Full Paper

Herb Formula “*Fufangqishe-Pill*” Prevents Upright Posture–Induced Intervertebral Disc Degeneration at the Lumbar in RatsQian-Qian Liang^{1,2}, Zhi-Jie Xi^{1,2}, Qin Bian^{1,2}, Xue-Jun Cui^{1,2}, Chen-Guang Li^{1,2}, Wei Hou^{1,2}, Qi Shi^{1,2}, and Yong-Jun Wang^{2,*}¹Department of Orthopaedics & Traumatology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, P.R. China²Institute of Spine, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, P.R. China

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Abstract. Degeneration of the lumbar spine plays an important role in most chronic low back pain. Prevention of lumbar intervertebral disc (IVD) degeneration is therefore a high research priority. Both our previous multicenter clinical trials and pharmacological research showed that *Fufangqishe-Pill* (FFQSP), a newly patented traditional Chinese medicine, could effectively relieve the symptoms of neck pain and prevent cervical degeneration. To clarify the effect of FFQSP on lumbar IVD degeneration, we applied a lumbar IVD degeneration rat model induced by prolonged upright posture. Pretreatment of FFQSP for one month prevented the histological changes indicating IVD disorganization; increased type II–collagen level, decreased type X–collagen protein level, and increased Col2 α 1 mRNA expression at all time points; and decreased Col10 α 1, matrix metalloproteinase (MMP)-3, MMP13, and Interleukin (IL)-1 β mRNA expression induced by upright posture for 7 and 9 months. These results suggest that FFQSP prevents lumbar IVD degeneration induced by upright posture. FFQSP is a promising medicine for lumbar IVD degeneration disease.

Keywords: chronic low back pain, *Fufangqishe-Pill*, lumbar intervertebral disc degeneration, extracellular matrix

Introduction

Degeneration of intervertebral discs (IVDs) in the lumbar spine plays a central role in the pathogenesis of discogenic pain, disc herniation, spinal instability, and stenosis (1 – 4). Prevention of lumbar IVD degeneration is therefore a high research priority.

Fufangqishe-Pill (FFQSP) was designed by a group of traditional Chinese medicine (TCM) doctors in China, entirely based on TCM theories, in which radix astragali is the principal element, whereas moschus, Rhizoma chuanxiong, and another 3 natural medicine materials are adjuvant components to assist the effects of radix astragali. Our previous multicenter clinical trials showed that treatment with FFQSP for 3 months could effectively relieve the symptoms of neck pain, upper extremity

numbness, and small-neck-motion degree of cervical disease (New drug clinical trial documents number: China State Drug Administration 2003L04416), with no adverse effects. Our previous research also proved that FFQSP could prevent cervical degeneration caused by excision of the static ligamentous and dynamic muscular cervical stabilizers at C2 – C7 (5). Those above results demonstrate the clinical efficacy of FFQSP against cervical disc degeneration. Because of the effectiveness of FFQSP on IVD degeneration, the effect of FFQSP on lumbar IVD degeneration deserved further research.

Previous research suggested that prolonged upright posture could induce the above degenerative changes in IVDs in rat lumbar spine (6). The pathologic changes of the upright induced rat model mimic human disc degeneration, including the abnormal calcification of vertebral endplate with up-regulated type X–collagen expression, formation of cleft in annulus fibrosus, the decrease of disc height, down-regulated expression of type II collagen, and augmented expression of matrix metalloprotein-

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ase (MMPs). Thus, we used this model to assess the in vivo effect of FFQSP in lumbar IVD degeneration induced by prolonged upright posture.

In this study, we aimed to examine the effect of FFQSP on lumbar IVD degeneration induced by long term upright posture. The effect of FFQSP on IVDs was tested by histological evaluation, immunostaining for type II collagen and type X collagen, and real-time PCR analysis for Col2 α 1, Col10 α 1, MMP3, MMP13, and Interleukin (IL)-1 β mRNA expression.

Materials and Methods

Preparation of drug

FFQSP, a new patent medicine (National Invention Patent NO. ZL01126559.0), was supplied by Shanghai Sundise Chinese Medicine Technology Development Co., Ltd. (Shanghai, China), and the lot number was 031201. The storage location of the specimen used in the study is the Institute of Spine, Shanghai Traditional Chinese Medicine University, China. The quality control of FFQSP is secured by HPLC analysis with standard solutions of active ingredients (7).

We did not use any positive control drug in our experiments since no drugs are used clinically for prevention of IVD degeneration diseases.

Animals and experimental design

We followed the Guiding Principles for the Care and Use of Laboratory Animals Approved by Animal Regulations of National Science and Technology Committee. Ninety one-month-old male Sprague-Dawley rats were obtained from the Shanghai laboratory animal center of the Chinese Academy of Science (SLACCAS), raised in the Specific Pathogen-Free (SPF) unit at the laboratory animal center of Shanghai Traditional Chinese Medicine University and were randomly assigned into 9 groups ($n = 10$ per group): three control groups [5-month non-operated (control) group and 7- and 9-month control group], three model groups [5-month operated (model) group and 7- and 9-month model group], and three FFQSP groups [5-month operated and FFQSP-treated (FFQSP) group 7- and 9-month FFQSP group). Rats in the “control” groups underwent no treatment and were raised in ordinary cages. Rats in the “model” and “FFQSP” groups were anesthetized and both forelimbs of each rat were amputated from the shoulder joints via an anterior approach. Then they were induced to keep an upright posture in custom-made cages for 5, 7, or 9 months. The food bucket and water bottle in custom-made cages can be freely adjusted. The length of rats in every cage was measured every week. The heights of food and water supplies were adjusted accordingly to

ensure all rats could get food and water by themselves. At the 4, 6, and 8th months after the real surgery, FFQSP groups were daily fed with FFQSP (5 g/kg) for 30 days. Rats from 5-, 7- and 9-month groups were respectively killed with pentobarbital at the 5th, 7th, and 9th month after the surgery, and their lumbar spines were harvested for analysis.

We chose another 12 7-month-old normal female Sprague-Dawley rats and randomly divided them into 2 groups (vehicle and FFQSP group, 6 rats in each group). Vehicle-group rats were treated with physiological saline, and the FFQSP-group rats were fed with FFQSP. Seven days later, rats from both groups were killed and lumbar discs were obtained for real-time PCR analysis.

Histological evaluation

L4 – L5 IVDs were fixed using 4% paraformaldehyde, decalcified, dehydrated, cleared with dimethylbenzene, and then embedded in olefin. At least 4 consecutive 7- μ m-thick sections were prepared and stained using Safranin-O/Fast Green. Sections were examined by using a photomicroscope set (Olympus DP71; Olympus, Tokyo), and morphometric study was performed by using an image auto-analysis system (CMIAS-99B; Okolab, Milan, Italy).

Immunohistochemical staining for type II collagen and type X collagen

Immunohistochemical localization of type II collagen and type X collagen was performed according to the method of Liang et al. (6) with rabbit polyclonal antibody to type II collagen and type X collagen (Cell Signaling, Inc., MA, USA; 1:100 dilution) were used. The color reaction was elicited by using 3,3'-diaminobenzidine (DAB) solution, visualized, and examined by using a photomicroscope set (Olympus DP71).

Real-time PCR analysis

The isolation of total RNA, the reverse transcription of RNA to synthesize cDNA, and the quantitative real-time PCR amplifications were performed by the method of Liang et al. (6). The primers for Col2 α 1, Col10 α 1, MMP3, MMP13, IL-1 β , and β -actin were designed by Takara Biotechnology Co., Ltd. (Otsu), and the target sites on genes and PCR product length are shown in Table 1. PCR was performed using the RotorGene real-time DNA amplification system (Corbett Research, Sydney, Australia). Gene expression was normalized against β -actin. PCR products were analyzed and quantified using RotorGene 6.0 analysis software (Corbett Research).

Table 1. Sequences of primers used in the real-time RT-PCR

Genes	Forward primer	Reverse primer
Col2 α 1 (112 bp)	5'-TCCTAAGGGTGCCAATGGTGA-3'	5'-GGACCAACTTTGCCTTGAGGAC-3'
MMP13 (142 bp)	5'-CCCTGGAGCCCTGATGTTT-3'	5'-CTCTGGTGTTTTGGGGTGCT-3'
Col10 α 1 (143 bp)	5'-TTCACAAAGAGCGGACAGAGA-3'	5'-TCAAATGGGATGGGAGCA-3'
IL-1 β (120 bp)	5'-AGGTCGTCATCATCCCACGAG-3'	5'-GCTGTGGCAGCTACCTATGTCTTG-3'
MMP3 (104 bp)	5'-TGGACCAGGGACCAATGGA-3'	5'-GGCCAAGTTCATGAGCAGCA-3'
β -Actin (150 bp)	5'-GGAGATTACTGCCCTGGCTCCTA-3'	5'-GACTCATCGTACTCCTGCTTGCTG-3'

Fig. 1. Safranin-O/Fast Green of IVDs. In the surgery samples, the heights of IVDs were lower compared to the control ones. FFQSP treatment increased the disc height at the 5th month after surgery. The arrow indicates the quantitative measurements on IVD height; Bar, 1000 μ m (A). Each value represents the mean $\pm$ S.D. of 6 sections. ** $P < 0.01$ vs. model group in the same month (B).

Statistical analyses

The one-way analysis of variance test was used when more than two groups were being compared followed by Dunnett's t -test, and unpaired Student's t -test was used for dual comparisons. A P -value of <0.05 was taken as statistically significant. Data are expressed as means $\pm$ S.D. SPSS 10.0 (SPSS, Chicago, IL, USA) was used for analysis of the statistics.

Results

Histological observation of the effect of FFQSP on IVD degeneration

In control samples, the IVD between L4 – L5 and adjacent endplates appeared to have a well-organized annulus fibrosus, endplate, and nucleus pulposus (Figs. 1 – 3). The disc height was decreased by prolonged up-right posture at all time points. The pretreatment of FFQSP for one month could increase the disc height of

Fig. 2. Safranin-O/Fast Green staining at the annulus fibrosus. In the surgery group, the lamellar structures of the annulus fibrosus were disorganized at all time points. FFQSP treatment almost completely reversed the disorganized structure of the annulus fibrosus at the 5th and 7th month after surgery and diminished the fissures at the 9th month. Bar, 100 μ m.

upright rats at the 5th month after surgery ($P < 0.01$), but has no effect at the 7th and 9th month. Prolonged upright posture could also induce a decrease of volume up to shrinkage of the nucleus pulposus. However, the pretreatment of FFQSP for one month could rescue the shrinkage change of the nucleus pulposus subjected to the upright posture for all 5, 7, and 9 months (Fig. 1).

The lamellar architecture of both the inner and outer parts of the annulus fibrosus became disorganized and fracture was induced by upright posture at all time points. FFQSP pretreatment almost completely reversed the fissures of the inner part of annulus fibrosus subjected to upright posture at the 5th and 7th month, and lessened number of fissures appear at all layers of the annulus fibrosus after surgery for 9 months (Fig. 2).

Immunohistochemical findings of the effect of FFQSP on type II-collagen and type X-collagen protein expression

Immunohistochemical study of the control samples revealed strong immunoreactivity for type II collagen in the nucleus pulposus. Much weaker immunostaining for type II collagen was observed at all time points in the model group. However, the density of the type II collagen-positive staining of the FFQSP group increased dramatically compared to the model group (Fig. 3).

Immunohistochemical staining for type X-collagen protein in the control endplates was very weak and limited to the outmost layer of the calcified section at all time points. In the 7- and 9-month model groups, type X

collagen-positive staining was observed at both calcified and non-calcified sections of the endplate. Interestingly, immunostaining for type X collagen was very weak and could not be observed at the non-calcified layer in the FFQSP groups (Fig. 4).

FFQSP inhibited the upright posture-induced down-regulation of Col2a1 and up-regulation of Col10a1 gene expression

The mRNA expression of Col2a1 was significantly reduced by upright posture at all three time points. In the contrast, the decrease of Col2a1 mRNA expression was completely recovered by the pre-treatment with FFQSP at all time points (Fig. 5A).

Compared with the vehicle group, FFQSP treatment for 1 week without any injury effect significantly decreased mRNA expression of Col2a1 in 7-month-old normal rats (Fig. 5B).

Upright posture increased mRNA expression of Col10a1 at all time points, while FFQSP could significantly reverse the up-regulation of Col10a1 gene expression induced by upright posture at the 7th month (1.6-fold decreased) and 9th month (2.5-fold decreased), compared with the model group (Fig. 5C).

FFQSP blocked the upright posture-induced up-regulation of MMP3, MMP13, and IL-1 β gene expression

Compared with the control groups at all time points, gene expression of MMP3 and MMP13 increased significantly in the model group. FFQSP pretreatment

Fig. 3. Immunohistochemical assessments of the protein level of type II collagen. Positive staining of type II collagen declined in the nucleus pulposus at all time points after the surgery. FFQSP treatment completely reversed the decreased trend of type II collagen at the nucleus pulposus. Bar, 100 μ m.

Fig. 4. Immunohistochemical assessments of the protein level of type X collagen. Type X collagen-positive staining reached into the non-calcified section of cartilage endplates after the surgery for 7 and 9 months, but disappeared at the cartilage endplates when treated with FFQSP. Bar, 100 μ m. NC, non-calcified section of cartilage endplates; CF, calcified section of cartilage endplates. Arrows indicate positive staining.

slightly increased MMP3 and MMP13 gene expression at the 5th month, but significantly depressed the upright posture-induced up-regulation of MMP3 and MMP13 gene expression at the 7th and 9th month (Fig. 6: A and B). Without any injury effect, one-week FFQSP-treatment could also significantly decrease mRNA expression of

MMP13 in 7-month-old normal rats (Fig. 6C).

FFQSP decreased the upright posture-induced up-regulation of IL-1 β gene expression

We also examined the gene expression of IL-1 β at the 5th, 7th, and 9th month and found that IL-1 β increased in

Fig. 5. The mRNA expression of Col2a1 and Col10a1. In the operated rats, the mRNA expression of Col2a1 decreased significantly at all time points, but up-regulated significantly when treated with FFQSP. $**P < 0.01$ vs. model group in the same month (A). The treatment with only FFQSP for 1 week significantly down-regulated Col2a1 mRNA expression in 7-month-old normal rats. $**P < 0.01$ vs. vehicle group (B). Col10a1 mRNA expression increased significantly at all time points, and FFQSP treatment could decrease significantly Col10a1 gene expression at the 7th and 9th month after surgery. $*P < 0.05$, $**P < 0.01$ vs. model group in the same month (C). The columns represent the mean $\pm$ S.D. of three independent experiments.

Fig. 6. The mRNA expression of MMP3 and MMP13. In the operated rats, the mRNA expression of MMP3 and MMP13 is significantly up-regulated at all time points, and FFQSP treatment could decrease significantly MMP3 and MMP13 gene expression at the 7th and 9th month after surgery. $**P < 0.01$ vs. model group in the same month (A and B). The treatment of FFQSP for 1 week without any injury effect could significantly decrease the mRNA expression of MMP13 in lumbar discs of 7-month-old rats. $**P < 0.01$ vs. vehicle group (C). The columns represent the mean $\pm$ S.D. of three independent experiments.

the model groups at all time points, compared with the control groups. FFQSP could significantly reverse the up-regulation of IL-1 β at the 7th and 9th month (Fig. 7: A – C). Without any damage effect, FFQSP treatment for 1 week could also significantly decrease IL-1 β mRNA expression in 7-month-old normal rats (Fig. 7D).

Discussion

The objective of this study was to assess the effects of FFQSP on the lumbar IVD degeneration induced by long term upright stance. The amputated model was chosen because it could stimulate the upright stance of human

Fig. 7. The mRNA expression of IL-1 β . Upright posture up-regulated the mRNA expression of IL-1 β at all time points, but FFQSP significantly decreased IL-1 β gene expression at the 7th and 9th month after surgery. * $P < 0.05$, ** $P < 0.01$ vs. model group in the same month (A–C). The 1-week treatment of FFQSP significantly decreased IL-1 β mRNA expression in 7-month-old normal rats. ** $P < 0.01$ vs. vehicle group (D). The columns represent the mean $\pm$ S.D. of three independent experiments.

beings and cause chronic degenerative changes at lumbar IVDs, such as the loosening and fragmentation of the annulus fibrosus, and the decreased height of the disc. On the other hand, the structural distortion of the annulus fibrosus subjected to upright stance was almost completely recovered at the 5th and 7th month, and the height of the disc was recovered at the 5th month with the FFQSP treatment. This result suggests that FFQSP is an effective treatment for the degenerative morphology changes of lumbar IVD.

It was reported that type II collagen is the most abundant collagen in cartilaginous tissues and is often referred to as the major collagen (8, 9); its content is crucial for proper disc function, particularly in the nucleus pulposus (10–14). In this study, a significant decrease of protein content and mRNA expression of type II collagen was observed in upright induced lumbar IVDs. However, the treatment with FFQSP led to a recovery of type II–collagen protein and mRNA expression at all time points. This study indicates that FFQSP can reversely modulate the inhibited effect of the upright posture on the type II–collagen expression at lumbar discs. In order to observe the effect of FFQSP on normal rats, we used FFQSP to treat 7-month-old normal rats for 1 week and found that FFQSP could decrease the mRNA expression of Col2 α 1, which suggested that the effect of FFQSP on the

mRNA expression of Col2 α 1 is injury-dependent.

It is well known that type II collagen could be degraded by MMPs. In the MMP superfamily, MMP13 is an enzyme that possesses higher affinity to cleave the triple-helical structure of the type II collagen (15), and MMP3 is a metalloproteinase with broad substrate specificity, capable of degrading proteoglycan, laminin, fibronectin, and the non-fibrillar collagens, activating other pro-MMPs, including MMP-1, -8, -9, and -13 (16). So we investigated the mRNA expression of MMP13 and MMP3 and found that upright posture up-regulated the expression of MMP13 and MMP3, while FFQSP effectively inhibited the up-regulation trend. We proposed that besides the increased expression of type II collagen, the inhibition of its degradation by MMP13 and MMP3 might also contribute to the response of FFQSP in the protection against the upright stance-induced lumbar IVD degeneration at the 7th and 9th month. From the investigation of the effect of FFQSP on the normal rats, we found that FFQSP could decrease the mRNA expression of MMP13, which suggested that the effect of FFQSP on the MMP13 mRNA expression is not injury-dependent.

It is known that both isoforms of IL-1 (IL-1 α and IL-1 β) and MMPs are produced by the chondrocyte-like cells of the nucleus pulposus and inner annulus fibrosus of non-degenerate and degenerate IVDs in human samples (17). IL-1 β could stimulate the expression of MMPs (18, 19) and inhibit collagen biosynthesis (17, 20). We also examined the mRNA expression of IL-1 β , and the results showed that IL-1 β was up-regulated at the lumbar discs of the model group, but down-regulated by the treatment of FFQSP at the 7th and 9th month. This study suggested that FFQSP could inhibit the expression of IL-1 β at the affected IVDs. On the normal discs, FFQSP could also decrease IL-1 β mRNA expression, which indicated that the effect of FFQSP on the IL-1 β mRNA expression is not injury-dependent.

In addition, we also observed the protein and mRNA expression of type X collagen, an important player in endochondral ossification and matrix calcification, the increase of which indicates the terminal stage of degeneration (21–25). We found that type X collagen was up-regulated by upright posture for 7 and 9 months, but FFQSP could inhibit the expression of type X collagen. This result indicated that FFQSP could inhibit the matrix calcification of lumbar IVDs induced by long term upright posture.

Because the lumbar IVD degeneration was observed in the upright-posture model at 5 months later after surgery, we believed that the effect of the treatment from the 6th month and the 8th month after surgery showed therapeutic efficacy. Our results indicated that in addition to the

preventive effect, FFQSP has a good therapeutic efficacy on the IVD degeneration at 7 and 9 months after surgery.

FFQSP has been a new patent drug, and our previous quality control study of FFQSP suggested that this drug comprises total astragaloside, sinomenine, ferulic acid, and others. Total astragaloside, an active component isolated from radix astragali (26), has anti-inflammatory (27–29), anti-virus (30), and antioxidant (31) actions. Sinomenine, an alkaloid isolated from *Caulis sinomenii*, has been clinically used as a therapeutic treatment on rheumatoid arthritis (RA) (32, 33) for its dramatic anti-inflammatory property (33). Ferulic acid, isolated from *Rhizoma chuanxiong* (34), has antioxidative and anti-inflammatory effects (35–37). From all those active components effects, we presume that FFQSP has anti-inflammatory actions, which is consistent with the result in the current study that FFQSP inhibits mRNA expression of IL-1 β in the lumbar IVDs induced by upright posture for 7 and 9 months, suggesting that anti-inflammatory property of FFQSP might play an important role in the protection against the lumbar IVD degeneration process.

In conclusion, FFQSP inhibits lumbar IVD degeneration induced by long-term upright posture of rats, and its protective effects are associated with stimulating type II–collagen expression and down-regulating the MMPs, IL-1 β , and type X collagen. FFQSP is a promising medicine for preventing lumbar IVD degeneration disease.

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