

Effects of psoralen on chondrocyte degeneration in lumbar intervertebral disc of rats

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Abstract: Discuss the internal mechanism of delaying degeneration of lumbar intervertebral disc. The cartilage of lumbar intervertebral disc of SD rats was selected *in vitro*, then cultured by tissue explant method, and identified by HE staining, toluidine blue staining and immunofluorescence. The optimal concentration of psoralen was screened by cell proliferation assay and RT-PCR method. The cells in third generation with good growth situation is selected and placed in 6-well plate at concentration of 1×10^5 /well and its expression was tested. Compared to concentration of 0, the mRNA expression of Col2a1 (Collagen α) secreted by was up regulated chondrocyte of lumbar intervertebral disc at the concentration of 12.5 and 25 μ M ($P < 0.0$ or $P < 0.01$). The aggrecan mRNA of psoralen group was higher than blank control group ($P < 0.01$); compared with IL-1 β induced group, the mRNA expression of Col2a1 was significantly increased but the mRNA expression of ADAMTS-5 was significantly decreased in psoralen group ($P < 0.01$). These findings suggest that, psoralen can remit the degeneration of lumbar intervertebral disc induced by IL-1 β to some extent, and affect the related factors of IL-1 β signaling pathway.

Keywords: Psoralen; IL-1 β ; chondrocyte; intervertebral disc, degeneration.

INTRODUCTION

With the speed up of social development and life pace, intervertebral disc degeneration has become one of the factors that threaten health of people and lower living quality. Intervertebral disc degeneration is an extremely complex pathological change process. A large amount of studies indicate that, intervertebral disc degeneration is closely related to cell apoptosis, abnormal calcification of end plate, vascular morphology, blood flow volume and abnormal stress of end plate or end plate injury, NO, inflammatory cytokines such as IL-1, IL-6 and TGF, MMPs. IL-1 β is one of the important inflammatory factors in intervertebral disc degeneration and can induce the degeneration of chondrocyte of intervertebral disc (Yu *et al.*, 2012). Fructus psoraleae with the function of nourishing liver and kidney is the common drug for curing degeneration of intervertebral disc, and psoralen is its important effective constituent. This study observed the effect of psoralen on mRNA expression of Col2a1 secreted by *in-vitro* intervertebral disc induced by IL-1 β , aggrecan, ADAMTS-5, IL-1 β , COX-2, in order to further clear the internal mechanism of psoralen in curing degeneration of intervertebral disc degeneration.

Materials, main reagents and instruments

Experimental materials, main reagents and instruments needed in experiment is as shown in table 1.

METHODS

Isolation and culture of primary generation chondrocyte

SD rats were killed by injecting 0.4ml ketamine

hydrochloride into enterocoelia. The spine was isolated in sterile conditions. Remove the muscle and ligament attached to spine. Cut vertebral plate. Break off centrum by rongeur to expose intervertebral disc. The intervertebral disc was isolated by ophthalmic operating scissors and tweezers and removed anulus nucleus pulposus. Finally obtain chondral plate. It was washed by PBS solution for three times, cut into small pieces ($< 0.3 \text{ mm}^3$) and then evenly put into 10cm \times 10cm culture dish. It was lightly backed off into a 5% CO₂ and 37 $^{\circ}$ C incubator. Observe cellular morphology and select the 3rd generation cells for experiment.

Discrimination and identification of chondrocyte cell HE staining

Put cover glass into 6-well plate. Place cells on the cover glass in the 6-well plate at concentration of 1×10^5 /cell. Use 2 ml DMEM contained 10%FBS and 1% penicillin-streptomycin to culture cells. After 80% of cell blending, take out the cover glass and add 4% paraformaldehyde for fixing. Then successively undergo gradient ethanol dehydration, haematoxylin staining, 1% ethanol-hydrochloride aqueous solution differentiation, ammonium hydroxide returning to blue, haematoxylin-eosin staining, ethanol dehydration to wax. Mount it by neutral balsam and heat. Observe it under microscope.

Cell toluidine blue staining

Put cover glass into 6-well plate. Place cells on the cover glass in the 6-well plate at concentration of 1×10^5 /cell. Use 2ml DMEM contained 10% FBS and 1% penicillin-streptomycin to culture cells. After 80% of cell blending, take out the cover glass and add 4% paraformaldehyde for fixing. Add 0.5% toluidine blue for 10 min of dip dyeing.

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Conduct ethanol dehydration to wax. Observe it under microscope and then dry. Mount it by neutral balsam.

Initial screening of psoralen concentration

Adopt CCK-8 method to detect cell proliferation. Place cells into 96-well plate at concentration of 1×10^4 /well. Culture it by 2ml DMEM contained 10%FBS and 1% penicillin-streptomycin. Absorb the culture solution after 1 day culture. Then culture it by serum-free medium overnight. Absorb culture solution and add psoralen (0, 12.5, 25, 50, 100, 150, 200, 400 μ M). Repeat 6 wells. Culture it by DMEM contained 0.5% FBS and 1% penicillin-streptomycin for 48 h. Add 10 μ M CCK-8 into each well and place it into 5%CO₂ and 37°C incubator. 2h later, detect OD value by micro plate reader at wavelength of 450 nm. Initially screen out concentration of psoralen according to OD value.

The effect of psoralen of different concentration on mRNA of Col2a1 of chondrocyte

According to the initial screening result of concentration of psoralen, select the intervertebral disc chondrocyte with good growth situation. Place cells into 6-well plate at concentration of 1×10^5 /well. Culture the cells by 2ml DMEM contained 10%FBS and 1% penicillin-streptomycin. 3 days later, culture it by serum-free medium overnight. Then culture it by DMEM contained 0.5% FBS and 1% penicillin-streptomycin for 48h. Adopt RT-PCR method to detect the effect of psoralen of different concentration on mRNA expression of Col2a1 to further confirm the optimal concentration of psoralen.

Detection of mRNA expression of Col2a1, Aggrecan, ADAMTS-5, IL-1 β , COX-2 by RT-PCR method

Select the 3rd generation intervertebral disc chondrocyte with good growth situation. Place it into 6-well plate at concentration of 1×10^5 /well. Divide them into blank control group, IL-1 β induced group, psoralen group, 3 well for each group. Culture cells by 2ml DMEM contained 10% FBS and 1% penicillin-streptomycin. 3 days later, use serum medium for culturing overnight. Then exchange into DMEM contained 0.5% FBS and 1% penicillin-streptomycin and added 0 (blank control group), IL-1 β 10ng/ml (IL-1 β induced group), IL-1 β 10 ng/ml + psoralen with optimal concentration (psoralen group) for 48h culture. Extract total RNA. Confirm its integrity of RNA after treated by agarose gel electrophoresis. Detect the content and purity of RNA by ultraviolet spectrometry photometer. Reversely transcribe RNA into cDNA according to the instructions of reverse transcription kit. The reaction conditions were 37°C 15min and 85°C 5s. Match 20 μ l reaction systems: sybgreen mix 10 μ l, Rnase free water 7 μ l, upstream primer 1 μ l and downstream primer 1 μ l, template cDNA 1 μ l. Repeat 3 wells for every sample and conduct degeneration at 95°C. Annealing temperature was confirmed according to the design requirements of every

primer. Expand at 72°C. The same sample were underwent β -actin internal reference PRC reaction. The primer searched SD rat gene name from Genebank database.

The sequence of primer is as follows:

ADAMTS-5: F-GCAT-CATCGGCTCAAAGCTACA; R-TCAGGGATCCTCA-CAACGTCAG;

Aggrecan: F-TCCGCTGGTCTGATGGACAC; R-CCAGATCATCACTACGCAGTCCTC;

Col2a1: F-TCCTAAGGGTGCCAATGGTGA; R-AGGACCAAC-TTTGCCTTGAGGAC; IL-1 β : F-AGGTCGTCATCATCCCACGAG; R-GCTGTGGCAGCTACCTATGCTTTG;

COX-2: F-GGAGCATCCTGAGTGGGATGA; R-AAGCAGTCTGGGTCGAACTTG;

β -actin: F-GGAGATTACTGCCCTGGCTCCTA; R-GACTCATCGTACTCCTGCTTGCTG.

The obtained Ct was processed according to 2^{- $\Delta\Delta$ ct} method and analyzed by statistical software.

STATISTICAL ANALYSIS

Data was analyzed using SPSS 19.0. Measure data was expressed by (Mean \pm SD). All values were underwent homogeneity of variance and normality test. Test level was $\alpha=0.10$. Comparison of mean between groups adopted one-way analysis of variance. Comparison between two means adopted q test.

RESULTS

Discrimination and identification of chondrocyte

Observe the growth process of cells cultured by tissue block culture method under inverted microscope. After 7 days, the cells gradually isolated from tissue, showing prismatic and triangle, then expanding to polygon. The nucleus was round or oval, sometimes we can see dicaryon. Also, there was vacuole and particle within cytoplasm. The cells covered the medium with 10cm diameter after 20 days. HE staining indicated that, the cell nucleus was blue and dielectial dyeing of amaranth or red appeared in cytoplasm and around cells. The cell nucleus treated by toluidine blue staining was purple and the cytoplasm was dark blue. There was no significant difference between the morphology and structure of primary cells and the 2nd generation cells. Immunofluorescence was green, indicating that, the detected cytoplasm and intercellular substance expressed a large amount of Col2a1.

Initial screening result of concentration of psoralen

Table 2 shows that, when exogenously add psoralen, cell activity gradually decreased as the concentration increased. At concentration of 100, 150, 200 and 400 μ M, cell activity significantly decreased compared to concentration of 0 (P<0.01).

Table 1: Materials, main reagents and instruments

5 one-month-old female SD rats, weight of 180±20g	Shanghai Slac Laboratory Animal Co., LTD
ketamine hydrochloride injection	Fujian Gutian Pharmaceutical Co., Ltd
CCK 8 cell proliferation and activity detection kit	Dojindo Company Japan
IL-1 β , dimethyl sulfoxide (DMEM)	Sigma Company USA
6-well and 96-well culture plate	Corning Company USA
four-channel Florescent real-time quantitative PCR instrument	Biorad Company Singapore
DP-71 inverted phase contrast microscope	Olympus Company Japan
psoralen	China Pharmaceutical Biological Products Analysis Institute (Shanghai)

The effect of psoralen of different concentration on Mrna expression of Col2al in chondrocyte

According to the initial screening result, select psoralen with concentration of 0, 12.5, 25, 50 μ M to interfere intervertebral disc chondrocyte. As shown in table 2, the mRNA expression of Col2al can be up regulated when the psoralen concentration was 12.5 μ M and 25 μ M and the difference between it and concentration of 0 was statistically significant ($P < 0.05$ or $P < 0.01$). It hinted that, psoralen with concentration of 12.5 μ M had good cell proliferation activity and mRNA expression of Col2al. Thus 12.5 μ M was considered as the optimal concentration.

Table 2: Comparison of OD value of chondrocyte after intervened by psoralen of different concentration for 48h (Mean±SD)

Psoralen concentration (μ M)	Sample size	OD value
0	6	0.91±0.07
12.5	6	0.93±0.13
25	6	0.93±0.20
50	6	0.79±0.11
100	6	0.76±0.08*
150	6	0.67±0.05*
200	6	0.69±0.07*
400	6	0.41±0.13*

The comparison of mrna expression of col2al, aggrecan, ADAMTS-5, IL-1B and COX-2

As shown in table 4, compared to blank control group, mRNA expression of Col2al decreased, mRNA expression of aggrecan and ADAMTS-5 increased in IL-1 β induced group, and the difference was statistically significantly ($P < 0.01$). mRNA expression of aggrecan in psoralen group was also higher than blank control group ($P < 0.01$). Compared to IL-1 β induced group, mRNA expression of IL-1 β increased and ADAMTS-5 decreased in psoralen group, and the difference was statistically significantly ($P < 0.01$). The comparison of mRNA expression of IL-1 β between groups was not statistically significant ($P > 0.05$). mRNA expression of COX-2 in psoralen group was lower than IL-1 β induced group, and the difference was statistically significant ($P < 0.05$).

DISCUSSION

Chondral plate is the important part of intervertebral disc, and its functions include supporting, shielding, nutrition channel and metabolic channel (YanJun *et al.*, 2013). Chonral plate is composed of chondrocyte and matrix. The matrix is mainly Col2al and protein polysaccharide. Col2al plays the function of fibrous framework. Protein polysaccharide plays the function of maintaining intervertebral disc structure, metabolism and biomechanical property. Specifically, it plays important roles in regulating the effective aperture between matrix macromolecule, controlling the distribution and transport of charged solvency, protecting the exchange of substance of intervertebral disc tissue with external world, preventing the calcification of end plate itself, etc (Zhe *et al.*, 2013). The abnormal expression of aggrecan can degrade a lot of aggrecan in intervertebral disc. There is evidence suggested that, the degeneration of intervertebral disc is result from degeneration of chondral plate; it can stimulate the further damage on adjacent joints and tissues (Kai *et al.*, 2012). Therefore, delaying the degeneration of chondral plate is of great importance to recovery and regeneration of degeneration of intervertebral disc (Xiang and Xinzhi, 2013).

Table 3: The effect of psoralen of different concentration on mRNA expression of Col2al of chondrocyte (Mean±SD)

Concentration of psoralen (μ M)	Sample size	Col2al mRNA
0	3	1.00±0.06
12.5	3	1.75±0.23*
25	3	2.15±0.13**
50	3	1.84±0.82

Note: compared to concentration of 0 of psoralen, * $P < 0.05$, ** $P < 0.01$

Intervertebral disc is the biggest blocking tissue with no blood vessel in human body with limited metabolism ability, insufficient self-repair ability, easy accumulation of injury, thus stimulate the easy occurrence of chronic inflammation mediated by inflammatory cytokines and proteolytic enzyme (Zorica *et al.*, 2011) Therefore, it is to

Table 4: Comparison of mRNA expression of Col2a1, aggrecan, ADAMTS-5, IL-1 β and COX-2 between groups (Mean \pm SD)

Groups	Simple size	Col2a1	Aggrecan	ADAMTS-5	IL-1	COX-2
Blank control group	3	1.01 \pm 0.13	1.03 \pm 0.27	1.00 \pm 0.02	1.00 \pm 0.06	1.00 \pm 0.08
IL-1 β induced group	3	0.67 \pm 0.13*	1.55 \pm 0.05*	1.39 \pm 0.05*	1.22 \pm 0.23	1.10 \pm 0.03
Psoralen group	3	1.68 \pm 0.10 $\square\square$	1.76 \pm 0.21*	1.04 \pm 0.30 $\square\square$	0.98 \pm 0.29	0.92 \pm 0.06 \square

Note: compared to blank control group, *P<0.01; compared to IL-1 β induced group, Δ P<0.05, $\Delta\Delta$ P<0.01.

say that, inflammation is not only the result of pathological change of intervertebral disc degeneration, but also the initial factors of secondary lesion of intervertebral disc. The expression of a large number of inflammatory cytokines deteriorates the survival environment of intervertebral disc cells and affects the normal development history and function exertion. Among these inflammatory factors it is believed that, IL-1 β plays important role in degeneration of intervertebral disc (Yu *et al.*, 2012). There is high expressed IL-1 β in chondrocyte of degenerated intervertebral disc that can stimulate or inspire the expression of other inflammatory factors such as IL-6, NO and COX-2, thus cause ischialgia (AJ. 2009). Induce apoptosis of intervertebral disc cells, inhibit the generation of protein polysaccharide, start multiple chondrocytes signal transduction cascade, up regulate the expression of MMPs, further induce the degradation of Col2a1. Therefore, the inhibition of IL-1 can prevent and even reverse degeneration of intervertebral disc (Hongxun and Chenglong, 2011).

Fructus psoraleae has the functions of warming kidney and enhancing yang, qi absorption and antidiarrheic. It is said that, fructus psoraleae can warm water viscus and is the drug for generating yang in ying, sthenic fire and tonify soil. Psoralen belonging to furan coumarin compounds is a kind of component with important biological activity as well as one of the phytoestrogen that can promote the proliferation and differentiation of rat osteoblasts (Mishan *et al.*, 2012), and inhibit the increase and expansion of resorption pits formed by isolated osteoclast on sclerite (Qiqing *et al.*, 2011). This study screened psoralen concentration that was with optimal cell proliferation activity and can enhance the mRNA expression of Col2a1. We found that, under this concentration, psoralen can up regulate the expression of Col2a1 and aggrecan of intervertebral disc chondrocyte induced by IL-1 β , down regulate the mRNA expression of ADAMTS-5 and COX-2, indicating that psoralen was of great importance to remit the degeneration of intervertebral disc chondrocyte induced by IL-1 β .

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