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The Effect of the Medium Viscosity on the Cells Morphology in Reaction of Cells to Topography- I

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ABSTRACT. The medium viscosity may alter the cellular reaction to topography which may affect cell growth, morphology and movement. This research had investigated the effect of the diffusion processes on the reactions of cells to topography, cells grew in medium with high viscosity on plane and grooved topography. The viscosity was changed by adding viscous macromolecules to the normal medium. Cells were examined in the presence of a variety of concentrations of macromolecules, such as dextran, ficoll, polyvinylpyrrolidone, Methylcellulose and Carboxymethylcellulose to the normal medium, which differed in their size and capacity to elevate fluid viscosity, and chemical nature. The medium viscosity was determined by measuring its flow time relative to water, using an Ostwald viscometer. A novel feature of these studies is combining measurement of the medium viscosity with the osmolarity of all studied media. The osmotic effect could be discounted. Diffusion was altered by raising viscosity of the medium, which affects the reactions of cells to topography. Results from this study had shown that cell morphology is directly affected when cells are grown in medium of high viscosity by decreasing cell length and reducing its mobility. It seems that medium viscosity affects the cytoskeleton by limiting its assembly and operation. Recovery experiments have shown that cells were still alive in high viscosity media and after they recovered in ordinary medium they spread and aligned again. In conclusion, these results support the hypothesis that diffusion effects around the cells may be important in cells reaction to topography, which suggest that elevated medium viscosity is pertinent to biochemical and cellular functions.

Key Words:
Viscosity, Topography, Cell Culture, Media and Tissue Engineering.

Introduction

The nature of the interactions of a cell with other cells and with a substrate is an important problem in Biology. To grow cells in tissue culture is one method of studying these interaetions. When mammalian cells are grown in vitro, most cells do not divide unless they are attached to a solid surface. This surface is commonly covered with an adsorbed layer of serum protein (Penttinen *et al,* 1958; Saxen & Penttinen, 1965; Giaever and Ward; 1978).

Therefore, it is possible to grow mammalian cells *in vitro*, where the cells act more like independent organisms with a generation time of approximately 24h. For many purposes, it is sufficient, cheaper and more practical to study cells in tissue culture rather than in an intact animal (Carrel, 1912; Giaever & Keese 1986). Early experiments by Harrison (1907), offered reproducible techniques that have been generally accepted as marking the true beginning of tissue culture.

Culture medium is used to maintain the cells. The medium is an aqueous solution of salts, cell growth factors, vitamins, antibiotic agents and possibly serum (Wilkinson and Schut, 1998).

On other hand, Biological cells are also strongly influenced by the topography of the surface on which they live, both in cell culture and in vivo. They are guided along micron sized grooves and change their shape becoming more elongated (Brunette *et al,* 1983; Clark *et al,* 1990; 1991; Wojciak *et al,* 1996). These effects can be used for cellular engineering to determine the behaviour of cells and in particular to make prostheses for medical purposes (Wilkinson and Schut, 1998).

Topographical Reactions

Cells naturally respond to chemical and topographie cues. The response of cells to topography has been recognised for many years.

Many types of cells react to surface topography (Table 1). One type of such reaction is termed contact guidance. Contact or topographic guidance refers to the reactions of cells to substrate surface morphology and includes alteration in their shape, orientation, adhesion, gene expression, and polarity of their movement (Wood, 1988; Curtis & Clark, 1990). There are at least two components to the reaction, first the alignment of the cells to the substratum and second the migration of the cells aligns along the substratum features (Wojciak *et al,* 1995).

The reaction of cells to topography occurs during development or in natural tissue regeneration, where extracellular matrix or other cells provide the topographical cues (Curtis & Wilkinson, 1997; Carrel, 1912; Giaever & Keese 1986).

For an overview of the types of cells, which react to topography and their reactions, see Tables 1 and 2.

Table (1). Cell types reacting to topography (Curtis & Wilkinson 1998).

Chondrocytes and Osteocytes
Endothelia and Epitena
Epithelia and Mesenchyme
Fibroblasts and Smooth muscle cells
Leucocytes, Lymphocytes and Macrophages
Neurons and Oligodendrocytes
Some tumour cells
Fungi

Table (2). Effects on cells of contacting topography (Curtis & Wilkinson 1998).

Orientation: align and normal to topography
Extension enhanced
Accelerated movement: Polarised movement
Capture (movement stopped at a particular feature)
Effect on adhesion: Activation of Tyrosine phosphorylation, actin polymerization, Vinculin accumulation over the groove edge, Phagocytic activity and Fibronectin m-RNA expression

Cell adhesion

Cell adhesion is a erucial for assembly of individual cells into the three-dimensional tissues of animals. A variety of cell adhesion mechanisms are responsible for assembling cells with their connections to the internal cytoskeleton, determining the overall architeeturc of the tissue.

Barry *et al.* (1996) riviewed the funetional units of cell adhesion as typically multiprotein eomplexes made up of three general classes of protein; the cell adhesion molecules/adhesion reeeptors, the extracellular matrix (ECM) proteins, and the cytoplasmic plaque/peripheral membrane proteins. The cell adhesion receptors are usually transmembrane glycoproteins that mediate binding interactions at the extracellular (EC) surface and determine thc specificity of cell-cell and ccll-ECM recognition. They include members of the integrin, cadherin, immunoglobulin, sclcctin, and proteoglycan superfamilies. At the EC surface, the cell adhesion receptors recognizc and interact with either other cell adhesion receptors on neighbouring cells or with proteins of the ECM. ECM proteins are large glycoproteins including the collagens, fibronectins, laminins, and proteoglycans that assemble into fibrils or other complex macromolecular arrays.

At the intracellular surface of the plasma membranc, cell adhesion receptors associate with cytoplasmic plaque or peripheral membrane protcins. Cytoplasmic plaque proteins serve to link the adhesion systems to the cytoskeleton to regulate the functions of the adhesion moleeules, and to transduce signals initiated at the cell surface by the adhesion receptors.

The adhcsive interactions betwecn a cell and its surrounding ECM regulatc its morphology, migration, growth, and differentiation (Burridge and Wodnika, 1996).

It was shown that surface topography could affect cell adhesion (Curtis & Clark, 1990; Chehroudi & Brunettc, 1995; Curtis & Wilkinson, 1998).

Cell shape

There is evidence that surfacc topography can alter cell shape (Weiss, 1945; Brunette, 1988; Clark, 1989; Cherhroudi *et al,* 1995). Two amongst the main effects that appear to control cell behaviour and morphology in tissue culture are, according to Curtis and Varde, 1964, the effeet of population density on both overlap and cell spreading (Abercrombie and Dunn, 1975) and secondly the effect of the topography on overlap behaviour, population density and cell spreading.

Since there have been many suggestions that topographieal reactions of cells may play a role in embryogcnesis, regeneration and wound healing, it is thought that topography can affeet cell morphology and eell behaviour (Bard & Higginson, 1977; Newgreen, 1989; Wylie *et al,* 1979; Webb *et al,* 1995; Curtis & Clark, 1990).

Clark, 1994 studied the role of the local environment in influencing the behavior of eells and found that the topography of a substratum could dramatically alter eell shape and guidancc. This was dependent on number of factors including step height or groove depth, the density of the cue, cell type and cell density.

Diffusion between cells

In general, matter diffuses from a region of higher to lower concentration. In inquiring how molecules move it is instruetive to review a property of the free diffusion of natural moleeules.

In his random-walk modcl Einstein (1908) showed that free diffusion and Fick's law are accounted for by the random thermal agitation of individual molecules.

A molecule does not need to 'know' what its neighbors are doing, it does not need to know of the existence of a gradient; by moving randomly to the right and to the left with equal probability biased by concentration differences it will make its contribution to the diffusion flux (Hille, 1971).

Passive diffusion

In thc ease of free diffusion of a neutral molecule, the gradient in potential energy is equal to the gradient in chemical potential, which is said to be "Fickian". Conversely, any departure from Fick's law for neutral molecules is often taken to indicate non-passive diffusion processes. The case of Fickian diffusion can be exploited further to dcfinc some additional useful terms and an important theoretical problem (Sjodin 1971).

The diffusion eocfficient (D_i) according to diffusion Fick's law is $D_i = U_i RT$, the proportionality coefficient U_i is thc mobility of speeies I, R is the gas constant and T is the absolute temperature.

Molecules in solutions move in a random fashion due to the continual buffeting that they receive in collisions with other molecules. This movcment causes molecules to diffuse over intracellular distances in a surprising short time (Alberts *et al,* 1994).

Diffusion in the extracellular space

Extracellular space is filled by an intricate network of macromolecules constituting the extracellular matrix. This matrix is composcd of a variety of versatile proteins and polysaccharides that are secreted locally and assembled into an organised meshwork in close association with thc surfacc of the cell that produced them (Alberts *et al,* 1994). In between lies the tissue fluid often called the tissue ultrafiltrate.

The matrix plays an active and complex role in regulating the behaviour of the cells that contact it, influencing thcir development, migration, proliferation, shape, and function. The extracellular matrix has a correspondingly complex molecular composition (Alberts *et al,* 1994).

The macromolecules increase the viscosity of the extracellular space. In most connective tissues the matrix macromolecules are secreted largely by fibroblasts. The two main classes of extracellular macromolecules that make up the matrix are 1. polysaccharide chains of glycosaminoglycans (GAC), which usually linked to proteins to form proteoglyocans, and 2. fibrous proteins of two functional type: mainly structural (e.g. collagen and elastin) and mainly adhcsive (e.g. fibronectin and laminin). The polysaccharidc rcsists compressive forccs on thc matrix, and thc collagen fibres provide tensile strength.

Thc aqueous phase of the polysaccharide gel permits the diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells (Alberts *et al,* 1994).

The polysaccharide chains fill most of the extracellular spaces, providing mechanical support to tissues while still allowing the rapid diffusion of water-soluble molecules and the migration of cells (Alberts *et al,* 1994). This is because of the extended nature of GAC chains, and their hydrophilic properties, which can restrict the movement of large molecules through the matrix but allow relatively free diffusion of small molecules (Boubriak *et al,* 2000).

The diffusion displacement distances are comparable with cellular dimensions, raising the possibility that the measurements of water diffusion might provide a means of exploring cellular integrity and pathology (Eriksson *et al,* 2001). Tissues with more hindered diffusion in one direction than another are said to be anisotropie whereas diffusion is the same in all directions in isotropic tissues.

Pathological processes that change the microstructural environment, increased or decreased extracellular space and loss of tissue organisation, result in altered diffusion and/or anisotropy. Increased diffusivity could be caused by a cell loss resulting in increased extracellular space (Eriksson *et al,* 2000).

Diffusional transport of solutes through matrix can be characterised by diffusion and partition coefficients (Marouds and Urban, 1983).

Boubriak *et al,* 2001 studied the effect of matrix composition on solute diffusion in rabbit sclera, they have found that (1) diffusion and partition coeffieients are sensitive to solute molecular weight, decreasing as MW increases; (2) diffusion and partition coefficients are sensitive to tissue hydration, increasing as hydration increases; and (3) removal of glycosaminoglycans has only a small effect on either diffusion or partition coefficient.

Viscosity

Liquids exhibit resistance to flow known as viscosity. In general, it is the property of resistance which opposes the relative motion of the adjacent portions of the liquid and can consequently be regarded as a type of internal friction (Glasstone, 1948).

Bryant Chase *et al,* (1998) suggested that increased viscosity of a solvent decreases chemical reaction kinetics.

The movement of an objeet under a given force is slowed as viscosity increases. Thus the movement of a cell through intercellular space may be retarded by high viscosity media. Are effects of high viscosity media due to this or to effects on diffusion?

The coefficient of viscosity η is defined as the force per unit area, dynes-seconds per sq. em.(dyne-sec/cm^2), required to maintain unit differences of velocity, i.e., 1 cm. per sec. (cm/sec), between two parallel layers 1 cm. apart (Glasstone, 1948). In order to maintain a uniform velocity a steady force must, therefore, be applied to overcome the influence of the viscosity of the liquid. It has been found (Stokes, 1850) that if a small sphere of a radius r travels at a velocity u through a fluid, gas or liquid, having a coefficient of a viscosity η , the force applied which just balances that viscosity, is given by Stokes's law

$$f = 6 r \eta u$$

Centipoise (Cp) = 0.01 poise (unit of absolute viscosity = gm/sec x em).

One method for the direct measurement of viscosity requires the determination of the rate of flow of liquid through a capillary tube of known dimension (Glasstone, 1948). The

method commonly employed involves an instrument known as a viscometer of which several types are available.

Fluid inhibiting internal friction (transport of momentum) is said to be viscous. The viseosity of medium is modified in many studies by the addition of various macromoleeules to the medium (Hovav *et al,* 1987; Yedgar & Reisfeld, 1990; Tuvia *et al,* 1997; Armitage & Packer, 1998).

Although variation of solvent viscosity is a useful probe applied in studies of protein dynamies, enzyme function, effect of plasma viseosity on blood cells, mechanism of muscle eells, and also in bacterial motility as illustrated below, it has not been used to study cell reaction to topography.

It is important to study the viscosity of the medium when study the reaction of cells to topography because gap formed between lower side of the cells and the topography. This gap may contain a highly viscous medium, which affects the movement of molecules into and out of the cell. This may be an explanation for the hypothesis that cells react with planar topography more easier than they can react to rough topography. Alternatively it may increase the cell energy expenditure so much that it can not move.

Viscosity effects have been studied in blood plasma as well as in the extracellular fluid. Plasma viscosity is elevated in various pathological states, due to inereased levels of protein and other macromolecules (Yedgar & Reisfeld, 1990). They found that secretion of lipoproteins and lysosomal enzymes by liver cells is inhibited as a function of the medium viscosity. Correspondingly, elevation of the plasma viscosity of hyperlipidemic rats reduced lipoprotein level. Elevated viseosity of blood and plasma is eonsidered a risk factor for cardiovascular diseases and has been studied mainly in relation to microcirculation and hemodynamics (Tuvia *et al,* 1997).

In addition there are many studies on the effect of medium or plasma viscosity on blood cells, Hovav *et al*'s study. They studied the effect of medium viscosity on lysis of red blood cells (RBC's) by snake venom phospholipase A2 (PLA2). PLA2 and Ca^{++} were added to cells suspended in viscous medium to induce hemolysis. It was found that hemolysis is inhibited in direct proportion to increasing viscosity of the extracellular fluid. This phenomenon was observed with aggregated as well as disaggregated RBC's. In the presence of Ca^{++}-cell-enzyme complex that was suspended in viscous medium, RBC lysis was also inhibited as the medium viscosity was increased. The authors proposed that the viscosity of the cell surface aqueous environment regulate the action of PLA2 on RBC's membranes.

Kon *et al* (1987) studied quantitatively the effect of shear force (depending on shear rate and the viscosity of the extracellular medium) and the hematocrit of erythrocyte suspension on RBC deformation using a cone-plate rheoscope with various kinds of cells. They found that the increase of suspension viscosity at higher hematocrits (Hts) generally enhanced the ellipsoidal deformation of cells, in the same manner as increasing the suspending medium viscosity of a diluted cell suspension.

Kullmann *et al* (1999) had shown that raising the viscosity of the extracellular medium could modulate the diffusion coefficient, providing an experimental tool to investigate the role of diffusion in activation the synaptic and extrasynaptic receptors in the brain.

One suggestion was cells may spread on topography readily because diffusion under them is altered. Medium must be pushed out as cells spread over a surface help the cells.

This could be expeeted because eells might fail to conform to a deep topography as well as they do to a planar surface. If diffusion is important, viseosity should be important as well because diffusion rates and the viseosity of the medium are closely linked.

Therefore the reaetion of cells to groove/ridge topography in media with different viscosity was examined in this project.

If diffusion processes are involved in the reactions high viscosity media should alter cell reaction to topography. The simplest feature of the reaction of cells we have examined is the morphological reaction of changes in cell length and width.

In general, the extracellular fluid viseosity may play an important role in regulation of cellular and biochemical proccsses, Thus viscosity may also affect othcr fundamental properties of the cells.

Therefore, viscosity needs to be carefully considered in experimental design and interpretation of reaction of eells to topography.

Materials and Methods

Cells and Cells Culture Materials

1. Epitenon cells:

Epitenon fibroblasts were isolated from rat flexor tendons of male Sprague dawley rats according to the method described by Wojciak and Crossan(1995). The eells were cultured in ECT medium.

2. Mouse Endothelial B10D(2) cells:

They werc isolated from mouse using the method reported by Curtis (1998) as described below:

Originally these werc isolated from murine B10D2 strain pulmonary cells by growing explants of lung tissue in the Ham's F10 medium until appreciable outgrowths had developed. The explants were then rcmoved manually. The outgrowth cells were grown to near confluency and then trypsinised to form ccll suspensions. Macrophages remained adherent at this stage and the cell suspension appeared to contain a mixture of fibroblasts, cpithelia and endothelial cells. When these cells had grown to confluency they were trypsinised and the cell suspension treated with Dynal magnetic beads (450 grade) bearing the *Swainsonia* leetin specific for mouse endothelia. These beads were prepared in-house. The cells wcrc incubated with a 100:1 ratio of beads to cells for 1 hour at 37^0 C. Then magnctic scparation was started and the fibroblasts and epithclia, which had not attached beads, were removcd. Thc beaded cells werc placed in a culture and as they dividcd they emerged from their bead coats and spread out as colonies of cells. Thcsc were grown to confluency, subcultured and thc bcad extraction repeated.

The cells were then subcultured again and thc following tests used to establish their identity. They were found to be LDL-receptor positivc, Swainsonia-lectin binding positive, Factor VIII positive, to havc Weibel-Palade bodies, to have normal chromosome counts and appearance, to be cytokeratin-ncgative and to have 'cobblestone' morphology at confluency though arcuate morphology at low density. Collagen types were consistent with an endothclial nature.

These tests were carried out by Professor A. Curtis and the line then maintained in the laboratory by repeated passage and/or storage at -70^0 C.

3. Media:

The media were used in this project are ECT media for epitenon cells and Ham's F10 for endothelial cells.

4. Reagents used to change medium viscosity:

Viscosity of the medium was varied by adding to the normal media (ECT or Ham'sF10) various macromolecules that differ in size, chemical nature, and in their capacity to increase fluid viscosity as:

Ficoll with molecular weight (400,000), Dextran Mw (503,000) and Dextran Mw (2,000,000) all with different concentrations low, mid and high. Same polymers with low molecular weight where used as a control this are Dextran Mw (9,000), Ficoll Mw (70,000).

5. Measurement Instruments:

Viscometer:

The U-tube Ostwald viscometer was been used to measure the medium viscosity.

Refractometer:

An Abbé Refractometer has used to measure the refractive index for all media used in IRM experiments.

The Abbé Refraetometer is a precision optical instrument comprising two glass prisms, between which the test liquid is sandwiched as a thin film, illuminated by white light source (a lamp).

Osmometer:

The GONOTEC digital micro-osmometer was used to measure the media osmolarity at freezing point depression.

6. Light microscopy examination and imaging analysis:

Each group of cells were plated onto grooved substrata with groove width 5μm-10μm in high viscosity media (medium viscosity was altered by adding high moleeular weight molecules to the normal media as mentioned before) and control media and incubated at 37^{0}C for 24h. Cells were fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1x PBS each for 5 min. Then cells were stained with Coomassie blue for 5-10 miu followed by repeated washing from tap water.

The cell length and great width of 50 cells each in experimental and as control were measured in each kind of media using an ocular micrometer and stage micrometer. Results have been calculated and compared with the corresponding values from the statistical tables.

Images typically were recorded using a CCD camera and saved in PC computer. Images processed using NIH Image 1.62n3 computer program.

The data was analysed using StateView 4.02, CA-Cricket GraphIII and Excel Microsoft computer programs.

Results

Viscosity measurements of the media used

Measurements shown in Table 3 indicate that the viscosity of the medium significantly increased as the concentration of high molecular weight molecules increased in the solution compared with the viscosity of water, ECT media and the low molecular weight molecules that were used as controls.

Table (3). Viscosity measurements.

Media name	**Viscosity [cP]**
Water as a control	0.69
ECT normal media	0.75
0.5%Dextran 9,000	0.75
Low 2%Dextran 9,000	0.82
Low 4%Dextran 9,000	0.88
Low 0.5%Ficoll 70,000	0.75
Low 2%Fieoll 70,000	0.82
Low 4%Ficoll 70,000	0.94
4%Dextran 503,000	2.58
2%Dextran 503,000	1.57
0.5%Dextran 2000,000	1.07
2%Dextran 2000,000	2.33
0.5%Polyvinylpyrrlidone 360k	1.26
0.5%Polyvinylpyrrolidone 40k	0.82
0.5 % Carboxymethylcellulose	48.09
2 %Methylcellulose(MC)	9.37
0.5 %Methylcellulose	1.57
2%Ficoll 400,000	1.07
4%Ficoll 400,000	1.32
8%Ficoll 400,000	2.58
Olive oil	31.43
Dimethylpolysiloxane (silicone oil)5 cm.st	3.52
Dimethylpolysiloxane 100 cetistok	76.88

Therefore, the possibility that the effect of medium viscosity on the reaction of cells is associated with the osmotic pressure must be considered. To examine this hypothesis the medium osmolarity was measured for all used media.

The real viseosities of the media were obtained (centi Poises) from Fick's law:

$$\eta / P = At$$

Therefore (η) is the viscosity, P is the density, A is a constant related to the viscometer tube dimensions and t is the flow rate of solution in the tube.

Osmolarity Measurements

The osmolarity measurements were measured using the Gonotec digital micro-osmometer (030) to measure freezing points depression, as shown in Table 4.

Table (4). The osmolarity measurements.

Media name	Concentration	Osmolarity (m Osm/kg)
Control ECT media		330
Ficoll 70,000	4%	338
Ficoll400,000	1%	334
Ficoll400,000	4%	356
Ficoll400,000	8%	358
Dextran 503,000	1%	330
Dextran 503,000	4%	330
Dextran 2000,000	0.5%	329
Dextran 2000,000	2%	335

Recovery experiments

Of interest is the finding that cells are still alive after incubated in high viscosity medium as obtained from the recovery experiments as shown in Figure 1 & 2.

Cells after recovery grow and appear to restore their validity. This confirms the suggestion that cells strong enough to live in a viscous conditions. Bares 2.5 μm.

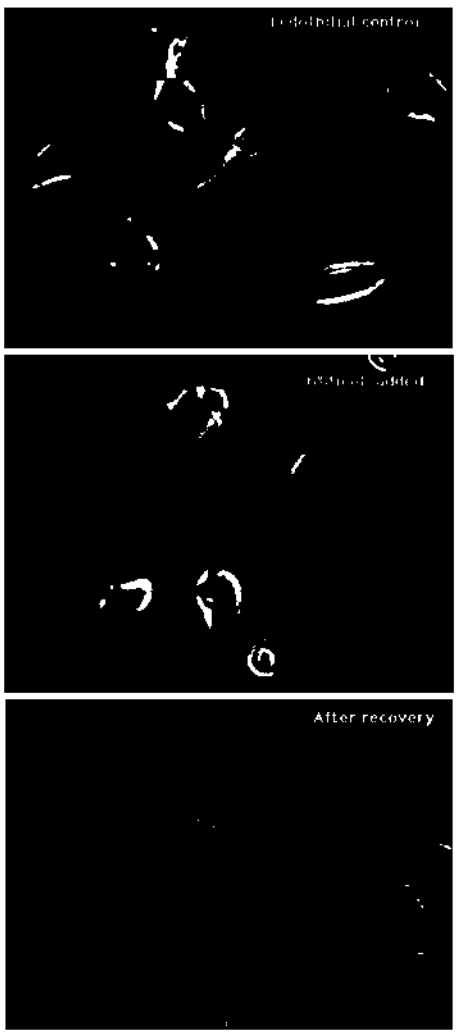

Fig. (1). Images show Endothelial cells B10D2.
1- Control Ham's F10 for 24h
2- 6% Ficoll 400,000 for 24 h
3- Ham's F10 as a recover for 24h.

Fig. (2). Images show Epitenon cells
1- Control ECT medium for 24h
2- 2%Dextran 2000,000 for 24h
3- 3- ECT as a recover for 24h

Morphological reactions

Morphometry:

The reaction of cells to topography is reduced so that the average of length of cells decreased as medium viscosity is increased for all reagents as shown in the related Figures and Tables.

Experiments were done on 5-10μm groove ridges and on planar controls. The media used are of various viscosities.

1. A study of the morphology of epitenon cells on grooved topography:

The effect of high molecular weight molecules:

By studying the effect of different concentrations of Dextran (with high molecular weight) on the length of the epitenon cells, it was found that the low concentration of Dextran did not affect significantly the viscosity of the media, and it did not affect the epitenon cell morphology significantly.

But the higher concentration of Dextran ($P<0.0001$ in all), the higher is the viscosity of the media and there is significant effect on the length of cells, but not on the width as shown in Table 5, Figure 3 and 9.

By studying the effect of different concentration of Ficoll (400,000) on the length of the epitenon cells. It was found that the length of the cells was affected by Ficoll concentration as shown in Table 6, Figure 4, 10 and 11.

The effect on the width of the epitenon cells:

While the width of the epitenon cells showed no effects when the medium viscosity increased using low or medium concentration of high molecular weight reagents, the width of the cells showed a similar significant increase when using high concentration of the high viscous reagents, cells in this medium appeared rounded, non-spreading with approximately same width and length.

In the very high concentration of reagents as seen with Ficoll (8%) the cells appear shrunken with small width (see Fig 11).

The effect of low molecular weight molecules used as a control:

By studying the effect of low molecular weight polymer (e.g. Dextran with mol.wt 9,000) on the length of epitenon cells we found that there is no significant effect on the length of epitenon cells incubated in low Dextran at different concentrations as shown in Table 7 and Figure 5 and 12.

Table (5). The effect of high molecular weight Dextran on epitenon cell length.

Dextran conc.	Average length μm	Standard deviation	P-value exp/ctrl
Control 1	81	42	
Dextran 1% 503,000	71	27	0.8391
Dextran 2% 503,000	70	27	0.8424
Control 2 Dextran 2m	96	33	
Dextran 4% 503,000	44	41	<0.0001
Dext0.5% 2000,000	51	26	<0.0001
Dextran 2% 2000,000	50	27	<0.0001

Table (6). The effect of Ficoll (mol. wt. 400,000) on epitenon cell length.

Conc.	Average length (μm) of 50 cells	Standard deviation.	P-value cont. &exp.
Control 1	94.42	43.2	
Ficoll 4%	35.13	15.4	<0.0001
Ficoll 6%	24.93	6.6	<0.0001
Ficoll 8%	21.81	8.4	<0.0001
Control 2	103	43	
Ficoll 0.5%	80	22	0.0008
Ficoll 2%	59	21	<0.0001

Table (7). Low mol. Wt. (9,000) Dextran used as a control.

Dextran conc.	Average length (μm) of 50 cells	Standard deviation	P-value
Control 1 normal ECT	93	39	
Dextran 0.5% Mol.wt 9,000	85	29	0.012
Dextran 2% Mol.wt 9,000	88.9	38	0.39
Dextran 4% Mol.wt 9,000	89	26	0.004

Table (8). Low molecular weight Ficoll (70,000) used as a control.

Ficoll conc.	Average length (μm) of 50 cells	Standard deviation	P-value
Control 2	87.4	43	
Ficoll 0.5% Mol. wt. 70,000	87.8	62	0.017
Ficoll 2% Mol. wt. 70,000	86.7	37	0.301
Ficoll 4% Mol. wt. 70,000	95	37	0.31

Table (9). Ficoll effect on endothelial cells.

Ficoll Conc.	Standard deviatiou.	Average length (μm) of 50 cells	P-value
Ham's F10	72.5	178.2	
Ficoll 2%	45.7	113.5	0.002
Ficoll 4%	53.6	50	0.037
Ficoll 8%	4.7	13	<0.0001

This indicates that the length of the endothelial cells decrease with the increasing of Ficoll concentration as shown in the Figure 7.

Table (10). Dextran effect on endothelial cells.

Dextran Conc.&Mw.	Standard deviation.	Average length (μm) of 50 cells	P-value
Control Ham's F10	81.4	177.9	
Dextran0.5% Mw.2000,000	73.6	142.9	0.485
Dextran 1% Mw.2000,000	50.6	94.5	0.001
Control Ham's F10	57	163.7	
Dextran2% Mw.503,000	62.3	127.7	.564
Dextran 4% Mw.503,000	43	87	.055

This indicates that the length of the endothelial cells decreases with increasing Dextran concentration as shown in the curve:

Table (11). Ficoll effect on endothelial cells on plane topography.

Ficoll Cone.	Standard deviation.	Average length (μm) of 50 cells	P-value
Ham's F10	38.9	100	
Ficoll 4%	37.8	55.9	0.883
Ficoll 8%	1.8	10.3	<0.0001

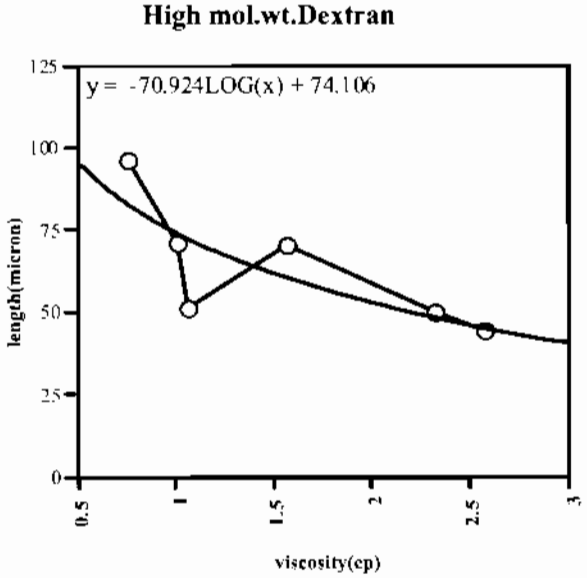

Fig. (3). The curve shows that the epitenon cell length decreases if the Dextran concentration increases. Standard deviation of the regression coefficient *(sb)* = - 0.781 and t = 25.18

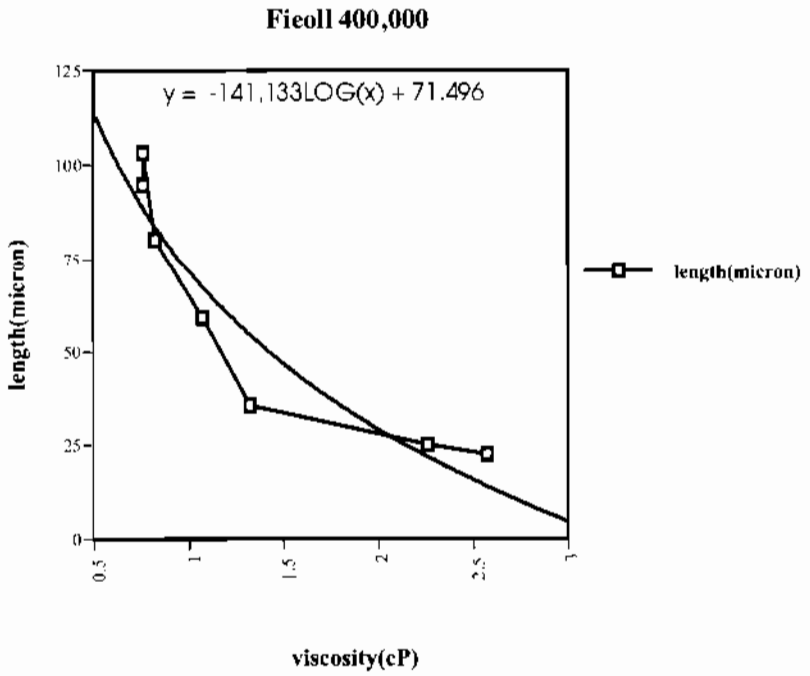

Fig. (4). The curve shows the relation between the cell length and the viscosity of ficoll medium. Standard deviation of the regression coefficient *(sb)* = - 0.888 and t = 53.05

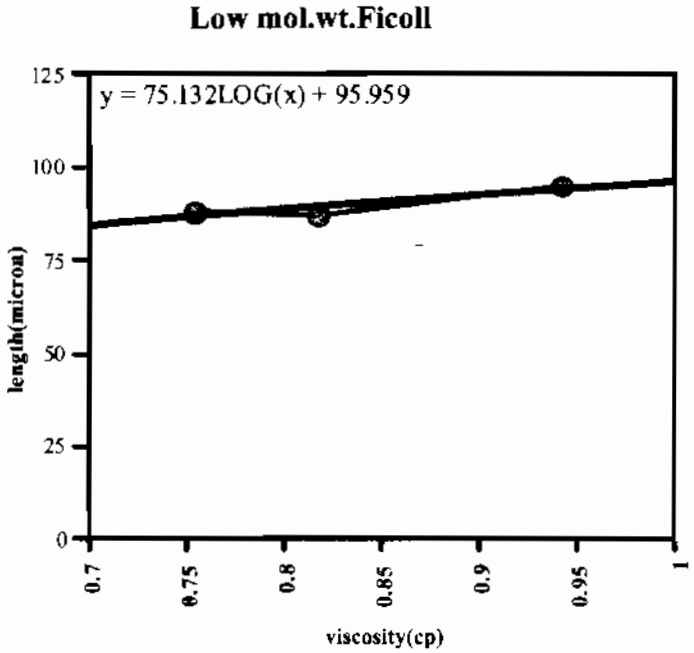

Fig. (5). The curve shows the relationship between viscosity (different concentration of low mol. wt. Dextran) and epitenon cell length. Standard deviation of the regression coeffieient *(sb)* = - 2.687 and $t = 0.054$.

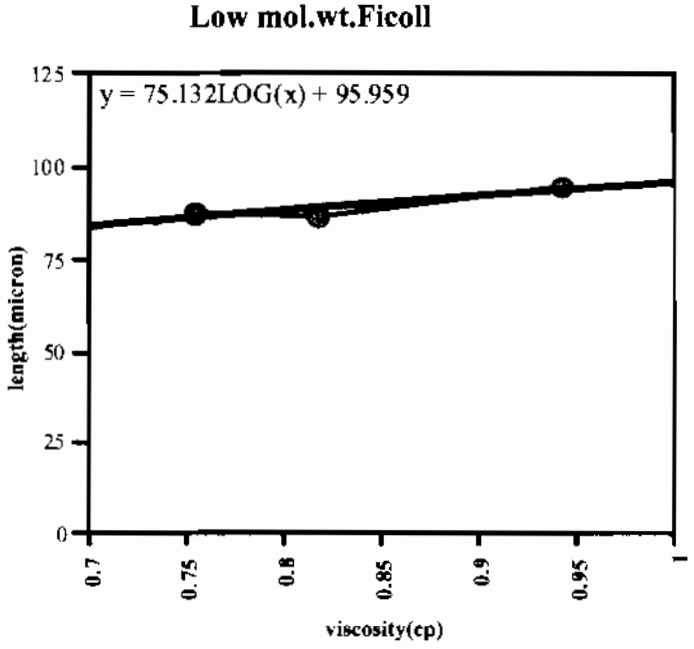

Fig. (6). The curve shows the relation between viscosity (with different concentrations of low mol. wt. Fieoll) and the Epitenon cell length. Standard deviation of the regression coefficient *(sb)* = 0.9000 and $t = 43.60$.

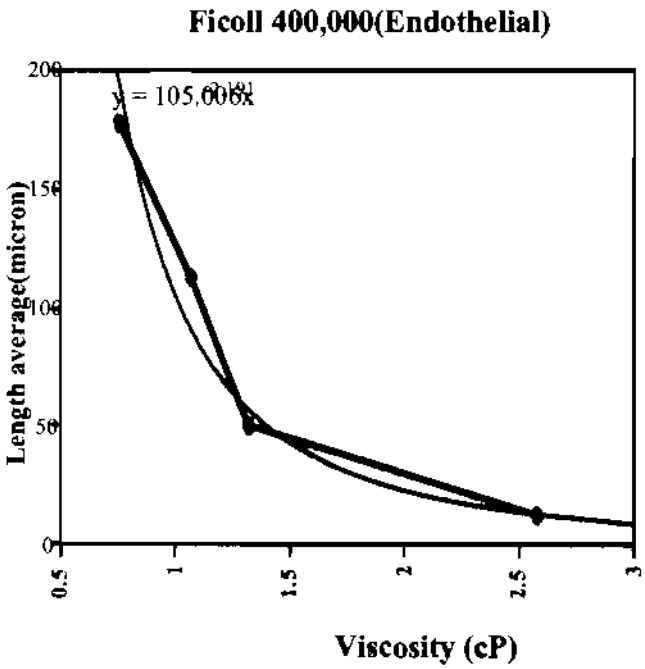

Fig. (7). The curve shows the decrease of endothelial length average with increase of medium viscosity. Standard deviation of the regression coefficient *(sb)* = - 0.873 and t = 90.72.

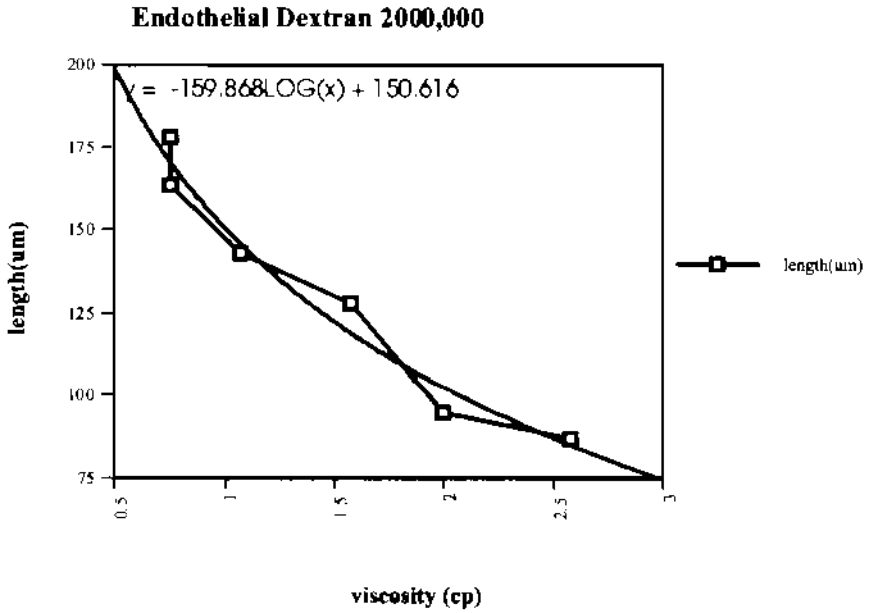

Fig. (8). The curve shows the decrease of endothelial length average with the inereasc of medium viscosity. Standard deviation of the regression coefficient *(sb)* = -0.795, and t = 53.05 is calculated from Snedecor and Coehran, 1980 as example:

$$t = \frac{b}{sb} = \frac{-42.171}{-0.795} = 53.05$$

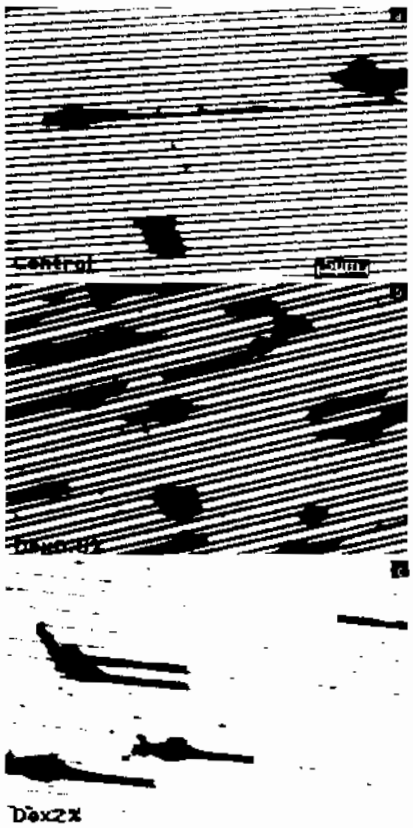

Fig. (9). Light microscopy images (20x-Objective) of Epitenon eells grown on quartz grooved topography with depth 5-8μm and 5μm width in Dextran with mol.wt 2000,000 and Control ECT medium for 24h.
Fixed and stained with Coomassie blue Control.
Dextran conc.0.5%
Dextran conc.2%
Scale bars 5μm for all images.
Number above scale bar indicates groove width.

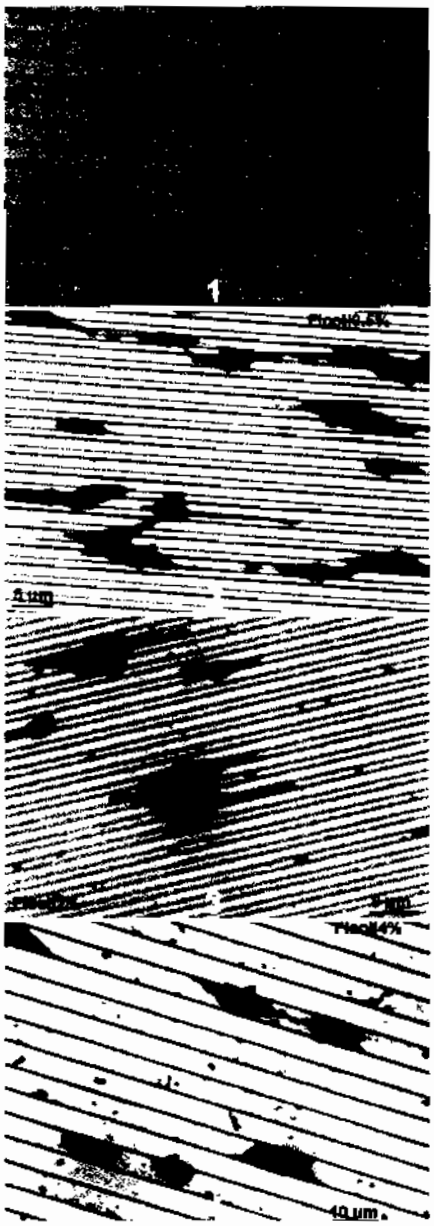

Fig. (10). Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 5-10μm width in Ficoll 400,000 and Control ECT medium for 24h.
Fixed and stained with Coomassie blue Control.
Ficoll conc.0.5%.
Ficoll conc.2%.
Ficoll conc.4%.

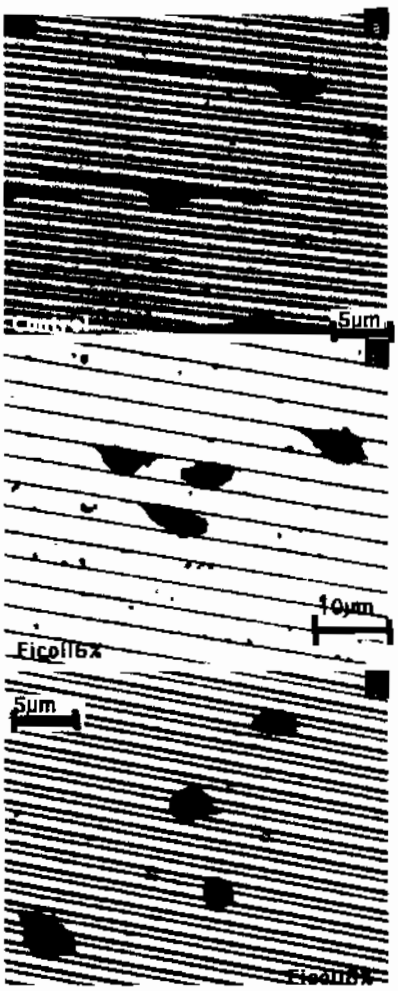

Fig. (11). Light microscopy images (20x-Objective) of Epiteuon cells grown on quartz grooved topography with depth 5-8µm and 5-10µm width in Ficoll 400,000 and Cootrol ECT medium for 24h.
Fixed and stained with Coomassie blue
a- Control
b- Ficoll conc.6%
c- Ficoll conc.8%

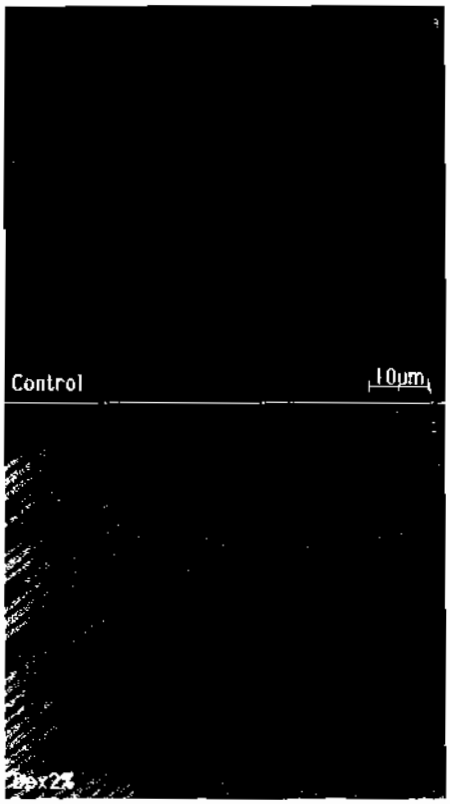

Fig. (12). Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5µm and 5µm width in Dextran with mol.wt 9,000 and Control ECT media for 24h.
Fixed and stained with Coomassie blue
1 Control
2 Dextran with eonc.2%

The effect of low molecular weight molecules used as a control:

By studying the effect of low molecular weight polymer (e.g. Dextran with mol.wt 9,000) on the length of epitenon cells wc found that there is no significant effect on the length of epitenon cells incubated in low Dextran at different concentrations as shown in Table 7 and Figure 5 and 12.

By comparing this experiment with the prcvious experimcnts on high molecular wcight dextran we show that the epitenon ccll length is affected by the medium viscosity and not by the chemistry of the polymers because all the fivc polymers of high molecular weight had the same effects at similar viscositics.

By studying the effect of low polymer (Ficoll with mol. Wt. 70,000) on the epitenon cells length, the results are similar to low molecular weight dextran, see Table 8 Figure 6 and 13.

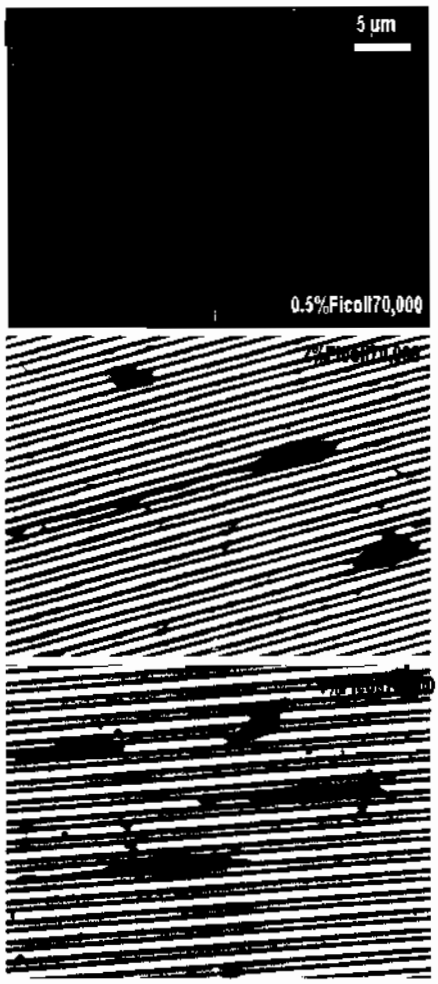

Fig. (13). Light microscopy images (20x-Objective) of Epitenon eells grown on quartz groove topography with depth 5µm and 5-10µm width in Ficoll with mol.wt 70,000 and Control ECT media for 24h.
Fixed and stained with Coomassie hlue
1. Ficoll 0.5%.
2. Ficoll 2%.
3. Ficoll 4%

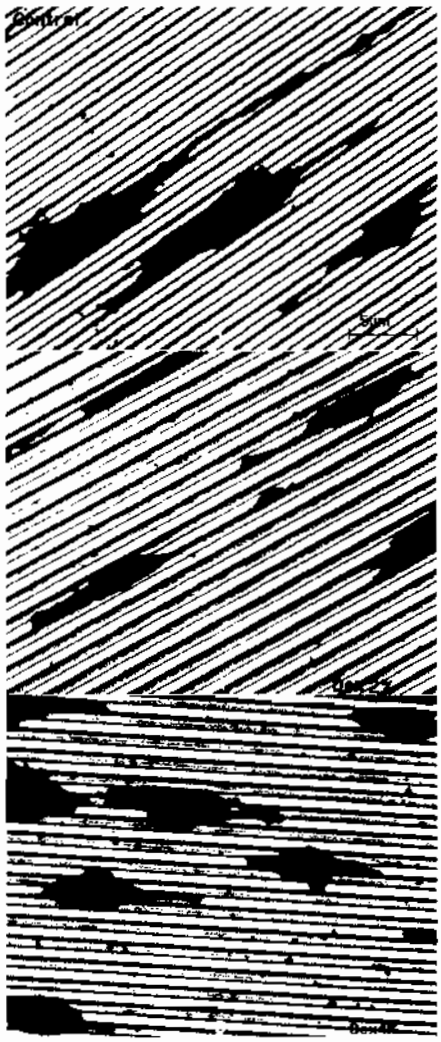

Fig. (14). Light microscopy images (20x-Objeetive) of Endothelial eells B10D(2) grown in Dextran with mol. wt. 503,000 for 24h on quartz grooved topography with depth 5µm and 5µm width.
Fixed and stained with Coomassie blue:
3. Control (Ham's F10)
4. Dextran eonc. 2%
5. Dextran conc. 4%

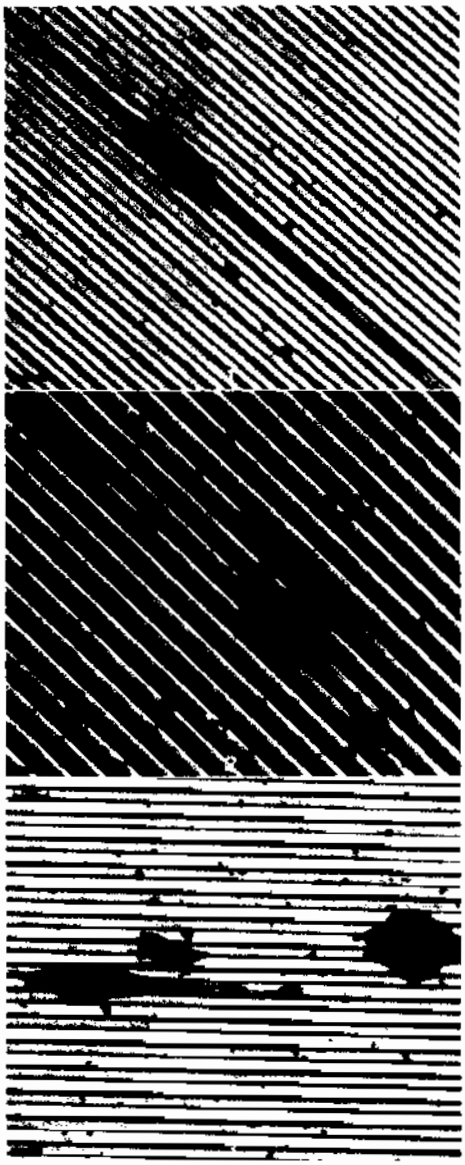

Fig. (15). Light microscopy images (20x-Objective) of Endothelial cells B10D(2) grown in Dextran with mol.wt. 2000,000 for 24h on quartz grooved topography with depth 5μm and 5μm width.
Fixed and stained with Coomassie blue:

3. Control (Ham's F10)
4. Dextran conc. 0.5%
5. Dextran conc. 1%

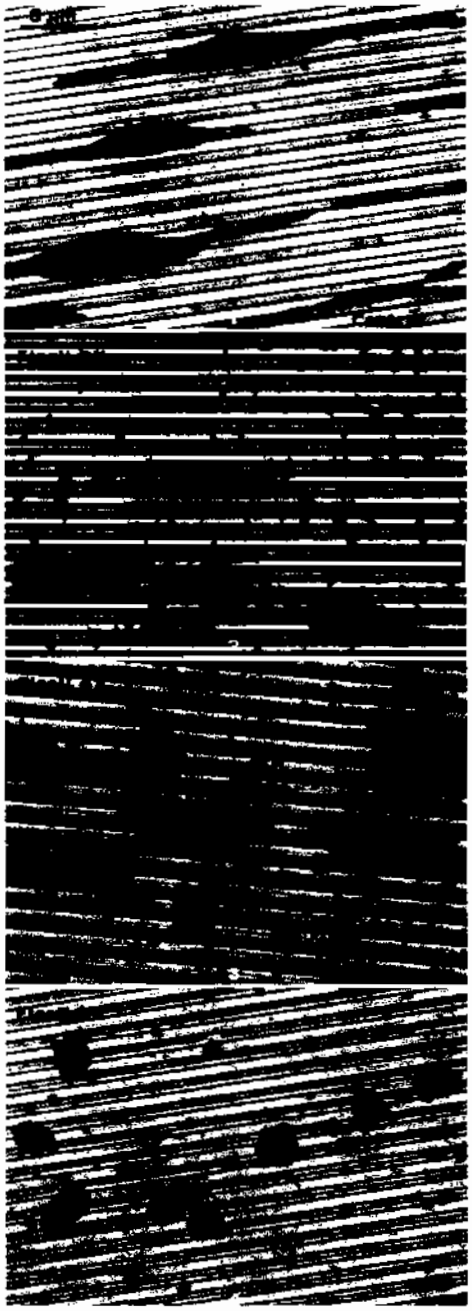

Fig. (16). Light microscopy images (20x-Objective) of Endothelial cells B10D (2) grown in Ficoll with mol.wt. 400,000 for 24h on quartz grooved topography with depth 5μm and 5μm width.
Fixed and stained with Coomassie blue:

1. Control (Ham's F10)
2. Ficoll conc.2%
3. Ficoll conc.4%
4. Ficoll conc.8%.

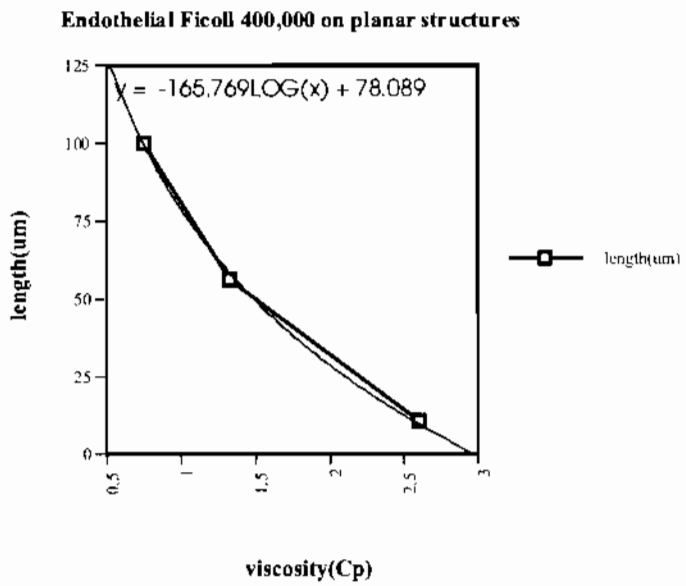

Fig. (17). The curve shows the decrease of endothelial length average with increase of medium viscosity. Standard deviation of the regression coefficient *(sb)* = - 0.961 and t = 22.3 for all Ficoll 400,000 results.

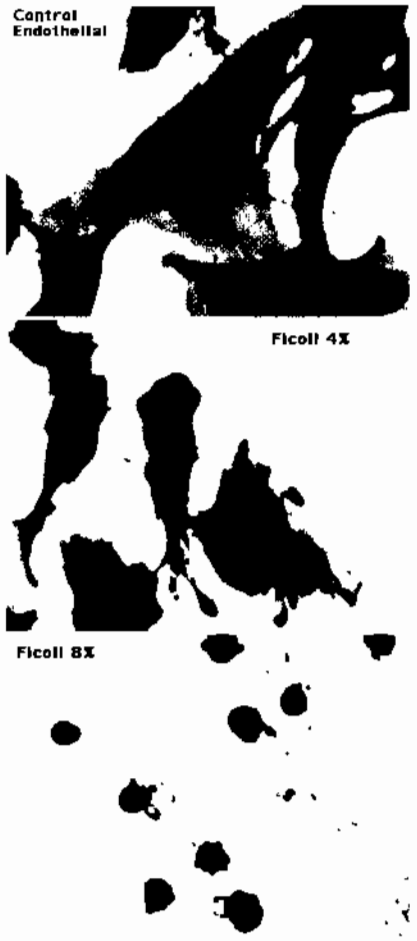

Fig. (18). Light microscopy images (20x-Objective) of Endothelial cells B10D2 grown on glass plane cover-slip in Ficoll 400,000 and control Ham's F10. Fixed and stained with Coomassie blue.

1. Control.
2. In Ficoll 4%.
3. In Ficoll 8%

It is clearly observed that there is a difference between control and experimental cells:

The control cells appear spread with well developed lamellipodia. While the cells were grown in 4% Ficoll appear to have some ability to spread and grow, cells were grown in Ficoll 8% appear to lose their ability to grow or spread to the substratum.

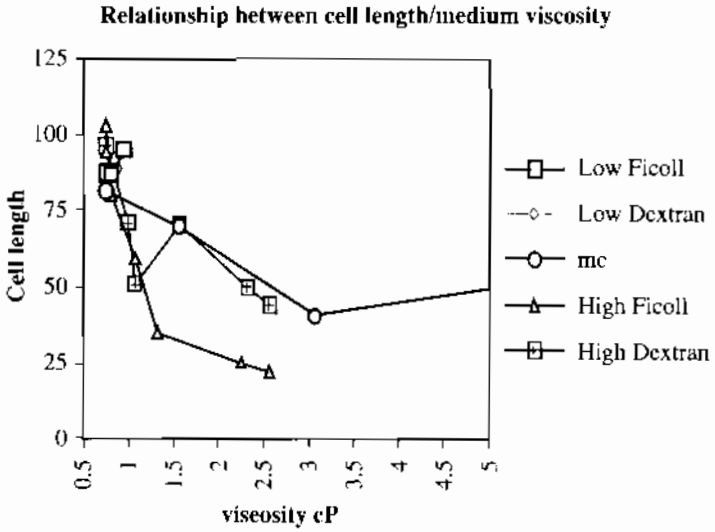

Fig. (19). The curves indicate that cell length decreases when the viscosity of the medium increases. The standard deviation of the regression coefficient ranged between 0.78 to 0.961. Comparing this to the length of the cells grown in low molecular weight reagents (low dextran standard deviation of the regression coefficient = - 2.687 and low ficoll Standard deviation of the regression coefficient = 0.900).

It is reasonable to assume that diffusion is being reduced when the viscosity is raised. The relation between viscosity and diffusion can be obtained from the Stokes-Einstein equation:

Student's *t* test was applied to compare the cell length of control and experimental cells. The test indicates that the effect of high viscosity medium on cell length was statistically significant ($P<0.0001$).

These results indicate that when there is no significant increase in the viscosity of the medium there is no effect on cell length. But when the viscosity was increased (viscosity ≥ 1c.P) appreciably the cell length was reduced ($P <0.0001$).

To confirm our results more we used another type of cells, murine capillary endothelial B10D2 and the same results that were observed with epitenon cells were shown. See Table 9-11 and Figures 7- 8 and 14-18.

For all subsequent results (Table 5-12) P values are obtained form StatView 4.02 Program and *t* values calculated according to Snedecor and Cochran(1980) as:

$$t = b / sb$$

Where, *b* is the regression coefficient of the viscosity and *sb* is the Standard deviation of the regression coefficient obtained by StatView 4.02 program.

2. A study of the morphology of Endothelial cells grown in highly viscous media on grooved topography

The effect of Ficoll (mol.wt.400,000) on endothelial cell length:

By studying the effect of different concentration of Ficoll on the length of the endothelial cells. It was found that the length of the cells was affected by Ficoll concentration, see Table 9 and Figures 7, 10, 11, 13 and 16.

The effect of Dextran (mol.wt.2000.000 and 503,0000) on endothelial cell length:

By studying the effect of different concentration of Dextran on the length of the endothelial cells. It was found that the length of the cells was affected by Dextran concentration, see Table 10 and Figures 8, 9, 12, 14 and 15.

3. A study of the morphology of Endothelial cells grown in highly viscous media on planar topography as a control

The effect of Ficoll (mol.wt.400,000) on endothelial cell length:

It was found that the length of the Endothelial cells (B10D2) was affeeted by Fieoll concentration when cells were grown on plane topography, see Table 11 Figures 17- 18.

Conclusion

Cell length decreased in medium with high viscosity see for example picture 3 & 4 in Fig 10 and picture b & c in Fig 11 for Epitenon cells and pictures 3 in Fig 15 and pictures 3 & 4 in fig 16 for Endothelial cells.

Whearse changes in the morphological reactions occurred less frequently when cells were cultured in low molecular weight Dextran 9,000 or Ficoll 7000 with low and high concentrations, see the pictures in Fig 13 & 14.

Equation : $D = kT / 6\pi\eta r$

Where D is diffusion coefficient, T is temperature, r is the radius of an ion, η is Coefficient of the viscosity by c.P. and k is Boltzmann constant=1.38066.

Diffusion in turn may affect the topographical reaction of cells.

Most of eells became less spread when grown in highly viscous medium (as shown in the curve figure 19 which summarises these results).

Of special interest is the finding that cells are still alive after incubated in high viscosity medium as obtained from the recovery experiments, see Fig 1 & 2.

The viscosity in turn affects diffusion around cells, and therefore may affect the morphology of the cells.

This study indieates that the reaction to topography is reduced as the medium viscosity is increased for the high concentration of all reagents used in the experiment and that can be tested by further experiments.

Discussion

This project has studied the effects of the viscosity of the medium on the reaction of cells to topography. The reasons for doing this are to:

1. Consider the role of diffusion in cellular reaction to topography.
2. Look at cells in an environment resembling intercellular conditions where medium viscosity is fairly high.
3. Control the condition that permits the correct growth of cells in the laboratory first and then inside the body.

4. Control cell adhesion, position and orientation which are vital to give correct growth and eell function.
5. Study conditions (ehemicals and topographical cues) that allow making constructs from living cells outside the body for subsequent implantation.

The medium viscosity has been changed by adding viscous macromolecules to the culture medium. The effects of viscosity appear to be linked to changes in cell morphology, spreading, movemcnt and on theoretical grounds to mechanical interactions with the substratc.

Because high concentrations of solutes were added to experimental media, it must be a concern that physiological effeets could bc due to altered osmotic or chemical activity.

It is clear from the data that the contribution of the reagcnts used to the total osmotic pressurc was small. Thus the osmolarity of the medium did not ehange too mueh after adding high molecular weight reagents. The fact that dcspite different chemistries the viscosity incrcasing reagents have similar effects argues that the chemistry of these reagents is not important in this study.

Thus viscosity but not colloid osmotic pressure is implicated in the response.

There may also be effccts resulting from the extra force needed to extcnd a cell into a high viscosity medium, but we have no evidence on this as yet.

In this study, only two cell typcs were used to invcstigate their reaetions in the presence of high viscons media. The cells demonstrated fairly similar reactions to topography. Both cell types can be affected by medium viscosity which results in changes of cell morphology and the rate of cell movement.

To understand the effects of medium viscosity wc must first analyse the meehanisms by which viscosity alters cell morphology or movements.

Contact guidance refers to the reactions of ccIls with the topography of their substratum, as discussed in thc introduction. The present investigations on the mechanism of contact guidance focus on the dynamic effect of high viscous media in relation to morphological, as explained below.

Viscosity as a probe of cell reaction to topography

The major result of this study is that the morphological reactions to topography and the movement of ccIls on topography were both markedly slowed when the medium viscosity is increased, thus viscosity is a pertinent variable. The inverse dependenee of reaction of cells to topography on viscosity strongly implies diffusional limitations to those reactions. As slower reactions were obtained for near-osmolar conditions of it is inferred that viseosity most likely affects the diffusional motions of moleeules in and out of thc spaee between cell and substratum. This in turn could nearly have exchange rates compared with a cell in suspension and make exchange rates similar to those in the body. The results suggest that such diffusional motions need to be more carefully considered in experimental design of cell reactions to topography because it will affect the space between cells and topography.

Cells are able to orient themselves in response to external signals. The sensitivity of cells to signal gradients may be extremely high. Cell polarisation initiated by gradients of an external signal is then stabiliscd by global reorganisation of the cytoskeleton. Change in the external conditions of cells may be affecting the cell polarisation by affecting the

redistribution of pseudopodia due to the reorganisation and orientation the whole cytoskeleton. Viscosity effects in narrow gaps on the outside of the eell may affect cell polarisation (papers of IRM study II and cytoskeleton study III, under construction).

Variation of medium viscosity

In these studies the medium viscosities were altered by adding small conceutrations of different macromolecules to the normal media (ECT and Ham, F10), such as Ficoll, Dextran, Mcthylcellulose and Carboxymethylcellulosc.

Tuvia *et al* (1997) studied the effect of mcdium macroviseosity on cell membrane fluctuations. They modified the solvent viscosity by the addition of high concentrations of small co-solvents such as glycerol and sucrose, producing relatively high viscosity levels. They considered the possibility that the effect of macroviscosity on cell function is transduced through a direct effect of extracellular fluid macroviseosity on cell membrane dynamics.

Tuvia in his study, determined the medium viscosity by measuring its flow time, relative to water, using an Ostwald viscometer at 25° C. The same method was used in this study to measure the medium viscosity but at more relevant temperature (37^{0}C). He also used Dextran and Carboxymethylcellulosc to increase the medium viscosity, the same macromolecules are being used in this study.

Chase et al (1998) also used an Ostwald viseometer to measure the viscosity of fibre solutions relative to that of water in order to correlate directly muscle fibre mechanisms with bulk solution viscosity (at 12^{0}C). In their study relative viscosity (η/η_0) was calculated according to:

$$\eta / \eta_0 = tp / t_0p_0$$

Where t is the flow time measured in the viscometer, p is density, and the subcript 0 indicates measurements on water. They estimated thc rclation of viscosity and diffusion by the equation

$$D = kT/6\Pi\eta r$$

Where k is Boltzmann's constant and T is absolutc temperaturc, r is the radius of a sphere and D is diffusion coefficient.

This is the same equation that is used to estimate the relationship between viscosity and diffusion in the present study.

Previously many workers suggested that many types of cells react to microtopgraphy and nanotopography by changes in important cell behaviour processes including: cell morphology, adhesion, changes in movement, contact guidance, and tissue organisation (Chehrouidi & Brunette, 1995; Brunette *et al,* 1988; Curtis & Clark, 1990; Singhvi *et al,* 1992), activation of tyrosine kinases (Nobes *et al,* 1995; Wojciak-Stothard *et al,* 1996), condensation of actin cytoskelcton (Rovensky & Samoilov, 1994; Oakley & Brunettc, 1993), and changcs in gene expression (Ohara and Buck, 1979; Oakley and Brunette, 1995; Webb *et al,* 1995; Meylc *et al,* 1994).

Since it is easy to observe the morphology of the cell, most of the data here refers simply to morphology and its concomitant orientation.

Other workers suggest that eells do not conform to many topographies as closely as they would to planar surfaces (Dunn & Brown, 1986; Brnnette, 1986; Clark *et al,* 1987; Curtis & Clark, 1990; Oakley & Brunette, 1993). Thc reason for this could bc that the eells

are under tension between attachments to groove edges and that this tension pulls the plasmalemma away from the groove bottom.

Effect of medium viscosity on extracellular matrix

The growth and development of an entire population society of cells could be eontrolled through struetural alterations of extracellular matrix (ECM) that produce assoeiated ehanges in eell shapc and assoeiated physieal foree redistributions (Ingber *et al,* 1981).

Ingber and Folkman (1987) stated that the extracellular matrix serves as a local "solid state" regulator of soluble growth factor action through its ability to modulate cell and nuelear structures. Another study from Maehesky and Hall (1997) mentioned that adherence of cells to extracellular matrix mediated through integrins is essential for normal cell development and movement.

Madri and Williams (1983), found that purified ECM molecules can modulate the growth and organisation of the capillary endothelial cells *in vitro.*

These suggestions seem important in the present study because the high viscosity medium could affect the thickness of the extracellular matrix. The matrix beeomes redueed in thickness by high viscosity molecule interpenetration by adding or removing water and in turn affects the diffusion of molecules into/ or out of the cells and also could affect the adhesion of cells to the matrix, which is reflected in the reduction of cell movements.

Boubriak *et al* (2000) found that the permeability coefficient of rabbit sclera of ECM decreases with an inerease in molecular weight solute (10 and 40 kD dextran). This finding could possibly be explained by the increase in asymmetry of the molecular shape with increase in dextran MW. Dextran are random coil extended polymers that do not have well-defined configuration, it can however take on asymmetric configurations. The resistance to motion of asymmetrical particles is characterised by the frictional eoefficients along the principle axes. Extended molecules would experience less resistance moving lengthwise than moving broadside, with the diffusion coefficient describing this 'end-on' movement dependent on the degree of asymmetry of the particle. In free solution the diffusion rate of asymmetrie molecules correlates predominantly with the long axis, because the molecules move randomly. In a matrix, an orientation effect causes restriction of broadside movement while offering little resistance lengthwise. The rate of diffusion of asymmetric molecules in a matrix appears to correlate mainly with the dimensions of the short axis, thus leading to an increase of diffusion rates in matrix in comparison with free solution.

It would therefore be anticipated that diffusion of solute through the ECM would decrease with the increase of matrix viseosity.

Explaining the morphological reaction results

These studies have reported changes in cell morphology or shape when cells were cultured in medium of high viscosity. Cell length was decreased when the concentration of high molecular weight reagent increased in the incubated medium.

There were several routes by which cells might respond to high viscosity of the medium one possibility is that cells shrink in presence of thick medium as shown in the morphology experiments.

General relevance to other aspects of cell biology.

The topography around cells may be that of surrounding cells, intercellular materials or biomaterials. The reactions to topography include cell orientation, rates of movement, and activation of the cells (Curtis and Wilkinson, 1997). Few studies have reported that the changes in cell shape can influence the gene expression (Chou *et al,* 1995), therefore it would seem reasonable to investigate the effect of medium viscosity on gene expression.

Cellular responses to medium viscosity may share common mechanisms involving cellular morphology; movements and responses to mechanical force (Abercrombie & Dunn, 1975; Heath & Dunn, 1978; Brunette *et al,* 1988, 1995; Elson, 1988; Curtis & Clark, 1990; Harris, 1994; Elson *et al,* 1998). For normal cells, this mechanism could play an important role during embryonic development and wound healing, allowing cellular proliferation or growth to be regulated in response to changes in chemical properties of the environment.

This study was the first report on how medium viscosity affects the reaction of cells to topography, which includes the morphological, locomotory responses, and the mechanism of these reactions. It is reasonable to continue this study by looking at the effects on cell function at the molecular level.

It would also be of interest to investigate the morphological reactions in greater detail, using electron microscopy. This would allow observation of cell spreading and polarisation at high resolution.

The adhesion pattern seems to be determined by the locomotory state of the cells (Verschueren, 1985). However, it is important to study the adhesion regions in the cells were they contact the substrate in the presence of medium of high viscosity because they are firm attachment structures that hold the cell in place and in its spread shape. This could be done using immunofluorescence of adhesion related proteins, such as integrins or vinculin.

Physical properties contribution possibility, rule out the importance to examine these properties in the presence of highly viscous medium e.g. forces.

One of the practical considerations stemming from this work is that viscosity needs to be carefully considered in both the design of reaction to topography experiments and interpretation of experimental data.

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تأثير لزوجة الوسط على شكل الخلايا أثناء تفاعلها مع السطوح الصناعية– I

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المستخلص. تؤثر لزوجة الوسط الذي تنمو فيه الخلايا الحية على تفاعلات الخلايا مع السطوح التي تنمو عليها مما قد يؤثر في نمو هذه الخلايا وشكلها الظاهري وحركتها.

الغرض من هذا البحث هو اكتشاف تأثير الانتشار على تفاعلات الخلايا مع السطوح حيث تمت تنمية الخلايا في وسط ذو لزوجة عالية على سطوح ناعمة وأخرى محززة. وقد تم تغيير لزوجة الوسط بإضافة جزيئات عالية اللزوجة مثل الديكستران، الفيكول، بوليفينايل بيرولدين، ميثيل سيليلوز وكربوكس ميثيل سيليلوزإلى بيئات طبيعية.

تمت التجربة باستخدام تركيزات مختلفة من الجزيئات عالية اللزوجة، والتي تختلف في حجم الجزيء وقدرته على تغيير لزوجة الوسط وطبيعته الكيميائية كما تم قياس لزوجة البيئات بقياس مدة سيولتها بالنسبة للماء باستخدام مقياس اللزوجة.

وأشتملت الدراسة على قياس لزوجة البيئات المستخدمة وأسموزيتها وثبت عدم تأثير الأسموزية على المحاليل.

كما ثبت تغير خاصية انتشار المواد بارتفاع لزوجة المحلول مما أثر على تفاعل الخلايا مع السطوح التي نمت عليها. وكانت النتائج تظهر التأثير المباشر على الشكل الظاهري للخلايا التي تحتضن في بيئات عالية اللزوجة بنقص أطوالها وتباطؤ حركتها.

ويبدو أن المحاليل ذات اللزوجة العالية تؤثر على الهيكل الخلوي فيصبح محدود التركيب والعمل.

وأظهرت تجربة المعالجة بأن الخلايا التي نمت في محاليل عاليـــة اللزوجـــة لاتزال حية وقد استعادت بتنميتها في بيئات ا عاديه خاصية الاستطالة والانتشار.

وتدعم هذه النتائج الفرضية بأن تأثير خاصية الانتشار حول الخلايا مهم فـــي تفاعل الخلايا مع السطوح النامية عليها. وتقترح اعتبار لزوجة البيئات عامل مهم في الوظائف الخلوية والكيموحيوية للخلايا.