Iranian J Biotech. 2019 January; 17(1): e1863 DOI: 10.21859/ijb.1863

Brief Report

Effect of Pressure Stresses on Cell Viability and Protein Expression of Fascial Fibroblast

Bo Chen [1,*], Ying Jia [2], Jiangyan Xiong [3], Lei Chen [1], Minghong Luo [1], Weiyin Cheng [1], Yalin Wang [1], Feng Liu [1,4], Sibu Ma [5,6]

[1] College of Acupuncture and Massage, Guiyang University of Chinese Medicine, Guiyang Shi, Guizhou Sheng, China
[2] Department of Stomatology, Guizhou Medical University, Guizhou, China
[3] College of Nursing, Guiyang University of Chinese Medicine, Guiyang Shi, Guizhou Sheng, China
[4] Department of Emergency, Affiliated Hospital of Jiujiang University, Jiujiang, China
[5] College of Humanities, Guiyang University of Chinese Medicine, Guiyang, China
[6] Institute of Traditional Chinese Medicine of Minorities, Central University for Nationalities

[*] *Corresponding author:* Bo Chen, College of Acupuncture and Massage, Guiyang University of Chinese Medicine, Dongqing South Road Guiyang, Guiyang 550025, Guizhou, People's Republic of China. Tel: +86 130 0784 3202; Fax: +86 0851 8565 2189; E-mail: chenbo_med@163.com

Background: Many physical and mechanical phenomena occur during the acupuncture and tuina regime, and pressure is one of the most basic mechanical phenomena.
Objectives: To understand the cellular bio-physical mechanism of basic mechanical stimulation via acupuncture and tuina by investigating the effect of different *in vitro* pressures on the cell viability and protein expression differences that originate from the facial fibroblasts around the meridians.
Materials and Methods: *In vitro* culture of the facial fibroblasts around the meridians was conducted using different pressures to perform single and multiple stimulation(s) on the cells. Thus, the changes in the fibroblast cell viability (cell viability rate and diameter) were tested, and changes in the fibroblast protein expression were observed.
Results: We found that the pressure stimulation may excite the fascial fibroblast viability at the acupoint and increase cell viability. Two interactive factors are involved: the pressure intensity and the number of pressure stimulations. In addition, we found that all three pressures lead to significant regulation effects on the protein expression of the meridian-related fascial tissue fibroblasts, and clustering analysis revealed that 100 kPa pressure stimulation exhibits the most evident effect on the protein expression which is the pressure inducing the most differentiated protein expression.
Conclusions: During the *in vitro* pressure process, the difference in the cell viability rate and protein expression of the facial fibroblasts around the meridians may (from a cell mechanics' point-of-view) reveal the cytobiological and therapeutic mechanism of the basic mechanical stimulation via acupuncture and tuina on the facial fibroblasts around the meridians.

Keywords: Cell Survival; Fascia; Fibroblasts; Meridians

1. Background

Many physical and mechanical phenomena occur during the acupuncture and tuina regime, and pressure is one of the most basic mechanical phenomena (1, 2). Our team's pilot study showed that cells that are under pressure, which was the basic mechanical factor that was applied during the *in vitro* simulation during acupuncture and tuina, may not only promote the release of multiple biochemically active substances, such as NO, PGE2, MMP-1, TIMP-1, IL-1, IL-6, etc., but also down-regulate and/or maintain the synthesis of certain biochemically active substances, such as IGF-1 (3-5). These changes in the biochemically active substances occur in the meridians' acupoint region where the fascial connective tissue fibroblast originated, and the region receives and transfers the pressure stimulation via the filaments within the β1 integrin and cytoskeleton. However, neurological functions and other complex body fluid factors are not affected. The potential therapeutic and regulatory functions of the functional proteins occur, and this is the effect of "meridian patency, vital energy and blood smoothing, and vital qi strengthening" (6, 7). Unfortunately, currently, it is unclear how pressure stimulation can influence the cell viability rate of the meridian-related fascial fibroblasts, what may occur to the synthesis of more functional proteins inside the cells after the cells receive the stress,

and how the stability is maintained and how down-regulation is inhibited.

2. Objectives

Therefore, the present study focuses again on the meridians' acupoint-related fascial connective tissue fibroblast. Different pressures will be applied to further explore the *in vitro* effect of this type of stress on the biological behaviors of the meridian-related fascial connective tissue fibroblasts by observing the difference in the meridian-related fascial connective tissue fibroblast viability rates and protein expression. The results may provide an experimental understanding of the cellulate bio-physical mechanism that occurs due to basic mechanical stimulation during acupuncture and tuina.

3. Materials and Methods

3.1. Cultures of Primary and Passage Facial Fibroblasts around the Meridians

Use of animals was approved by the Ethic Committees of Guiyang University of Chinese Medicine and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

Pregnant (14 days pregnant) Kuming mice (Chongqing Tengxin Biotechnology Co., Ltd., China animal license No.: SCXK (Army) 2012-0011) were adopted. The pregnant mice were sacrificed via cervical dislocation, the abdomen was dissected, the uterus was cut open, and the fetus was exposed. The skin and subcutaneous tissue on and within 2 mm of the governor meridian (posterior midline, from neck to lumbosacral area) of the fetal mouse were taken, the fascial connective tissues were scraped with a scalpel, and the fat and blood vessels were removed. All of these operations were performed under a dissecting microscope. The subcutaneous tissues were placed in a culture dish and cut into slurries with an ophthalmic scissor. Three milliliters of 0.1% type I collagenase were added. We followed the general *in vitro* experimental protocols for extraction, cultivation, and passage to amplify the fibroblast.

3.2. Pressure Experiment on the Meridian-related Fascial Connective Tissue Fibroblast

The 5th to 8th generations of the meridian-related fascial connective tissue fibroblasts were used, and 0.25% pancreatin and 0.5 mL of EDTA were added to prepare the cellular suspension. The cell density was adjusted to $1\times10^4/mL^{-1}$ for inoculation in the 6-orifice plate, and then they were placed in the incubator. After 2-4 hours, 2 mL of serum-free DMEM was added to the 6-orifice plate, and the incubator cultivation continued for another 24 hours. We replaced the two ml of DMEM with 10% of FCS after cell synchronization.

The cell protein expression was investigated as a function of the load magnitude. We used 0 kPa (control group) and 50 kPa, pressure stimulation100 kPa, pressure stimulation and 200 kPa (different pressure stimulation groups) with six samples in each group. Moreover, the cell viability rate of each mechanical stimulation group was investigated as a function of the number of loads applied load into a single load group (i.e., load applied once) and a multiple load group (i.e., load applied three times) with three samples in each group.

The cells from the pressure stimulation groups were placed into a pressure vessel, and then moved into the incubator. A gas pump was installed in the incubator, and the incubator was pressurized with a mixed gas of 5% CO_2 + 95% air. The pressures within the vessel were maintained at 50 kPa, 100 kPa, or 200 kPa, for two hours for each applied load. The interval between the load applied in the multiple load group was 24 hours, during which time the culture was kept in the incubator for regular cultivation. Thus, the culture was maintained until after the load pressure experiments ended for inspection at a later time. The control was kept in the incubator for regular cultivation without any stimulation, and it was maintained for later inspection in addition to cells taken from each pressure group after the pressure experiments ended.

3.3. Sample Preparation and Tests for the Fibroblast Viability Rate

The stressed cells were placed into the incubator for another four hours of cultivation. The culture medium was absorbed and removed, and three cycles of PBS washing was performed for five min/cycle. Then, 0.25% pancreatin and 200 μL of EDTA was added, and the cell culture plate was gently shaken to sufficiently cover the entire bottom of the culture plate. The culture was then placed in the incubator for five minutes for digestion. Then, we added 800 μL of 10% FBS and DMEM to stop the digestion. We evenly distributed the cells on the culture plate and moved it into the viability rate meter-specified measuring cup, which was then placed into the cell viability rate meter for inspection.

3.4. Sample Preparation and Protein Expression of the Fibroblast

As shown above, the culture was then placed into the incubator for five minutes of digestion, and we added 800 μL of 10% FBS and DMEM to stop the digestion in addition to three cycles of PBS washing and centrifugation (1000 rmp/min for 8 min). The resulting cells were collected and frozen for later inspection. After the six samples from each group were mixed evenly, the AAM-BLM-1 antibody chip assays (**Fig. S1**; **Table S1**) by RayBiotech (Georgia, USA) were used. Detailed protocols referred to the Instruction for Use (IFU) of the assay kits.

3.5. Real-time Quantitative PCR

Total RNA was isolated from cells by RNA isolation Kit (Tiangen, Beijing, China) and reverse-transcribed into cDNA by M-MLV reverse transcriptase (Promega, Madison, WI). Real-time PCR was quantified by SYBR green mix (Takara, Dalian, China). Glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. The sequence of primers used are: IFN-gamma, F, 5'-GCCACGGCACAGTCATTGA-3', R, 5'-TGCTGATGGCCTGATTGTCTT-3'; CD30, 5'-CCTTCCCAACGGATCGACC-3', 5'-CCCGTCTTCATTGACGTAGTAGT-3'; EDAR, 5'-CCAACTGTGGTGAGAACGAAT-3', 5'-TCGTCGTCTTTAGTGCCGTAT-3'; NOV/CCN3, 5'-AGTGCCCCAGTATATCACCGA-3', 5'-TGCGGTCACAGTAGAGACCA-3'; P-Selectin, 5'-CCCTGGCAACAGCCTTCAG-3', 5'-GGGTCCTCAAAATCGTCATCC-3'; GAPDH, 5'-AGGTCGGTGTGAACGGATTTG-3', 5'-GGGGTCGTTGATGGCAACA-3'.

3.6. Statistical Analysis

All data are expressed as means ± SD. We used factorial ANOVA in the SPSS19.0 statistics software to determine if there was any statistical significance between the experimental group and the control. In the fibroblast protein expression investigation, we determined and counted the proteins that changed more than two magnitudes (up-regulation) and less than 0.5 magnitude (down-regulation) using a chemiluminescence imaging system. We used radiographic images that were obtained from a laser confocal scanner to quantify the grey scale for the original signal value, which, was standardized after correction with a positive protein to obtain the standard value. For the calculated data, we used the SPSS19.0 statistical software to perform row × column $\chi 2$ inspections to compare the cell protein expression of each group under different stimulation intensities and determine if there was any statistical difference for the inter-group changes. Finally, the clustering analysis software, Treeview, was used to export the clustering analysis chart to analyze the effect of different pressure stimulation intensities on the biological behaviors of the facial fibroblasts around the meridians.

4. Results

4.1. Effect of In vitro Pressure Stimulation on the Meridian-related Fascial Connective Tissue Fibroblast Viability Rate

As shown in the factorial ANOVA analysis, for the two 0 kPa groups that involved single and multiple stimulation(s), the cell viability rate was kept stable during the experiment. As the pressure was increased, the cell viability rate trends for each single stimulation group first increased and then decreased, and the cell viability rate for the 100 kPa group was the highest. For the multiple stimulation groups, the cell viability rate of the 50 kPa group was the highest, the cell viability rate of the 100 kPa decreased, and the cell viability rate of the 200 kPa group was the lowest. The different pressures are the reason why the viability rate of the meridian-related fascial connective tissue fibroblasts were evidently higher than that of the control group ($P < 0.01$). The different number of stimulations had a significant effect on the viability rate of the meridian-related fascial connective tissue fibroblasts ($P < 0.05$), and the intensity of pressure stimulation combined with the number of stimulations had a significant effect on the viability rate of the meridian-related fascial connective tissue fibroblasts ($P < 0.05$) (**Tables 1 and 2**).

Table 1. Effect of *in vitro* pressure stimulation on the meridian-related fascial connective tissue fibroblast viability rate

Groups	1 stimulation		3 stimulations		Total	
	n	Viability rate (%)	n	Viability rate (%)	n	Viability rate (%)
0 kPa	3	76.3333 ± 3.75411	3	76.2667 ± 1.79536	6	76.3000 ± 2.63211
50 kPa	3	82.4000 ± 1.21655	3	83.7667 ± 1.06927	6	83.0833 ± 1.26873
100 kPa	3	85.8333 ± 1.65025	3	80.1000 ± 2.00749	6	82.9667 ± 3.54439
200 kPa	3	82.8667 ± 2.20530	3	76.8333 ± 3.18172	6	79.8500 ± 4.11278

Table 2. Tests of between-subjects effects Dependent Variable: viability rate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	291.580 (a)	7	41.654	7.995	0.000	0.778
Intercept	155719.260	1	155719.260	29888.534	0.000	0.999
The times of stimulation	41.082	1	41.082	7.885	0.013	0.330
The intensity of stimulation	184.863	3	61.621	11.827	0.000	0.689
The times * the intensity	65.635	3	21.878	4.199	0.023	0.441
Error	83.360	16	5.210			
Total	156094.200	24				
Corrected Total	374.940	23				

a R Squared = 0.778 (Adjusted R Squared = 0.680).

4.2. Effect of in vitro Pressure Stimulation on the Diameter of the Meridian-related Fascial Connective Tissue Fibroblasts

As shown in the factorial ANOVA analysis, for the two 0 kPa groups that involved single and multiple stimulation(s), the cells kept growing and the diameter increased during the experiment. As the pressure increased, the cell diameter trend for each single stimulation group first increased and then decreased. The cell diameters in the 100 kPa group were the highest. As the pressure increased, the cell diameters in the multiple stimulations groups gradually increased, and the cell diameters in 200 kPa group were the highest. However, when compared with the control group, the different pressures pressure stimulation did not have a significant effect on the diameters of the meridian-related fascial connective tissue fibroblasts (P>0.05). Additionally, the different number of stimulations did not have a significant effect on the diameter of the meridian-related fascial connective tissue fibroblasts (P>0.05). The pressure and the number of stimulations combined did not result in any significant differences in the diameters of the meridian-related fascial connective tissue fibroblasts (P>0.05) (**Tables 3 and 4**).

Table 3. Effect of *in vitro* pressure stimulation on the diameter of the meridian-related fascial connective tissue fibroblasts

Groups	1 stimulation		3 stimulations		Total	
	n	Cell diameter (μm)	n	Cell diameter (μm)	n	Cell diameter (μm)
0 kPa	3	14.4933 ± 0.45982	3	14.8067 ± 0.34530	6	14.6500 ± 0.40214
50 kPa	3	14.5967 ± 0.65577	3	14.9267 ± 0.26633	6	14.7617 ± 0.48276
100 kPa	3	14.7033 ± 0.33171	3	14.9467 ± 0.38837	6	14.8250 ± 0.32390
200 kPa	3	14.5433 ± 0.20744	3	15.0200 ± 0.33151	6	14.7817 ± 0.35963

Table 4. Tests of between-subjects effects dependent variable: cell diameter

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	0.884 (a)	7	0.126	0.811	0.591	0.262
Intercept	5242.466	1	5242.466	33674.808	0.000	1.000
The times of stimulation	0.508	1	0.508	3.260	0.090	0.169
The intensity of stimulation	0.230	3	0.077	0.492	0.693	0.084
The times * the intensity	0.147	3	0.049	0.314	0.815	0.056
Error	2.491	16	0.156			
Total	5245.841	24				
Corrected Total	3.375	23				

a R Squared = 0.262 (Adjusted R Squared = -0.061).

4.3. Effect of the in vitro Pressure Stimulation on the Protein Expression of the Meridian-related Fascial Connective Tissue Fibroblasts

For this experiment, we chose a biotin-marked mouse antibody chip that can detect 308 proteins simultaneously. The antibody chip detection results were shown in **Figure 1A**. In detail, the positive control points are visible, whereas the negative control points are not detected, and the remaining protein points are irregularly presented, indicating that the quality control is accurate and the result is confident.

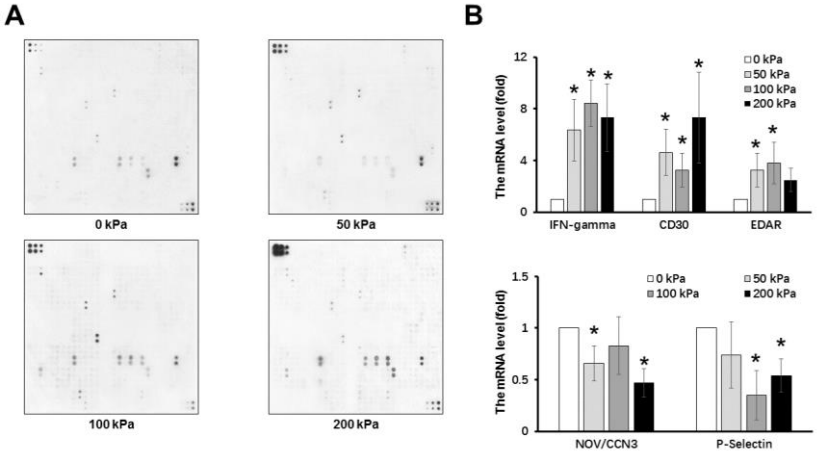

Figure 1. Effect of the *in vitro* pressure stimulation on the protein expression of the meridian-related fascial connective tissue fibroblasts. (A). A biotin-marked mouse antibody chip was chosen for this study, positive control strains were visible, and negative control strains were not detected. The remaining proteins were irregularly presented. (B). Real-time quantification PCR was used to verify the chip screening to confirm the observed variation in cell protein expressions. In all 3 strain groups, we observed upregulated proteins IFN-gamma, CD30, and EDAR; additionally, we also observed down-regulated proteins NOV/CCN3 and P-Selectin.

The three pressures resulted in a clear regulatory effect on the protein expression of the meridian-related fascial tissue fibroblasts; the comparison of the three inter-regulation groups showed a χ^2=59.005 with P<0.0005.

The overall regulation (including up-regulation and down-regulation) in the 50 kPa group was the smallest, and up-regulation prevailed. The magnitude of regulation in the 100 kPa group was the highest with the amount of protein that was up-regulated and down-regulated was the most of the three groups, and the amount of up-regulation was the highest. For the 200 kPa group, the magnitude of regulation was in between the other two groups, and up-regulation also prevailed. However, for the 200 kPa group, the amount of down-regulation was the lowest of the three groups (**Table S2**, **Table S3** **and** **Table S4**).

We used real-time quantitative PCR to verify the mRNA levels in these three groups. The results showed that compared to the control group the expression of the IFN-gamma, CD30, and EDAR in the pressure groups increased, whereas the expression of the NOV/CCN3 and P-Selectin in the pressure groups were significantly lower, which further confirmed the accuracy of the chip results (**Fig. 1B**).

4.4. Clustering Analysis of the Effect of Different Pressures on the Protein Expression of the Fibroblasts

The clustering analysis revealed pressure stimulation that the 200 kPa pressure had the lowest influence on the regulation of the fibroblast protein expression, followed by the 50 kPa pressure. The influence of the 100 kPa pressure stimulation on the protein expression was the highest, which is the pressure inducing the most differentiated protein expression (**Fig. 2**).

5. Discussion

The primitive stimulation approach is to press, squeeze, massage, and rub with the hands or fingers the body's meridians and acupuncture points. Therefore, pressure stimulation is one of the most primitive mechanical stimulations in acupuncture and tuina. From the perspective of modern medical physics and its mechanical analysis, no matter how the acupuncture and tuina approaches have evolved, pressure stimulationhas remained the most basic mechanical stimulation. Cell viability refers to the biological status and functions of the cells. There are various parameters that define the cell viability, such as vital cell ratio, cell size, oxidation-reduction potential of the cell mass, integrity of the cytomembrane, the enzymatic activity of the cells (e.g., esterase), etc. These parameters that provide a measurable and comparative metric for the health of the cells may be adopted separately or combined into cell viability research. Modern investigations show that the mechanical factor is also one of the major factors that impacts cell viability. In this study, we chose the cell viability rate and cell diameter as the metrics for cell viability. The results showed that w pressure stimulation can produce an overall increase in the acupoint fascial fibroblast viability by increasing the cell viability rate. Moreover, single (i.e., immediate effect) pressure stimulation and multiple (i.e., accumulative effect) pressure stresses resulted in different cell viability rate trends. For single load stimulation, the cells are sensitive to a medium load, whereas for multiple load stimulations, the cells are sensitive to a light load. The pressure stimulation encouraged the overall growth (i.e., increase in diameter) of the acupoint fascial fibroblast, but the load intensity, number of independent load stimulations, and the two factors combined all resulted in a statistical difference in the cell growth. Therefore, the pressure can increase the cell viability, and its effects on the cell viability are primarily due to the increase in the cell viability rate.

The clustering analysis revealed pressure stimulation that the 200 kPa pressure had the lowest influence on the regulation of the fibroblast protein expression, and the influence of the 100 kPa pressure stimulation on the protein expression was the highest.

Meanwhile, we used AAM-BLM-1 antibody chips from RayBiotech to detect the expression changes of 308 proteins. The results showed that a low pressure stimulation (50 kPa) up-regulated the expression of 106 proteins, maintained the expression of 130 proteins, and down-regulated the expression of 72 proteins. The medium pressure stimulation (100 kPa) up-regulated the expression of 170 proteins, maintained the expression of 83 proteins, and down-regulated the expression of 55 proteins. The 200 kPa pressure up-regulated the expression of 161 proteins, maintained the expression of 128 proteins, and down-regulated the expression of 19 proteins. The pressure stimulation of all three groups imposed evident regulatory effects on the expression of the facial fibroblasts around the meridians protein. The lowest pressure caused the lowest overall regulation (including up-regulation and down-regulation), with up-regulation prevailing. The medium pressure caused the maximum regulation, and there were more proteins that were up-regulated than in the other two groups. With this group, up-regulation also prevailed. The regulation of the heavy pressure group was similar to that of the medium pressure group. In addition, the clustering analysis revealed pressure stimulation that the medium pressure stimulation had the most influence on the protein expression, being the greatest differentiated protein expression among the clustering analysis.

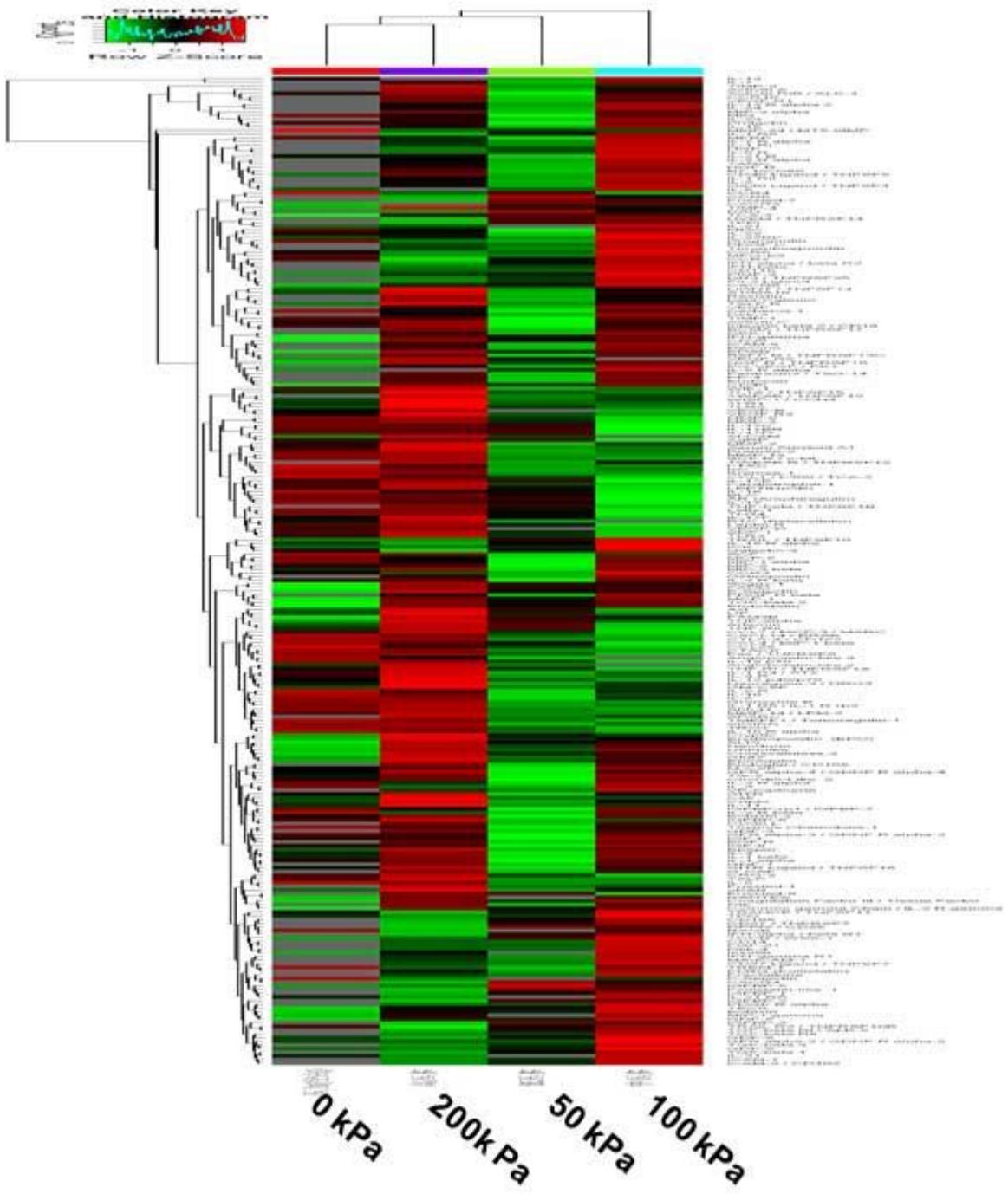

Figure 2. Clustering analysis of the effect of different pressures on the protein expression of the fibroblasts

According to our experiments, during *in vitro* pressure stress, the cell viability and protein expression of the meridian-related fascial connective tissue fibroblasts may change accordingly. All of these changes may (from a cellular mechanics' point-of-view) reveal the cytobiological and therapeutic feedback mechanism that occurs the meridian-related fascial connective tissue fibroblasts via simple mechanical stimulation (pure pressure stress) that occurs during acupuncture and tuina. Based on the mechanical intensity tests of this study, we observed that a medium pressure stimulation may activate the cell viability and protein synthesis/regulation functions the most. However, our study also shows that low and medium pressures contribute to the accumulated activation effects of pure pressure stimulation on the cell viability, whereas medium and heavy pressures contribute to the accumulated regulation effects on the cell protein expression. This difference requires further investigation to determine the cause of this phenomenon.

Acknowledgements

This work was supported by the Guizhou Science and Technology Foundation (Guizhou Science Cooperation J (2014) No. 2030), National Natural Science Foundation of China (No. 81160456) and Construction project of Key Laboratory of higher education in Guizhou Province (Guizhou Education KY [2014] No. 218).

Disclosure of Conflict of Interest

None.

References

1. Chen B, Jiang Y, Zhang X, Tian W, Wu Y. A new thinking on the mechanism of action of acupuncture and massage in the treatment of non-nerve tissue. *J Guiyang Coll Trad Chin Med*. 2012;**34**(1):9-12.
2. Chen B. Study on the Basic Micromechanics of Acupuncture. *J Guiyang Coll Trad Chin Med*. 2011;**33**(2):32-35.
3. Chen B, Luo YF, Cui J, Feng LM, Yang XF, Feng L. [Comparative study on effects of static pressure stimulation on release of PGE2 and IL-6 in fibroblasts in the rat "Zusanli" and its adjacent areas]. *Zhongguo Zhen Jiu*. 2007;**27**(2):135-140. pmid: 17370500
4. Chen B, Xinggui W, Xiaoyu L, Huang X, Jialin L. Effects of extracorporeal pressure stimulation on release of tumor necrosis factor-α and interferon-β by fascia tissue cells in the" Zusanli. *Lishizhen Med Mater Med Res*. 2011;**22**(3):734-736.
5. Chen B, Cui J, Xie X, Li J, Li X. Effects of external pressure stimulation on matrix metalloproteinase-1, matrix metalloproteinase-1 inhibitor, prostaglandin E2 and insulin-like growth factor 1 in rat fascia fibroblasts derived from "Zusanli". *Chin J Clin Rehabil Tissue Eng*. 2010;**14**(15):2756-2760.
6. Chen B, Xie X, Jia Y, Mingzhuang L. Effect of extracorporeal pressure stimulation on release of nitric oxide and interleukin-1ß by rat fascia tissue cells. *Chin Arch Trad Chin Med*. 2008;**23**:622-624.
7. Liu F, Hu T, Luo M, Chen B. Effects of Different pressure Stimulation on the Expression of Integrin ß1 Subunit and Microfilaments of Fibroblasts. *Guiyang Coll Trad Chin Med*. 2016;**38**:6-10.