The effects of different hydrostaticpressures on gene expression in vertebral cartilage endplate cells

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Research article

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Abstract

Background : The nutrients of the intervertebral disc are mainly provided through penetration from the cartilage endplate, and the stable functional status of the cartilage cells directly influences the function of the cartilage endplate. However, there is no study on gene expression in endplate cells under varying pressure conditions. Knowledge of anabolic/catabolic metabolic alterations of cartilage endplate cells in response to various magnitudes and durations of compression is important to alleviate IVD degeneration through maintenance of extracellular matrix homeostasis and the repair of endplate cartilage. The purpose of this study was to examine the effects of compression on gene expression in vertebral cartilage endplate cells in vitro through a hydrostatic pressure system.

Methods : Rabbit vertebral cartilage endplate cells were exposed to hydrostatic pressure at 0.1, 0.7, 2 and 4 MPa for 4 or 24 hours. Real-time polymerase chain reaction was performed to analyze the gene expression of inflammation (iNOS, COX-2), matrix metabolism (MMP-3), anticatabolic metabolism (TIMP-1), and anabolic metabolism (aggrecan).

Results : Decreased magnitude and duration showed more anticatabolic/anabolic metabolism gene expression, whereas increased duration resulted in increased catabolic gene expression.

Conclusions : Low magnitude or short duration of compression demonstrated more anabolic and anticatabolic gene expression, while increased magnitude and duration showed more procatabolic gene expression.

Background

Degenerative spinal diseases are frequently found in recent years. Currently, there are many studies focused on the nucleus pulposus and the annulus tissue. Although it is well known that intervertebral disc (IVD) degeneration is the main cause of low back pain and sciatica due to protrusion of IVD, the exact mechanism is still unclear. Several studies have shown that IVD degeneration may originate from endplate change [1, 2]. The cartilage endplate provide the mainly nutrients of the IVD and the metabolite exclusion channel. The function of the cartilage endplate was influences by the cartilage cells while its mechanism is still unclear. It has been reported that the cartilage endplate, bony endplate and trabecular bone below the endplate start to show deformation under axial pressure before irreversible changes occur in the structures. An increase in the magnitude or in the repeated load has a risk of irreversible injuries and even a fatigue fracture of the cartilage endplate [3]. The cartilage endplate maybe plays a significant role in the nutrient supply to human IVD. Calcification of the cartilage endplate significantly reduces the nutrient levels and excretion of metabolites in human IVD [4]. Previous studies have shown that IVD degeneration may originate from endplate change[5–7]. We thought the cartilage endplate was disturbed by hydrostatic pressurization in human IVD. There is a little apparent study on the gene expression of cartilage endplate cells with the application of hydrostatic pressurization[8, 9]. Therefore, we aimed at anabolic/catabolic metabolic alterations of cartilage endplate cells in response to various magnitudes

and durations of compression. By this way, we tried to alleviate IVD degeneration through the maintenance of extracellular matrix homeostasis and repair of endplate cartilage. We initially investigated the gene expression profiles of inflammatory cytokines and catabolic metabolism and anabolic metabolism in response to various magnitudes and durations of pressure using an in vitro model of rabbit intervertebral cartilage endplate cells to understand how mechanical signaling regulates the expression of genes involved in IVD degeneration. We hypothesized that various pressures result in alterations in key gene expressions of cartilage endplate cells.

Our methods are to test the gene expression in the model of rabbit intervertebral cartilage endplate cells with hydrostatic pressurization ideally, which was firstly used in this study. Based on the results, it might be possible to discuss the important effect of cartilage endplate cells in IVD degeneration.

Methods

Ethical statement

We get the ethical review permission from Capital Medical University, Beijing, China and obey the guidelines for the care and use of animals. There is a statement of the ethics committee indicating approval of the research (2014SB-011-01).

Design of the hydrostatic pressurization chamber

The hydrostatic pressurization chamber was manufactured by the company (Beijing Century Senlong Experimental Apparatus Co., Ltd) was used in this study. The machine is a convenient and completely used in i*n vitro*-operated hydrostatic pressure system (Fig. 1) and the same system was used in previous studies [10,11]. The major components include the pressure container, smart temperature control device, pneumatic supply and pressure indicator, as well as a piston device; distilled water is also used. The experimental system has a stable pneumatic supply, and it can monitor and regulate pressure in real time through a pressure gauge. The designed pressure range is 0-5 MPa. This system not only has the advantage of a general liquid chamber, but also the hydrostatic pressure can be altered as needed; therefore it can provide conditions of hypoxia and constant temperature which are similar to that in the human IVD, without changing ion composition and osmotic pressure in response to pressure.

Isolation and culture of rabbit intervertebral cartilage endplate cells

Intervertebral cartilage endplate cells were isolated from the adult Japanese white rabbit, Jw-Nibs strain, with an average weight of 2.5 kg and 50% male and 50% female (Beijing Vital River Lab Animal Technology Co., Ltd.). We can get sample from 4 discs in every rabbit. The number of animals in each experimental group was 2. The experiment is divided into 4 groups, each group has two times, so a total

of 16 rabbits was required. The housing of animals is cages with wood floor and hay. The number of cage companions is 4. The light/dark cycle is 12 with 22℃. They were feed with fresh water and forage in our animal center building. The animals are uthanized during the experiment by air embolization after injecting the phenobarbital sodium in rabbit ear veins. The second generation of cartilage cells cultured *in vitro* and the culture medium were randomly divided with random number table into 4 groups: the control group (0.1 MPa), the low pressure group (0.7 MPa), the medium pressure group (2 MPa) and the high pressure group (4 MPa). Culture medium was added to each group and then the cells were placed in the pressure vessel (loaded with 37°C distilled water) using a 20ml-plastic injector. Hydrostatic pressure was applied at 0.7 MPa, 2 MPa, and 4 MPa for 4 or 24 hours. The pressure vessel temperature was maintained at 37°C during loading by using the smart temperature control device. After the experiment, the animals are put into the special plastic bags and handed over to the animal center. Another researchers of our team assess results of the experiment independently.

Gene expression

Immediately after pressure application, the cartilage cells were removed from the sodium citrate dissolution. mRNA was isolated using an mRNA extraction kit (R2050; Zymo Research Corp., Irvine, CA). According to the kit instructions, OD260/OD280 was measured and RNA was detected by 1% agarose gel electrophoresis. For cDNA synthesis, 1 μg total RNA in each sample was used according to the instructions of Bios Script First Strand cDNA Synthesis Master Mix (Thermo Fisher Scientific KK, Tokyo, Japan). The designed primers are shown in Table 1. Real-time polymerase chain reaction (RT-PCR) was used for the analysis of gene expression. The tested genes included markers of inflammation (iNOS, COX-2), matrix metabolism (MMP-3), anticatabolic metabolism (TIMP-1), structural component (aggrecan) and reference gene (GAPDH). The ΔΔCt method was used to calculate relative gene expression. The primary experimental outcomes are the gene expression of aggrecan and Cox-2, and the secondary experimental outcomes are the expression of iNOS, MMP3 and TIMP.

Statistical analysis

The relative gene expression was calculated as a percentage in order to compare the effect of different intervention factors on the same gene. In the figures, the positive and negative values respectively represent augmentation and inhibition of gene expression compared to that in the control group. Values represent the average of three trials ± standard error, with 95% confidence intervals calculated to determine the statistical significance. The confidence interval was calculated based on the *t* distribution due to the small sample size. One-way ANOVA was performed to compare groups. All data were analyzed with SPSS18.0 and $p<0.05$ was considered as statistically significant.

Results

Percent of gene expression was compared with control cells after exposure to 4 and 24 hours of pressure at 0.7 MPa, 2 MPa and 4 MPa (Fig. 2). After 4 hours, gene expressions of aggrecan and COX-2 were first increased and then decreased as the pressure was increased (Fig. 2a). Aggrecan gene expression reached a peak at 2 MPa (111% higher than that at 0.7 MPa) and subsequently it was decreased at 4 MPa but was 38.3% higher than that at 0.7 MPa (Fig. 2a); COX-2 gene expression reached a peak at 2 MPa (33.0% higher than that at 0.7 MPa) and afterward it was decreased at 4 MPa (Fig. 2a). MMP3 and TIMP-1 gene expressions were gradually increased with the increased in pressure, and MMP 3 gene expression reached a peak at 4 MPa (122% higher than that at 0.7 MPa, 68.8% higher than that at 2 MPa), but the range of TIMP-1 gene expression was not as obvious as that of MMP3 gene expression (70.4% higher than that at 0.7 MPa, 38.9% higher than that at 2 MPa) (Fig. 2a).

At 24 hours, the variation of gene expression was distinct from those observed at 4 hours, except for TIMP-1 gene expression. The gene expression of aggrecan and iNOS was first suppressed (at 0.7 MPa), then augmented (at 2 MPa) and finally suppressed (at 4 MPa) (Fig. 2b). While that of COX-2 and MMP3 was augmented at low magnitude and suppressed at high magnitude; TIMP-1 gene expression did not show any change (Fig. 2b).

Discussion

After previus studies focused on IVD cells, we noticed the cartilage endplate is the vital organ for IVD [1,2,8,9]. There are two nutritional pathways for the IVD: the annulus fibrous pathway and the cartilage endplate pathway which is much more important for nutrition supply [12,13]. Cartilage endplate is the exchange passageway for nutrition and metabolites between the nucleus pulposus and the outside tissues.

Studies suggested the degeneration of IVD begin from cartilage endplate degeneration [14,15]. It seems protect or repair cartilage endplate may help to IVD [16]. Our research focus on cartilage endplate which was applied compression to elucidate the mechanism of disc degeneration.

It is well known that excessive pressure may result in disc degeneration, while moderate pressure is beneficial for the repair of intervertebral disc [5,6,17,18]. However, the signaling pathways initiated by mechanical stress and the relevant thresholds have not been precisely elucidated. In 1981, Nachemson reported a classical study on the *in vivo* measurement of intradiscal pressure [19]. The recent *in vivo* measurements indicate that lying prone results in an intradiscal pressure of 0.1 MPa, and bending down to lift a 20-kg weight results in 2.3 MPa [20]. As little as 1 MPa has been shown to have an effect on matrix gene expression in annulus fibrosus in vivo [21]. Wang et al found that during the weight lifting extension, the L3-4 disc endured a maximum shear load of about 230 N at the flexion position and maximum compressive load of 1500 N bodyweight at the upright position[22]. Fields et al showed that the endplate biochemical composition has a significant influence on its equilibrium tensile properties and that the presence of endplate damage is associated with a diminished composition-function relationship[23]. The data indicated that the anabolic and catabolic gene in cartilage endplate appeared

magnitude- and duration-dependent changes in response to pressure. The present data (Fig. 2 showed the inflammatory response and anabolic metabolism were augmented firstly and then inhibited with the increase of compressive stress at 0.1 MPa for 4 hours, while the catabolic and anticatabolic metabolisms were gradually increased. However, the increase amounts in catabolic genes were much larger than anticatabolic genes, indicating the more catabolic metabolism than anabolic metabolism. After 24 hours, the alteration of gene expressions was distinctly different than those observed at 4 hours. Anabolic metabolism, inflammatory responses and catabolic metabolism peaked at 2 MPa and were rapidly suppressed at 4 MPa. However, the expression of anticatabolic genes was gradually augmented with the increase of compressive stress. It was indicated that a low magnitude or short duration of compression in anabolic and anticatabolic gene expression. Since to date there is no similar study for referral, the innovative method of this study seems to be beneficial.

The limitations of our study is animal model which didn't use the mice because we need precisely to get the cartilage endplate cell. But we don't think rabbits are less reliable than mice and we think the rabbits are less reliable than human beings disc cells.

This study shows that moderate pressure in favor of the matrix homeostasis, and sustained pressure is good for the promotion of catabolic genes and is harmful to cell repair. We considered that vertebral endplate chondrocytes have a dependency of "load-time". It provided the basis for further exploration of the restoration of endplate cartilage that was a new idea of effective prevention of intervertebral disc degeneration.

If we collect the samples during spinal surgery in the future, the reliability of this experiment will be further improved.

Conclusions

The gene expression of cartilage endplate appeared magnitude- and duration-dependent changes in response to pressure. That facilitate development of drugs targeted towards delaying intervertebral disc degeneration.

Abbreviations

IVD: intervertebral disc; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; MMP-3: matrix metabolism-3; TIMP-1: anticatabolic metabolism-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Declarations

Ethics approval

There is a statement from our research ethics committee indicating approval of the research (2014SB-011-01).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

None.

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Authors' contributions

QS wrote the manuscript and provided data for Table 1, GZL planed and designed the experiment, Jiang Chen conducted all statistical analyses and YGZ searched literature and completed experiment. All authors reviewed the final manuscript. QS performed the data acquisition, assisted with the statistical analysis, interpreted the data, and drafted the manuscript. YGZ performed all statistical analyses and critically revised the manuscript. JC contributed to the data acquisition and critically revised the manuscript. GZL initiated and supervised the study, performed data acquisition, interpreted the data, and co-drafted the manuscript.

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Not applicable.

References

1. Shirazi-Adl A, Taheri M, Urban JP. Analysis of cell viability in intervertebral disc: Effect of endplate permeability on cell population. J Biomech. 2010;43:1330-6.

2. Haschtmann D, Stoyanov JV, Gédet P, Ferguson SJ. Vertebral endplate trauma induces disc cell apoptosis and promotes organ degeneration in vitro. Eur Spine J;17:289-99.
3. Cinotti G, Della Rocca C, Romeo S, Vittur F, Toffanin R, Trasimeni G. Degenerative changes of porcine intervertebral disc induced by vertebral endplate injuries. Spine (Phila Pa 1976). 2005;30:174-80.
4. Wu Y, Cisewski S, Sachs BL, Yao H. Effect of cartilage endplate on cell based disc regeneration: a finite element analysis. Mol Cell Biomech. 2013;10:159-82.
5. Moore RJ. The vertebral endplate: disc degeneration, disc regeneration. Eur Spine J 2006;15(Suppl 3):333-7.
6. Cao Y, Liao S, Zeng H, Ni S, Tintani F, Hao Y, Wang L, Wu T, Lu H, Duan C, Hu J. 3D characterization of morphological changes in the intervertebral disc and endplate during aging: A propagation phase contrast synchrotron micro-tomography study. Sci Rep. 2017;7:43094.
7. Rutges JP, Jagt van der OP, Oner FC, Verbout AJ, Castelein RJ, Kummer JA, Weinans H, Creemers LB, Dhert WJ. Micro-CT quantification of subchondral endplate changes in intervertebral disc degeneration. Osteoarthritis Cartilage. 2011;19:89-95.
8. Montagne K, Onuma Y, Ito Y, Aiki Y, Furukawa KS, Ushida T. High hydrostatic pressure induces pro-osteoarthritic changes in cartilage precursor cells: A transcriptome analysis. PLoS One. 2017;12:e0183226.
9. Cheleschi S, De Palma A, Pecorelli A, Pascarelli NA, Valacchi G, Belmonte G, Carta S, Galeazzi M, Fioravanti A. Hydrostatic pressure regulates microRNA expression levels in osteoarthritic chondrocyte cultures via the Wnt/β-Catenin pathway. Int J Mol Sci. 2017;18. pii: E133.
10. Handa T, Ishihara H, Ohshima H, Osada R, Tsuji H, Obata K. Effects of hydrostatic pressure on matrix synthesis and matrix metalloproteinase production in the human lumbar intervertebral disc. Spine (Phila Pa 1976). 1997;22:1085-91.
11. Liu G, Ishihara H, Osada R, Kimura T, Tsuji H. Nitric oxide mediates the change of proteoglycan synthesis in the human lumbar intervertebral disc in response to hydrostatic pressure. Spine (Phila Pa 1976). 2001;26:134-41.
12. Katz MM, Hargens AR, Garfin SR. Intervertebral disc nutrition. Diffusion versus convection. Clin Orthop Relat Res. 1986;(210):243-5.
13. Hamilton DJ, Seguin CA, Wang J, Pilliar RM, Kandel RA; BioEngineering of Skeletal Tissues Team. Formation of a nucleus pulposus-cartilage endplate construct in vitro. Biomaterials. 2006;27:397-405.
14. Boyd LM, Carter AJ. Injectable biomaterials and vertebral endplate treatment for repair and regeneration of the intervertebral disc. Eur Spine J. 2006;15 Suppl 3:S414-21.
15. Cinotti G, Della Rocca C, Romeo S, Vittur F, Toffanin R, Trasimeni G. Degenerative changes of porcine intervertebral disc induced by vertebral endplate injuries. Spine (Phila Pa 1976). 2005;30:174-80.
16. Neidlinger-Wilke C, Würtz K, Urban JP, Börm W, Arand M, Ignatius A, Wilke HJ, Claes LE. Regulation of gene expression in intervertebral disc cells by low and high hydrostatic pressure. Eur Spine J.

2006;15 Suppl 3:S372-8.
17. Chen J, Baer AE, Paik PY, Yan W, Setton LA. Matrix protein gene expression in intervertebral disc cells subjected to altered osmolarity. Biochem Biophys Res Commun. 2002;293:932-8.
18. Ishihara H, McNally DS, Urban JP, Hall AC. Effects of hydrostatic pressure on matrix synthesis in different regions of the intervertebral disk. J Appl Physiol (1985). 1996;80:839-46.
19. Nachemson AL. Disc pressure measurements. Spine (Phila Pa 1976). 1981;6:93-7.
20. Wilke H-J, Neef P, Caimi M, Hoogland T, Claes LE. New in vivo measurements of pressures in the intervertebral disc in daily life. Spine (Phila Pa 1976). 1999;24:755-62.
21. LeMaitre CL, Freemont AJ, Hoyland JA. Localization of degradative enzymes and their inhibitors in the degerate human intervertebral disc. J Pathol. 2004;204:47-54.
22. Wang S, Park WM, Kim YH, Cha T, Wood K, Li G. In vivo loads in the lumbar L3-4 disc during a weight lifting extension. Clin Biomech (Bristol, Avon). 2014;29:155-60.
23. Fields AJ, Rodriguez D, Gary KN, Liebenberg EC, Lotz JC. Influence of biochemical composition on endplate cartilage tensile properties in the human lumbar spine. J Orthop Res. 2014;32:245-52.

Table 1

Table 1 Primer sequences used

ene	Sequence
ggrecan	For: ’-GCTACGGAGACAAGGATGAGTTC-3’
	Rev: 5’-CGTAAAAGACCTCACCCTCCAT-3’
OX-2	For: 5’-CACGCAGGTGGAGATGATCTAC-3’
	Rev: 5’-CAGGCACCAGACCAAACACTT-3’
NOS	For: 5’-CCCCTTCAACGGCTGGTA-3’
	Rev: 5’-TCTGTGACGGCCTGATCTTTC-3’
MP-3	For: 5’-AGCCAATGGAAATGAAAACTCTTC-3’
	Rev: 5’-CCAGTGGATAGGCTGAGCAAA-3’
IMP-1	For: 5’-AGCAGAGCCTGCACCTGTGT-3’
	Rev: 5’-CCACAAACTTGGCCCTGATG-3’
APDH	For: 5’-GATGCTGGTGCCGAGTAC-3’
	Rev: 5’-GCTGAGATGATGACCCTTTTGG-3’

Figures

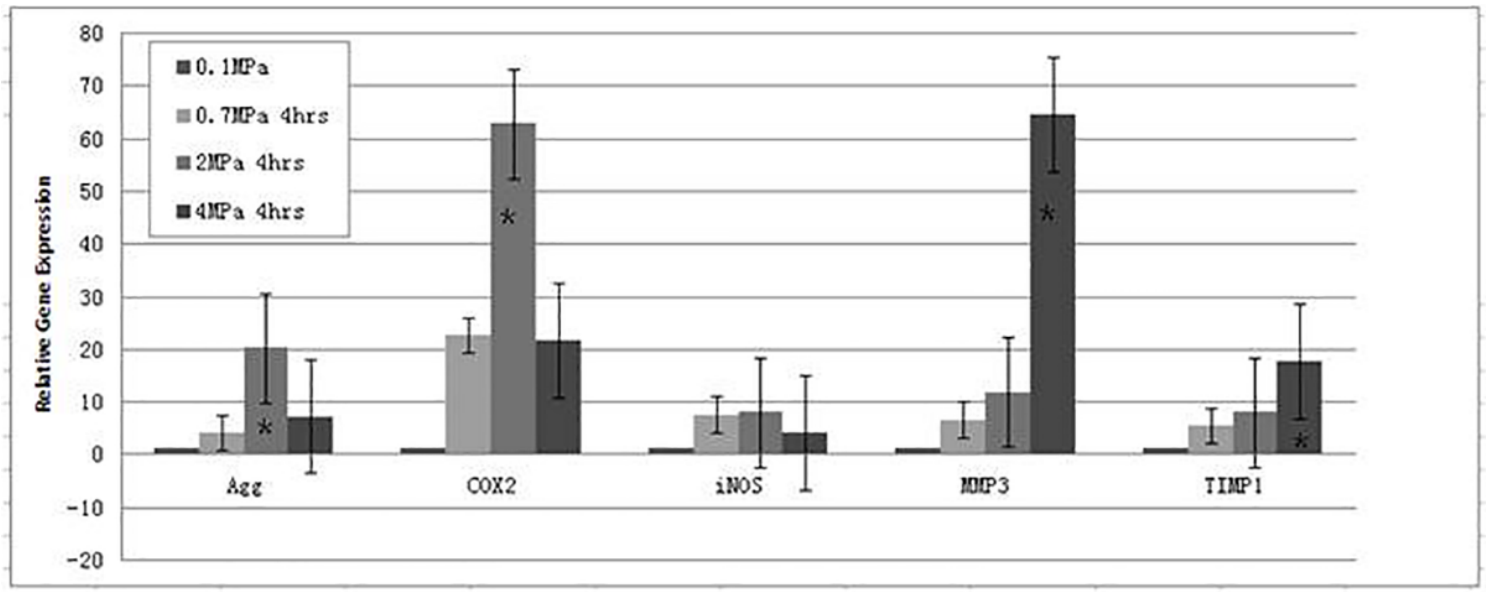

Figure 1

Schematic and photograph of the hydrostatic pressurization chamber

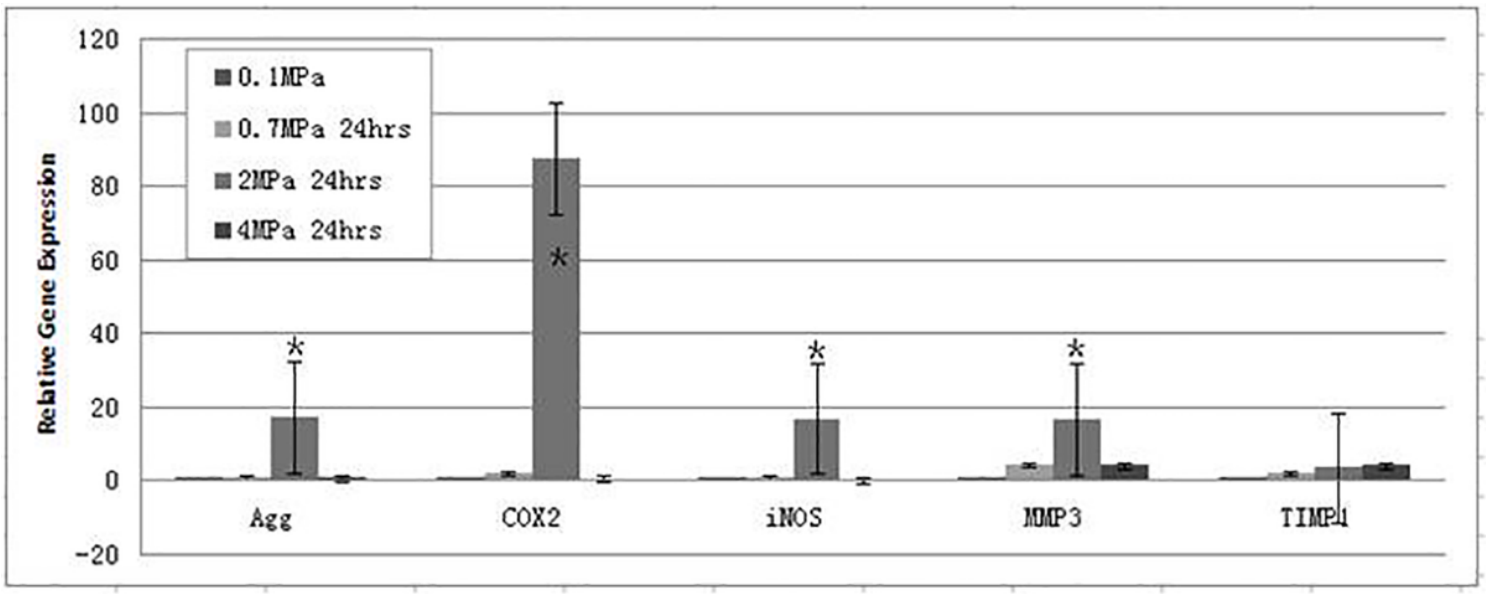

Figure 2

Percent gene expression compared with control cells after exposure to 4 hours of compression. (a) and 24 hours of compression (b) at 0.1 MPa, 0.7 MPa, 2 MPa and 4 MPa. Data are reported relative to control cell gene expression average ± standard error.

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