10^{-9} N/cell on the 3-D culture. The 3-D culture faces a slightly weaker gravitational force. Instead, the 2-D culture uniquely encounters an overall static hydraulic force 1.964×10^{-3} N/cell, or a static hydraulic pressure 37.51 N/cm^2. Normally, the 2-D culture is accompanied with microenvironment diffusion control, which may change into kinetic control when the intracellular consumption rate is extremely lower than the sufficiently high transport balance coming from the extracellular compartment.

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6. Conflict of Interest

The authors declare that there are no conflicts of interest among them.

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