



Resveratrol could reverse the expression of SIRT1 and MMP-1 *in vitro*

J.W. Wu¹, J.J. Wang², J.B. Chen¹, Y.L. Huang¹, H. Wang¹, G.H. Liu¹, L.F. Li³, M. Kang¹, X.G. Wang¹ and H.H. Cai¹

¹Department of Spine Surgery, HuiZhou Municipal Central Hospital, HuiZhou, Guangdong, China

²Department of Ophthalmology, HuiZhou Municipal Central Hospital, HuiZhou, Guangdong, China

³Department of Emergency, HuiZhou Municipal Central Hospital, HuiZhou, Guangdong, China

Corresponding author: H.H. Cai

E-mail: honhuacai@126.com

Genet. Mol. Res. 14 (4): 12386-12393 (2015)

Received April 26, 2015

Accepted August 15, 2015

Published October 16, 2015

DOI <http://dx.doi.org/10.4238/2015.October.16.5>

ABSTRACT. Intervertebral disc degeneration is the main cause of lumbago disease, in which the extracellular matrix structure and moisture in the nucleus pulposus is lost continuously. In this study, we aimed to detect differential expression of silence mating type information regulation 2 homolog 1 (SIRT1) and matrix metalloproteinase-1 (MMP-1) in human intervertebral disc nucleus pulposus cells and to explore the effects of SIRT1 and MMP-1 on the development of the intervertebral disc degeneration. Intervertebral disc nucleus pulposus specimens from 41 patients who underwent lumbar protrusion resection at HuiZhou Municipal Central Hospital, during the period from October 2011 to December 2013, were studied in comparison with 23 control cases from patients who underwent fractured lumbar resection. In degenerated human intervertebral disc nucleus pulposus cells, the expression of SIRT1 is decreased and MMP-1 is increased compared with that of the control cells.

Resveratrol could reverse these effects, thereby increasing the expression of *SIRT1* (0.87 ± 0.07 vs 0.54 ± 0.04), *Coll2a1* (0.90 ± 0.08 vs 0.38 ± 0.01), and *aggrecan* (0.69 ± 0.07 vs 0.42 ± 0.05) and decreasing the expression of *MMP-1* (0.61 ± 0.03 vs 0.93 ± 0.08). These results suggest that resveratrol could possibly reverse the process of intervertebral disc degeneration and thus could be applied as a potential drug for the disease.

Key words: Resveratrol; SIRT1; MMP-1; Immunohistochemistry; Intervertebral disc nucleus pulposus

INTRODUCTION

Lumbago disease, which has a high incidence and disability rate, has become a serious problem affecting the quality of life of people worldwide (Becker et al., 2012). About 80% of the aged population have lumbago disease (lower back pain), and intervertebral disc degeneration is the main cause of lumbago diseases, such as prolapse of lumbar intervertebral disc, lumbar spondylolisthesis, and lumbar discogenic pain (Brisby, 2010). During the occurrence and development of nucleus pulposus degeneration, extracellular matrix structure and moisture is lost continuously; hence, the normal intervertebral disc structure changes resulting in abnormal function thereafter (Shen et al., 2012). Many factors and genes are associated with this process (Liang et al., 2012). Current studies show that there is a close relationship between the intervertebral disc diseases and matrix metabolism imbalance (Matrisian, 1990; Boileau et al., 2011; Chen et al., 2011). Silence mating type information regulation 2 homolog 1 (SIRT1), by inhibiting the expression of matrix metalloproteinase-1 (MMP-1), promotes cartilage extracellular matrix synthesis (Wang et al., 2012). In this study, we detected the expression of SIRT1 and MMP-1 in degenerative human nucleus pulposus intervertebral disc specimens and analyzed the functions of SIRT1 and MMP-1 in the development of intervertebral disc degeneration, in order to find a novel target for the therapy of intervertebral disc degenerative diseases.

MATERIAL AND METHODS

Clinical material

This study was approved by the Ethics Committee of HuiZhou Municipal Central Hospital, and informed consent was signed by the patients. The intervertebral disc nucleus pulposus specimens obtained from 41 patients who had undergone lumbar protrusion resection at HuiZhou Municipal Central Hospital during the period from October 2011 to December 2013 were designated as the experimental group, while the nucleus pulposus specimens obtained from 23 patients who had undergone fractured lumbar resection were assigned to the control group. The experimental group comprised the following: 25 males and 16 females, aged 22 to 75 years old with an average age of 47.25 ± 5.62 years; and specimens obtained from the L4-5 position (nucleus pulposus) of 24 patients and the L5/S1 position from 17 patients. The experimental group inclusion criteria were typical clinical symptoms and signs such as back pain or/and lower limb pain, reduced intervertebral space and lumbar disc hernia-

tion; significantly reduction of intervertebral space by X-ray; MRI displayed prolapse of lumbar intervertebral disc; and low TW2 nucleus pulposus signal. The control group comprised 14 males and 9 females, aged 18 to 27 years old with an average age of 21.76 ± 1.04 years; specimens obtained from the L4-5 position of 13 patients and the L5/S position of 17 patients; and Pfirrmann grade level III to IV. The control group inclusion criteria were burst fracture of the lumbar vertebra before the onset of the disease and no history of lumbar and leg pain; MRI detection of intervertebral disc without prolapse; increased TW2 nucleus pulposus signal (Hong and Xu, 2010).

Cell preparation, culture, and passage

The nucleus pulposus tissue specimens were processed into 1-cm sections and then were incubated in 10 mL 2% type II collagenase (Beijing Zhong Shan Biological Co. Ltd. Beijing, China) and stirred with a magnetic stirrer until the tissue was dissolved completely. Then, cells were centrifuged at 1200 g for 10 min, the supernatant was removed, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad CA, USA) to obtain a suspension with a cell concentration of 1×10^5 /mL nucleus pulposus cells. Static cultures were maintained in an incubator at 37°C with saturated humidity and 5% CO₂. Every 3 days, the medium was changed, and after 15 days, the medium was changed to 20% FBS. When cells grew to 95% confluence, 1 mL 0.25% trypsin digestion solution with 0.01% EDTA was added for 5 min, and cells were centrifuged at 1000 g for 10 min to isolate the P2 cell suspension from the supernatant. The suspension was cultured continuously with 10% FBS to a concentration of 1×10^5 /mL nucleus pulposus P2 cells (Hu et al., 2011).

Immunohistochemistry

The nucleus pulposus cell specimens were fixed with 4% polyformaldehyde solution, embedded in paraffin, and then sliced to 4- μ m thickness. Then, the slice was dewaxed, dehydrated, and incubated at 40°C with 3% H₂O₂ blocker and rabbit anti-human SIRT1 monoclonal antibody (Expro (Shanghai) Trading Co., Ltd., Shanghai, China) or rabbit anti-human MMP-1 polyclonal antibody (Santa Cruz Biotech Inc., Dallas, TX, USA) overnight. Chromogenic detection by the 3,3'-diaminobenzidine method was performed using a two-step immunoassay kit (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China) according to kit instructions.

Quantitative fluorescent polymerase chain reaction (PCR) detection

Total cellular RNA was extracted using the RNAiso Plus kit (TaKaRa Biotechnology (Dalian) Co., Ltd., Japan) according to the manufacturer instructions. RNA was reverse-transcribed into cDNA, and quantitative fluorescent PCR was carried out using SYBR reagent (Toyobo (Shanghai) Biological Technology Co., Ltd., Japan). Primers were synthesized by the Shanghai Biological Engineering Co., Ltd. (Shanghai, China). Primer sequences were as follows: *SIRT1*, upstream 5'-ccagaacatagacacgctgaac-3', downstream 5'-ctcctcgtacagcttcacagtca-3'; *MMP-1*, upstream 5'-ggctgaaagtgacfgggaac-3', downstream 5'-ttggcactggcgtgt-3';

collagen type II, alpha 1 (Coll2a1), upstream 5'-ctcaagtcctcaacaaccaga-3', downstream 5'-ggggtcaatccagtagtctccac-3'; proteoglycan (*aggrecan*), upstream 5'-gccagcaccaccaatgtaagt-3', downstream 5'-agtaacaccctccacgaactcag-3'; internal reference *glyceraldehyde 3-phosphate (GAPDH)*, upstream 5'-ctttggtatcgtggaaggactc-3', downstream 5'-gtagaggcaggatgatgttct-3'. The reaction conditions were as follows: 95°C pre-denaturation for 3 min, 95°C denaturation for 20 s, 58°C annealing for 20 s, and 72°C extension for 20 s, for 35 cycles (Shen et al., 2013).

Resveratrol (RES) intervention experiment

When the cultured degenerated human nucleus pulposus P2 cells had grown to 90% fusion or higher, cells were divided randomly into intervention and non-intervention groups. The intervention group was treated with 50 mM RES (Hubei Yuancheng Pharmaceutical Company Ltd., Wuhan, China), while the non-intervention group was treated with DMSO only. After 24 h, the expression levels of *SIRT1*, *MMP-1*, *Coll2a1*, and *aggrecan* in the two groups were detected by quantitative fluorescent PCR.

Consequence determination

Consequence determination was performed as described previously (Chen et al., 2011, Shen et al., 2012, 2013).

Statistical analysis

A database of the results was established using Excel 2007 (Redmond, WA, USA). Data analysis was carried out using the SPSS v.18.0 statistical analysis software (Chicago, IL, USA), and data are reported as means \pm standard deviation. The Student *t*-test was used for comparison between two groups, and Pearson correlation analysis was used to determine the correlation between groups. A P value less than 0.05 indicates statistical significance.

RESULTS

Expression of *SIRT1* and *MMP-1* in nucleus pulposus cells

As shown in Table 1, the results of immunohistochemical examination suggest that the positive expression of SIRT1 in the nucleus pulposus cells of the experimental group was significantly lower than that of the control group ($P < 0.05$), whereas the expression of MMP-1 was significantly higher ($P < 0.05$) than that of the control group.

Expression of *SIRT1* and *MMP-1* in nucleus pulposus P2 cells

As shown in Table 2, the results from quantitative fluorescent PCR suggest that the positive expression of *SIRT1* in nucleus pulposus P2 cells of the experimental group was significantly lower than that of the control group ($P < 0.05$), whereas the expression of *MMP-1* was significantly higher ($P < 0.05$) than that of the control group.

Table 1. Differential expression of *SIRT1* and *MMP-1* in two groups of nucleus pulposus cells of immunohistochemical examination.

Group	Number of cases	<i>SIRT1</i>	<i>MMP-1</i>
Experimental	41	29.61 ± 3.05*	37.83 ± 3.94*
Control	23	53.74 ± 3.86*	12.45 ± 4.63*
<i>t</i> -test		3.752	5.194
P		0.038	0.025

SIRT1: silencing mating type information regulation 2 homolog 1; *MMP-1*: matrix metalloproteinase-1. *Values were normalized expression (means ± SD).

Table 2. Differentiated expression of *SIRT1* and *MMP-1* in two nucleus pulposus P2 cell groups of RT-PCR.

Group	Number of cases	<i>SIRT1</i>	<i>MMP-1</i>
Experimental	41	0.58 ± 0.03*	0.95 ± 0.08*
Control	23	0.91 ± 0.07*	0.62 ± 0.02*
<i>t</i> -test		4.307	4.285
P		0.032	0.033

SIRT1: silencing mating type information regulation 2 homolog 1; *MMP-1*: matrix metalloproteinase-1. *Values were normalized expression (means ± SD).

Expression of *SIRT1*, *MMP-1*, *Coll2a1*, and *aggrecan* after RES intervention

As shown in Table 3, after RES intervention, expression levels of *SIRT1*, *Coll2a1*, and *aggrecan* increased significantly compared with those of the non-intervention group ($P < 0.05$), whereas the expression of *MMP-1* decreased remarkably ($P < 0.05$) in the intervention group compared with that of the non-intervention group.

Table 3. Expression changes of *SIRT1*, *MMP-1*, *Coll2a1*, and *aggrecan* after RES intervention.

Group	<i>SIRT1</i> mRNA	<i>MMP-1</i> mRNA	<i>Coll2a1</i> mRNA	<i>aggrecan</i> mRNA
Intervention	0.87 ± 0.07*	0.61 ± 0.03*	0.90 ± 0.08*	0.69 ± 0.07*
Non-intervention	0.54 ± 0.04*	0.93 ± 0.08*	0.38 ± 0.01*	0.42 ± 0.05*
<i>t</i> -test	3.416	3.588	5.726	3.534
P	0.035	0.033	0.028	0.034

SIRT1: silencing mating type information regulation 2 homolog 1; *MMP-1*: matrix metalloproteinase-1. *Values were normalized expression (means ± SD).

Correlation analysis between the expression of *SIRT1* and *MMP-1*

By Pearson correlation analysis, a negative correlation (correlation coefficient = -0.0714-0.0785, $P < 0.05$) was observed both between the experimental and control groups and between the intervention and non-intervention groups.

DISCUSSION

Intervertebral disc degeneration is a major cause of lumbar disc herniation and lumbago disease, which is triggered by the abnormal changes in the chemical composition and structure of the extracellular matrix of the intervertebral disc nucleus pulposus cells, which

gradually age and undergo apoptosis (Gries et al., 2010; Alini et al., 2013). Development of nucleus pulposus degeneration is characterized by loss of the extracellular matrix structure and water content, which affects the normal structure and function of the intervertebral disc (Clouet et al., 2009). According to the existing studies, lumbar disc degeneration may be correlated with an inadequate nutrition supply to the lumbar intervertebral disc tissue, causing apoptosis of the nucleus pulposus cells, inflammation, biomechanical changes, autoimmune response, and changes in matrix enzyme activity, cytokines, and many other factors (Yuan et al., 2011a; Xu et al., 2013), especially type II collagen and proteoglycan. Nucleus pulposus cells are cartilage-like cells, and the main components of the extracellular matrix are type II collagen and proteoglycan (Wang et al., 2013). When intervertebral disc degeneration occurs, type I collagen replaces type II collagen, proteoglycan content decreases, and the nucleus pulposus cells' moisture content decreases, among other intervertebral disc extracellular matrix changes (Wang et al., 2010). The proteoglycans are replaced by fibrous tissue, nucleus pulposus fibrosus, and further losing moisture, prone to outburst from a weak point of the fiber ring. Therefore, studies of the biological characteristics of intervertebral nucleus pulposus cells are of significant importance to elucidate the mechanism and clinical treatment of intervertebral disc degeneration.

SIRT1 is an NAD⁺-dependent protein deacetylase (Denu, 2005; Couzin-Frankel 2011). Numerous researches show that enhancing the expression of SIRT-I in human articular chondrocytes could promote the expression of the extracellular matrix genes. Gagarina et al. (2010) demonstrated that SIRT1 could inhibit the apoptosis of chondrocytes and delay apoptosis of articular cartilage cells and other various cell types in patients with osteoarthritis (Takayama et al., 2009; Ota et al., 2010). Immunohistochemistry of nucleus pulposus tissue and quantitative fluorescent PCR analysis of nucleus pulposus P2 cells by Shen et al. (2013) showed that SIRT1 gene and protein expression in nucleus pulposus cells of degenerative lumbar disc herniation significantly decreased and were accompanied by a decrease in matrix Coll2 α 1 and aggrecan in nucleus pulposus cells.

MMP-1 is a collagenase in the MMPs family, which are distributed widely in various human tissues. MMP-1 is secreted by fibroblasts and mainly degrades type II collagen, but also degrades type VII and VI collagens (Hall et al., 2011). MMP-1 plays an important role in the degradation of type II collagen, which is the main content of the cartilage matrix. Roberts et al. (2011) studied 49 cases of intervertebral disc degeneration by implementation of immunohistochemical analysis of various MMPs and found that the positive rate of MMP-1 reached 91%, but the results did not explain whether the specimens came from the nucleus pulposus tissue or the fibrous ring. Other experimental results (Zhang et al., 2012) showed that the positive expression rate of MMP-1 in degenerative intervertebral disc nucleus pulposus tissue was 93.3%, which was similar to the results obtained by Roberts et al. (2011). Thus, the molecular biology mechanism of intervertebral disc degeneration may include reduction in the number of cell factors and an increase in inflammatory factors that cause an increase in MMP-1 expression (Razaq et al., 2010; Yuan et al., 2011b).

RES is an anthraquinone terpenoid, which is widespread in nature and found in grapes, peanuts, giant knotweed rhizome, mulberry, and other plants. RES acts as a SIRT1 agonist, but the specific mechanism is still not clear. Recent research showed that RES increases the activity of SIRT1, but does not have a direct effect on SIRT1 (Zhong et al., 2013).

In this study, immunohistochemical detection suggested that positive expression of *SIRT1* in nucleus pulposus cells in the experimental group was significantly lower than that

of the control group, while the positive expression of *MMP-1* was significantly higher than that of control group. These results were validated by quantitative fluorescent PCR in nucleus pulposus P2 cells. After RES intervention, the expression of *SIRT1* in degenerative intervertebral disc nucleus pulposus P2 cells increased significantly, while *MMP-1* expression decreased significantly and negatively correlated with *SIRT1* expression. The expression of *Coll2a1* and *aggrecan* increased significantly, illustrating that the intervertebral disc degeneration occurrence and development were reversed. These results were similar to those obtained by others (Gagarina et al., 2010; Wang et al., 2012).

In conclusion, SIRT1 may enhance the human intervertebral disc nucleus pulposus cell activity, promote the synthesis of the extracellular matrix of nucleus pulposus cells, and inhibit the expression of MMP-1 to reverse the occurrence and development of intervertebral disc degeneration, which may be a new approach for the treatment of human degenerative disc disease.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

- Alini M, Li W, Markovic P, Aebi M, et al. (2013). The potential and limitations of a cell-seeded collagen/Hyaluronan scaffold to engineer an intervertebral disc-like matrix. *Spine* 28: 446-454.
- Becker A, Held H, Redaelli M, Chenot JF, et al. (2012). Implementation of a guideline for low back pain management in primary care: a cost-effectiveness analysis. *Spine* 37: 701-710.
- Boileau C, Amiable N, Martel-Pelletier J, Fahmi H, et al. (2011). Activation of proteinase-activated receptor 2 in human osteoarthritic cartilage upregulates catabolic and proinflammatory pathways capable of inducing cartilage degradation: a basic science study. *Arthritis Res. Ther.* 9: 121-128.
- Brisby H (2010). Pathology and possible mechanisms of nervous system response to disc degeneration. *J. Bone Joint Surg. Am.* 88: 68-71.
- Chen G, Yang JS and Zhang GY (2011). The expression of MMP-1 and TIMP-1 in the degenerative nucleus pulposus cells. *Dang Dai Yi Xue* 16: 46-47.
- Clouet J, Vinatier C, Merceron C, Pot-Vaucel M, et al. (2009). The intervertebral disc: from pathophysiology to tissue engineering. *Joint Bone Spine* 76: 614-618.
- Couzin-Frankel J (2011). Genetics. Aging genes: the sirtuin story unravels. *Science* 334: 1194-1198.
- Denu JM (2005). The Sir 2 family of protein deacetylases. *Curr. Opin. Chem. Biol.* 95: 431-440.
- Gagarina V, Gabay O, Dvir-Ginzberg M, Lee EJ, et al. (2010). SirT1 enhances survival of human osteoarthritic chondrocytes by repressing protein tyrosine phosphatase 1B and activating the insulin-like growth factor receptor pathway. *Arthritis Rheum.* 62: 1383-1392.
- Gries NC, Berlemann U, Moore RJ and Vernon-Roberts B (2010). Early histologic changes in lower lumbar discs and facet joints and their correlation. *Eur. Spine J.* 9: 23-29.
- Hall MC, Young DA, Waters JG, Rowan AD, et al. (2011). The comparative role of activator protein 1 and Smad factors in the regulation of TIMP-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J. Biol. Chem.* 278: 10304-10313.
- Hong ZC and Xu J (2010). The expression and significance of MMP-2, MMP-14, TIMP-2 in the degenerative nucleus pulposus cells. *Fujian Yi Yao Za Zhi* 32: 90-92.
- Hu M, Ma YZ, Huang FS, Li DW, et al. (2011). *In vitro* culture and morphological observation of adult degenerative nucleus pulposus cells. *Shi Yong Gu Ke Za Zhi* 17: 902-904.
- Liang WG, Ye DP, Li FS, Dai LB, et al. (2012). Experimental study on the effects of BMP-2 on the degenerative nucleus pulposus cells. *Zhong Guo Jiao Xing Wai Ke Za Zhi* 20: 1782-1785.
- Matrisian LM (1990). Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet.* 6: 121-125.
- Ota H, Eto M, Kano MR, Kahyo T, et al. (2010). Induction of endothelial nitric oxide synthase, SIRT1, and catalase by statins inhibits endothelial senescence through the Akt pathway. *Arterioscler. Thromb. Vasc. Biol.* 30: 2205-2211.

- Razaq S, Wilkins RJ and Urban JP (2010). The effect of extracellular pH on matrix turn over by cells of the bovine nucleus pulposus. *Eur. Spine J.* 12: 341-349.
- Roberts S, Evans H, Trividi J and Menage J (2011). Histology and pathology of the human intervertebral disc. *J. Bone Joint Surg. Am.* 88: 110-141.
- Shen JL, Hu ZM, Zhong XM, Zhang R, et al. (2013). SIRT1 regulates extracellular matrix synthesis through both AKT pathway and ERK1/2 pathway. *Zhong Guo Lao Nian Xue Za Zhi* 33: 1811-1814.
- Shen KL, Hu ZM, Zhong XM, Zhang XJ, et al. (2012). Resveratrol stimulates extracellular matrix synthesis in degenerative nucleus pulposus cells via upregulation of SIRT1. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 28: 1146-1150.
- Takayama K, Ishida K, Matsushita T, Fujita N, et al. (2009). SIRT1 regulation of apoptosis of human chondrocytes. *Arthritis Rheum.* 60: 2731-2740.
- Wang DW, Hu ZM and He J (2012). SIRT1 inhibits the human degenerative nucleus pulposus cells apoptosis through Akt/PKB pathway. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Jin Zhan* 39: 563-573.
- Wang FZ, Chen JM and Wu XT (2013). Morphological and activity observation of *in vitro* plate cultured human degenerative nucleus pulposus cells. *Zhong Guo Jiao Xing Wai Ke Za Zhi* 21: 1346-1351.
- Wang YT, Wu XT and Wang F (2010). Regeneration potential and mechanism of bone marrow mesenchymal stem cell transplantation for treating intervertebral disc degeneration. *J. Orthop. Sci.* 6: 707-719.
- Xu D, Sun Y, Bao G, Liu W, et al. (2013). MMP-1 overexpression induced by IL-1 β : possible mechanism for inflammation in degenerative lumbar facet joint. *J Orthop Sci.* 18: 1012-1019.
- Yuan W, Wang HR and Dong J (2011a). The research progress of lumbar intervertebral disc nucleus pulposus tissue engineering. *Zhong Guo Ji Zhu Ji Shui Za Zhi* 21: 154-158.
- Yuan Y, Zhao J, Li YM, Wang X, et al. (2011b). The expression and significance of NF- κ B, MMP-1/TMP-1 in lumbar intervertebral disc degenerative tissue. *Shan Dong Yi Yao* 51: 4-5.
- Zhang B, Yang SH, Lin SZ, Chen W, et al. (2012). The expression and significance of MMP-1 in differently degenerated nucleus pulposus cells and fibrous rings. *Zhong Guo Zu Zhi Gong Cheng Yan Jiu* 16: 2346-2349.
- Zhong XM, Hu ZM, Shen KL, Liu SL, et al. (2013). The effect of resveratrol intervention on the SIRT1 expression in the degenerated intervertebral disc end plate chondrocyte. *Zhong Guo Lao Nian Xue Za Zhi* 33: 1304-1307.