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Measurement of the Viscosity of Mousse Myoblasts Modified with the αβ Cristalline through the Technique of Micromanipulation by the Means of Micropipette

A. Sako, A. Memeledje and P. Assamoi
UFR Sciences, Structure de la Matière et Technologique,
Laboratoire de Physique de l'Atmosphère et de Mécanique des Fluides,
Université de Cocody, Abidjan 22 BP 582 Abidjan 22, Cote d'Ivoire

Abstract: In this study, we measured directly through the technique of micromanipulation by micropipette the viscosity of mouse muscle cells modified by transfection with the αβ-crystalline in order to highlight the role played by the changes in the cytoskeleton. Two vectors were used, the αβ-crystalline of wild type and R129G αβ-crystalline mutant. The medium and intrinsic cell viscosities were determined and compared. For this purpose, two populations of muscle cell clones were used: WT5, WT6 for cells transfected with the αβ-crystalline of wild type and Mut9, Mut11 for cells transfected with R129G αβ-crystalline mutant. There is a difference between the medium and intrinsic viscosities of these different types of muscle cells of mice.

Key words: Viscosity, micromanipulation, micropipette, skeletal muscles, rheology, αβ-cristalin

INTRODUCTION

The shape of the cell and their mechanical properties are strongly related to the structure and properties of their cytoskeleton. The cytoskeleton is a component of the cell membrane. When we want to study the role of a component of the cell or a particular ingredient of the membrane, such as the intermediate filaments that play an important role in the rheological behavior of the cell, we use a process of transfection. Transfection involves the use of a vector that will serve to introduce into the DNA of the target cell one or more gene(s). Proteins are then secreted and will allow to ex-press the character you desire.

So, Djabali *et al.* (1997) and Perng (1999) have altered cells to increase the expression of certain cytoskeletal elements to highlight their role. To see some of these interactions, we have worked on myoblasts of mouse modified by transfection with the αβ crystalline through the technique of the micromanipulation by the means of micropipette to measure the cell viscosity. The Myoblasts of mouse are used as animal model for human muscle cells. The αβ crystalline is known as one of the most important components of the cytoskeleton. Present study aimed at measuring directly the viscosity of mouse Myoblasts though the means of manipulation by micropipette in order to show the impact of the organization of the cytoskeleton on cell viscosity.

Corresponding Author: Aboubakar Sako, UFR Sciences, Structure de la Matière et Technologique, Laboratoire de Physique de l'Atmosphère et de Mécanique des Fluides, Université de Cocody, Abidjan 22 BP 582 Abidjan 22, Cote d'Ivoire

The technique of micromanipulation through micropipette, although known as an old method (Evans and Kukan, 1984; Evans and Young, 1989) suits well in the mechanical properties of individual cells determining. We used it to directly determine the viscosity of human muscle cells or modified mouse muscle cells.

Before this study, we used it to us to measure the viscosity of the human red cells. Other authors used it to determine the viscosity of other types of cells like the chondrocytes (Hochmuth, 1999), the chick embryo fibroblasts, the neutrophils (Doncho and Needham, 1994), the granulocytes (Evans and Young, 1989). It is however the first time that it is used to determine the viscosity of human muscular cells and of modified mice.

MATERIAL AND METHODS

This study started in January 2008 ended in March 2009.

Biological Material

The biological material used is mainly composed of mouse muscle cells (These cells have been provided by the laboratory of the Cytoskeleton and Development, Medical School Pierre and Marie Curie, site Pitié-Salpêtrière CNRS UMR 7000. 105 Boulevard of the Hospital 75013 Paris. France (Faculté de Médecine Pierre et Marie Curie site Pitié-Salpêtrière and CNRS UMR 7000, 105 Boulevard de l'Hôpital), 75013 Paris or by the BTR (Banque de Tissus pour la Recherche: AFM-GENETHON), Hospital Pitié-Salpêtrière, Bulding Babinski, 83 Boulevard of the Hospital 75013 Paris. France (Hôpital Pitié-Salpêtrière bâtiment Babinski, 83 boulevard de l'hôpital), 75013 PARIS).

Five types of mouse myoblasts (muscle cells of mice) were used:

- Test myoblasts
- Clones of myoblasts transfected with αβ crystalline of wild-type
- The corresponding clones of cells are called WT5 and WT6
- Clones of myoblasts transfected with the R129G αβ mutant crystalline
- The corresponding clones of cells are called Mut9 and Mut11

The R129G mutant αβ crystalline was known for its aggregates generating action of desmin; consequently it impaired the intermediate filaments therefore, the mechanical behaviour of muscle cells (Carlsson and Fischer, 2002; Fardeau *et al*., 2000; Goldfarb *et al*., 2004; Hans and Fardeaub, 2004; Li *et al*., 1999; Vrabie, 2005).

Apparatus for Manufacturing Micropipettes

To make the micropipettes, the following devices are used:

- A vertical stretch (NARISHIGE), to stretch the glass tube to get a tube, more or less tapered according to the settings adopted
- A microforge (ALCATEL), to form the closed end of the tapered tube

Bench of Micromanipulation

The chassis of micromanipulation was the following:

- A monitor (Hitachi Denshi Ltd Hitachi Tokyo, Japan) to monitor the experiments
- An inverse microscope (Olympus TO41 Olympus Optical Corp. Ltd., Tokyo, Japan) equipped with a video camera (HV 720 F Hitachi Denshi Ltd, Tokyo, Japan) whose images are transferred to the monitor

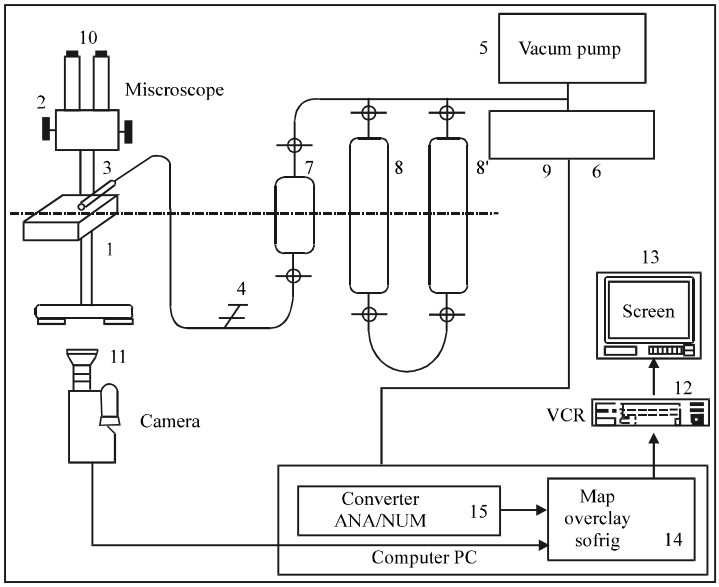

Fig. 1: Schematic overview of the bench of micromanipulation. 1: The transparent measuring chamber of 500 μL capacity, 2: The plate inverted microscope (OLYMPUS T041), 3: The micropipette, 4): The hydraulic micromanipulator (NARISHIGE), 5: The pump (KNF NEUBERGER MiniPort), 6: The pressure sensor reading digital (FC016 METER), 7: The adjustment flask, 8 and 8': the two identical water tanks, 9: The pressure sensor (VALIDYNE DP103) connected to a packer (VALIDYNE CD 15), 10: The microscope, 11: The camera, 12: The VCR, 13: The display screen, 14: The map overlay and 15: The converter ANA/NUM

- A hydraulic micromanipulator (Narishige micromanipulator, Tokyo, Japan) to move with precision the micropipette in the operative field.
- An orifice with a pressure transducer (Validyne, DP 103, Validyne Engineering Corp., Northridge, CA) to apply the necessary pressure to stick the cell to the micropipette or to cause its suction
- A VCR (JVC HR-D650MS Victor Company of Japan Ltd, Tokyo, Japan) to record experiences in real time
- A PC P2, with an acquisition software and image processing (SOFRIG Société Française d'Informatique et de Graphisme, Bobigny, France) for mixing images and informations about the experiment under way (Fig. 1)

Bench Scanning and Calculation

It consists of 3 elements:

- A PC DELL Optiplex GX280

It is structured around a motherboard 915G INTEL Pentium microprocessor with an Intel Pentium IV at 2.8 GHz, a 512 MB RAM, a graphics card Matrox Marvel G450 eTV, an internal hard drive WDC WD205 BA 80 GB capacity. This computer contains the software scan images (MATROX PC-VCR), the program AVIMECA and the macro used for the determination of the viscosity.

- An external hard drive MAXTOR One Touch 1394SBP2 IEEE 240 GB capacity for storing the digitized images
- A VCR SONY SLV-SE710 SMART ENGINE for the playback of video tapes on which are recorded the images of different experiments

Methods

Preparation of Cells

Populations of myogenic cells were isolated from muscle biopsies using Decary *et al.* (1997) method and that of cell raising. A piece of muscle biopsy was finely minced, 25 to 30 explants were cultivated in a petri dish with a diameter of 60 mm containing serum. The explants were tied and set in a humid atmosphere of about 37°C containing 5% CO_2 for 24 to 25 h. The above experimental environment was then changed with DMEM/F12 HAM (1/1) (GIBCO) containing 20% FCS. The transfection was done using the method of Schevzov *et al.* (1992). Four to five days later, the first mononuclear cells migrated out of explants, these cells are then trypsinised (trypsin-EDTA, GIBCO) collected by centrifugation and counted.

Process of Micromanipulation Experiment

The suspension containing the individual muscle cells was placed in a transparent measuring chamber of 500 μL capacity, designed to minimize the phenomenon of evaporation and yet to allow of an easy positioning of the micropipette.

The measuring chamber was on the deck of an inverted microscope. The Micropipette, filled with the suspension was inserted in the tank and connected to a hydraulic micromanipulator to perform micrometric displacement along 3 orthogonal axes with a sensitivity of 0.1 m. The pressure in the circuit can be established through a micrometric screw, accurate to 1/100 mm, which control the relative vertical displacements of two identical water tanks. Two systems for measuring the pressure could be used according to the experimental conditions. The pressure can on the one hand, be controlled with a pressure sensor (VALIDYNE DP103) connected to a packer (VALIDYNE CD 15) for measuring pressure between 0 and 500 Pa. On the other hand, the circuit of the pipette can be connected via a buffer tank, a pump (KNF NEUBERGER MiniPort) (5) connected to a pressure sensor reading digital (FC016 METER) to measure pressures between 0 and 20 000 Pa with an accuracy of about 1%.

Before the experiment itself, the pressure in the pipette is adjusted to that of the suspension finely adjusting the level of water in the adjustment flask. The calibration of device is made very carefully before each measurement. The tip of the micropipette is then positioned near the selected cell in the sample and the suction pressure set. Images of the suction are recorded and stored on the VCR via the acquisition software and image processing. The information about the experiment was embedded at the beginning of the experiment. The videotapes were labelled and numbered for their future use.

Digitization of Images and Calculation of the Viscosity

This process consisted in transferring the video images of an experiment from the magnetic stripe (where they are in analogical form) onto the hard disk of the computer of analysis (where they are in digital form).

The film is cut with a frequency of 20 Hz to meet the principle of persistence of vision. A picture is taken from the tape every 0.02 sec and then encoded (translated in bits) by the software (PC-MATROX VCR) of the scanning card. A name giving the characteristics of the

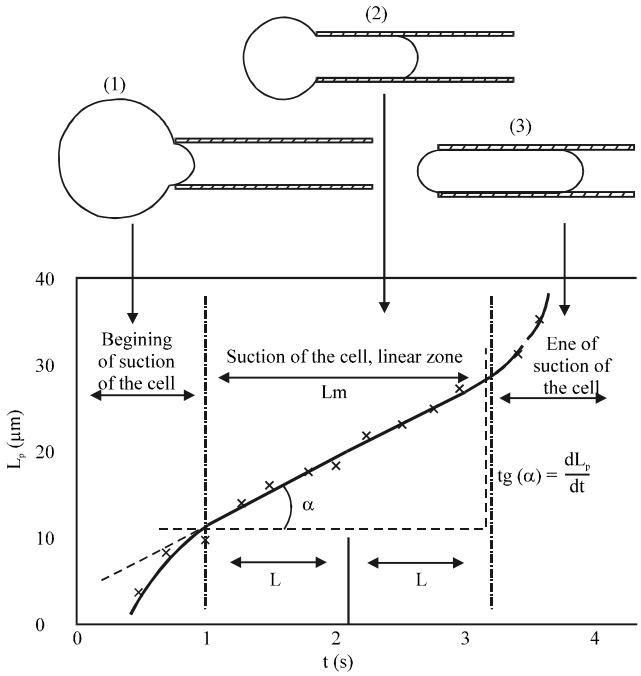

Fig. 2: The suction curve of a cell. $\underline{L} = L/R_p$ with L = Lm/2

experiment (number of the cell, cell type, number of the tape) can save the scanned images of the experiment to analyze on the external hard disk of large capacity (240 GB). A name is given to the created file in order to save it and it is performed at the location with the given name. After the images of the experiment have been digitized, we can proceed to their analysis.

For the analysis of these experiments, three programs were used:

- The pointing program which allowed the measuring of the cell suctioned length according to the time
- The Macro analysis program which allowed the calculation of the viscosity
- The synthesis program which provided an Excel spreadsheet for any information about the cell (cell type, date, experimental conditions, the cell radius, radius of the pipette, applied pressure ...) and its viscosity

The three programs are linked by written instructions also macro in Excel. The suction curve was plotted (Fig. 2).

$$\underline{Lm} = \frac{Lm}{R_P} \text{ (With Lm = Lp maximum)} \quad (1)$$

Lp maximum was the maximum suctioned length (Fig. 3). $\underline{Lm}$ was determined by the suction curve $\underline{L} = L/R_P$, was the middle of the linear portion of the suction curve (Fig. 2).

The suction curve has three parts. The first part corresponds to the entry phase of the cell in the pipette. The part of the cell outside the pipette and the tip of the pipette in the cell were spherical (Eq. 2).

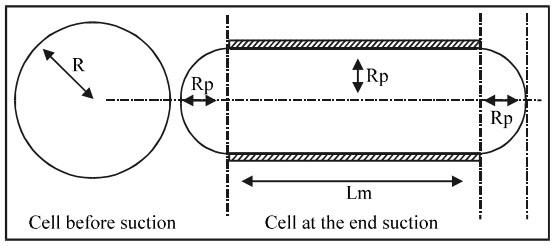

Fig. 3: Conservation of the volume during the suction of the cell

The second part of the curve corresponds to the suction phase of the cell at constant volume (Eq. 2). The last part of the curve corresponds to the end of the suction. The cell is almost entirely within the pipette (Eq. 3).

The viscosity is then calculated (Barthes-Biesel *et al*., 2006; Carin *et al*., 2003; Diaz *et al*., 2000; Doncho and Needham, 1994; Evans and Kukan *et al*., 1984; Evans and Young, 1989; Hochmuth, 1999, 2000; Lac *et al*., 2004; Lac and Barthes-Biesel, 2005, 2008; Lefebvre and Barthes-Biesel, 2007; Lefebvre *et al*., 2008; Lim *et al*., 2006; Thoumine *et al*., 1999) from the relationship 1:

$$\mu = \frac{\Delta P.R_p}{6\frac{dL_p}{dt}\left(1-\frac{R_p}{R}\right)} \tag{2}$$

The use of this relationship was related to the verification of the relationship which imposed the conservation of volume, relation 2, (Fig. 3).

$$\underline{R}^3 - 1 + (\underline{R}^2 + \frac{1}{2})(\underline{R}^2 - 1)^{1/2} = \frac{3}{2}(\underline{Lm} - \underline{L}) \tag{3}$$

In this relationship:

$$\underline{R} = \frac{R}{R_p} \tag{4}$$

where, R is the radius of the cell before suction and Rp the radius of the pipette

RESULTS AND DISCUSSION

Goldfarb *et al.* (2004) showed that transfection of mouse myoblasts with the αβ-crystalline of wild-type provokes a good organization of filaments in the cytoskeleton; on the contrary a transfection with the αβ-cristalline of mutant type causes the appearance of aggregates of desmin in the cytoskeleton. This is for instance the case of DRM (Desmine Related Myopathy). Thus, as cells transfected with the αβ-crystalline of wild-type we used myoblasts WT5 and WT6. For cells transfected with the αβ-crystalline of mutant-type we used myoblasts mut 9 and mut 11. The research-works (Sako, 2009) have also shown that it is possible to determine the viscosity of myoblast and that there is a difference of viscosity between myoblasts from healthy subjects and subjects with DRM.

Figure 4 shows the succession of images obtained during the suction of a mouse muscle cells (mouse myoblast). The cell is pressed against the micropipette by applying a

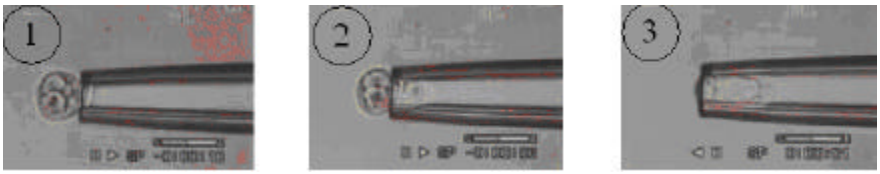

Fig. 4: Progression of a mouse muscle cell in the micropipette

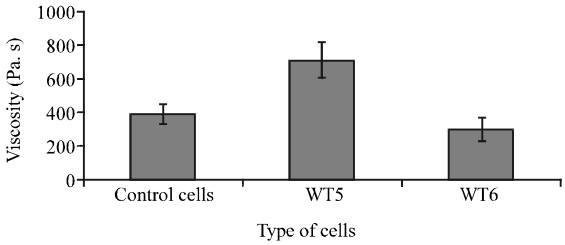

Fig. 5: Histogram of the cell viscosity according to the type of cells case of cells WT5 and WT6

Table 1: Cell type, viscosity, standard deviation of the viscosity and number of cells WT5 and WT6 treated

Type ofcells	Viscosity (Pa.s)	SD	No. of treated cells
Controls cells	391	65	15
WT5	710	110	16
WT6	301	71	13

vacuum p (1). The cell begins to enter the pipette. The suction of the cell has begun and is well advanced, the part of the cell outside the micropipette is still spherical and much of the cell was aspirated (Eq. 3). The suction is completed and the whole cell is inside the micropipette (Eq. 4). The curve of this suction shows the same curve as that obtained with other cell types previously studied (Fig. 2). There is a linear zone on the curve of the suction, the beginning and end of suction. The model (Eq. 2) is applicable to normal mouse myoblasts and the transfected ones.

Average value of the viscosity of cells transfected with the αβ-crystalline of wild-type WT5, WT6.

Table 1 gives the results of viscosity measurements of the control cells, WT5 and WT6 transfected with the α β -crystalline of wild type.

Figure 5 shows the evolution of the viscosity of muscle cells of control mice, WT5 and WT6. The viscosity of cells transfected with the αβ crystalline of wild-type (WT5, WT6) ranges from 400 to 700 Pa.s. It is about (391 65) Pa.s for control cells, (710 110) Pa.s for cells WT5and (381 71) Pa.s for cells WT6.

The compatibility diagram (Fig. 6) shows that there is no compatibility of viscosity between the control cells and WT5 on the one hand and between WT5 and WT6 on the other hand, whereas the viscosity values between control cells and cells WT6 are compatible. However these viscosities are of the same order of size. On average 467 Pa. s for control cells, WT5 and WT6.

Average value of the viscosity for cells transfected with R129G αβ mutant crystalline, Mut 9 and Mut 11.

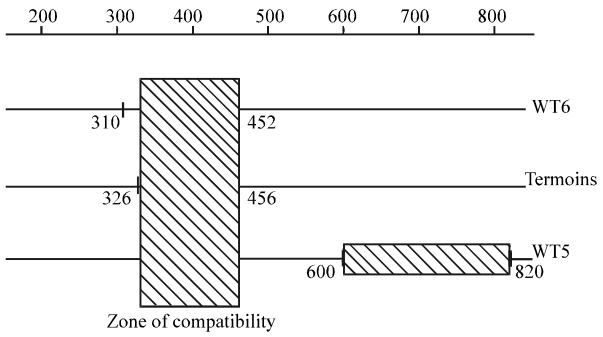

Fig. 6: Diagram of compatibility of viscosities for control transfectants, WT5 and WT6

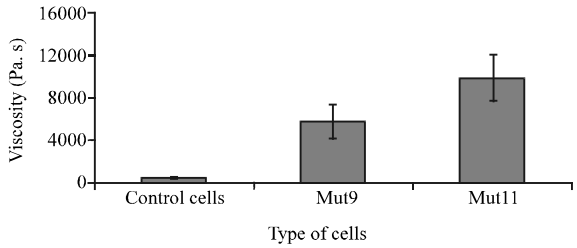

Fig. 7: Histogram of the cell viscosity according to the type of cells case of cells Mut9 and Mut11

Table 2: Cell type, viscosity, standard deviation of the viscosity, number of the Mut9 and Mut11 treated cells

Type ofcells	Viscosity (Pa.s)	SD	No. of treated cells
Controls cells	391	65	15
Mut9	5690	1502	29
Mut11	9790	2259	20

Table 2 gives the results of viscosity measurements for the control cells, cells Mut 9 and Mut 11 transfected with the αβ crystalline of mutant-type.

Figure 7 shows the evolution of the viscosity of muscle cells of control mice, Mut 9 and Mut 11.

For cells transfected with the αβ crystalline of mutant-type (Mut9, Mut11) the viscosity ranges between 5690 and 9790 Pa.s. It is about (391 65) Pa. s for control cells, (5690 1502) Pa.s for cells Mut 9 and (9790 2259) Pa.s for cells Mut 11. The compatibility diagram below (Fig. 8) shows that there is no compatibility of viscosity between control cells and Mut9 and Mut11 on the one hand and between the Mut9 and Mut11 on the other hand. The viscosity values for cells Mut9 and Mut11 however are of the same order of size.

Comparison of Viscosities Between Muscle Cells WT5, WT6 and Mut9, Mut11

The histogram in Fig. 9 shows the evolution of the viscosity of different types of cells. The medium value of the viscosity is about 467 Pa.s for the control cells, WT5, WT6 and that of the cells Mut9, Mut11 is about 7740 Pa.s. The difference of viscosity is clearly between cells of mice transfected with the αβ crystalline of wild type (WT5, WT6) which induces a good organization of the cytoskeleton and the cells of mice transfected with R129G αβ mutant crystalline (Mut9, Mut11) which provokes aggregates of desmin in the cytoskeleton.

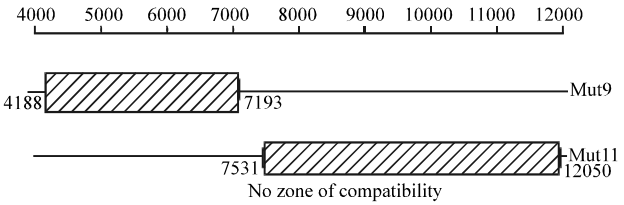

Fig. 8: Diagram of viscosities compatibility of transfectants Mut9 and Mut 11

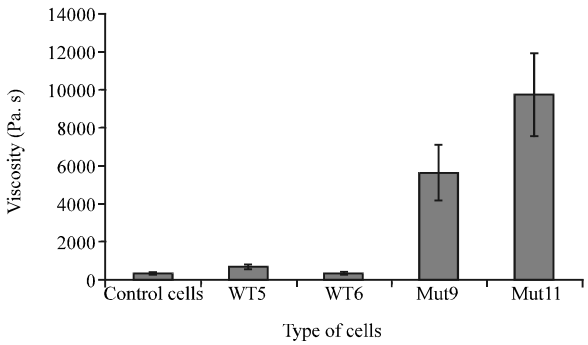

Fig. 9: Histogram of the cell viscosity according to the type of cells

A t-crossed test between the medium values of viscosity shows that:

- The cell viscosity of Mut 9 and Mut 11 is different at a level of 1%
- The cell viscosity of Mut 9, Mut 11 and the control group, WT5, WT6 is different at the level of 1%
- The cell viscosity of WT5 and the controls cells and WT5 and WT6 is different at the level of 5%
- The cell viscosities of the control muscle cells and the muscle cells WT6 are compatible
- Evolution of the viscosity according to the time, intrinsic viscosity
- Experimentally we notice that the viscosity depends on the maximum time of suction. A linear empirical law is proposed to connect the two measure

$$\mu=\mu_t.t+\mu_o \quad (5)$$

where, μ_t and μ_o are determined through linear regression by the method of least squares. μ_o is called intrinsic viscosity and μ_t coefficient of variation of viscosity according to the time.

Figure 10 shows the evolution of the viscosity of the muscle cells of mice WT6 according to the time

Table 3 gives the values of the intrinsic viscosity, the coefficient of variation of viscosity according to the time and the correlation coefficient of the control cells, cells WT5, WT6 and Mut9 and Mut11.

We notice that the intrinsic viscosity of cells WT5 (489 Pa.s) is greater than that of the control cells (280 Pa.s) and cells WT6 (120 Pa.s). This same trend is found when comparing the medium viscosities (Table 4) (710 Pa.s for WT5, 391 Pa.s for control cells and 301 Pa.s for cells WT6). However, the viscosity of cells WT6 (201 Pa.s/s) increases faster according the time than that of the control cells (20 Pa s/s) and WT5 (166 Pa. s/s).

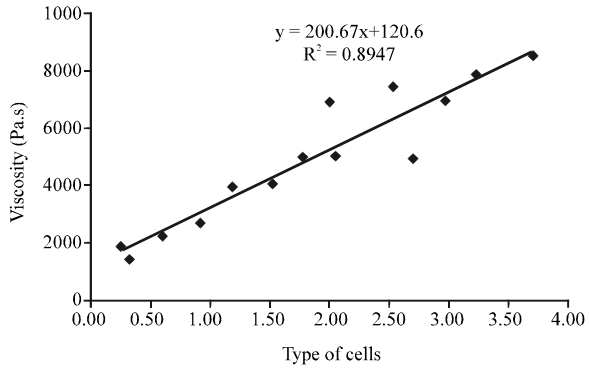

Fig. 10: Evolution of the viscosity of the cells WT6 according to the maximum time suction

Table 3: Intrinsic viscosity, coefficient of variation of the viscosity according to the time and determination coefficient of different types of cells

Type of cells	Viscosity (Pa.s)	SE
Controls cells	391	65
Mut9	5690	1502
Mut11	9790	2259

Table 4: Average viscosity and ES of control cells WT5, WT6

Type of cells	Viscosity (Pa.s)	SE
Controls cells	391	65
WT5	710	110
WT6	301	71

Table 5: Average viscosity and ES cells of control Mut9, Mut11

Type of cells	Viscosity (Pa.s)	SE
Controls cells	391	65
Mut9	5690	1502
Mut11	9790	2259

Table 6: Evolution of the diameter of cells according to their type

Type of cells	Diameter of cells (μm)	SD
Controls cells _PcDNA3(*)	16.50±0.64	0.64
WT5	17.32±0.80	0.80
WT5	16.20±0.66	0.66
Mut 9	17.26±0.64	0.64
Mut 11	16.80±0.44	0.44

(*) The control cells are cells transfected with the vector alone. They serve as control cell

The intrinsic viscosity of cells Mut9 (2814 Pa. s) is greater than that of cells Mut11 (1900 Pa. s), control cells (280 Pa.s), WT5 (489 Pa.s) and WT6 (120 Pa.s) . The cells Mut11 have the highest average viscosity, 9790, whereas there is 5690 Pa.s for cells Mut9 (Table 5). In terms of growth of the viscosity according to the time, the cells Mut11 have the highest coefficient (254 Pa.s/s)

Effect of Transfection on the Size of the Muscle Cells

The Table 6 gives the diameter of cells according to their type. A t-cross test applied to these medium values of diameters shows that they are not significantly different at the level of 1%. The difference in viscosity observed between muscle cells of mice WT5, WT6 and Mut9, Mut11 is not related to the size difference between cells.

CONCLUSION

The technique of micromanipulation by the micropipette was used to determine directly the viscosity of mouse myoblasts modified or not by transfection. The medium viscosity increased when myoblasts were transfected with R129G αβ crystalline which caused desmin aggregates in the cytoskeleton. The intrinsic viscosity followed the same trend. The rate of change of the viscosity according to the time of myoblasts transfected with the αβ crystalline of wild type is the smallest. The transfection of muscle cells with the αβ crystalline of wild type that induces a good organization of intermediate filaments of the cytoskeleton seemed to cause a decrease in the viscosity of muscle cells. The difference between the medium and the intrinsic viscosity of different types of mouse muscle cells did not seem to be related to the size of the cells. Cell diameters are compatible whatever the type of transfectant.

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