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A study of the influence of Guizhi plus Gegen Decoction on the mechanism of signal transductions occurring in cervical intervertebral disc cells of adult rats.

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Abstract

The relationship between the CaM-CaMK-CREB signal pathway and the treatment for cervical spondylosis by guizhi plus gegen decoction was studied. Group intervention on fibrous ring cells of cervical intervertebral disc of adult rats by medicine serum mixed with Guizhi plus Gegen decoction, blank serum and fetal calf serum respectively was made. Detect by MTT colorimetric assay the growth curve of fibrous ring cell under the adoption of Guizhi-plus-Gegen-decoction serum; Conduct western blotting to detect the impact of herbal medicine serum on expression and quantification of CaM, CaMK and CREB; Perform data processing and statistical analysis on the results. The five groups showed no significant difference between each other during the first day after the intervention of Chinese medicine serum($p > 0.05$); from the second to seventh day after the intervention, there were significant difference between the blank serum group and other groups($p < 0.05$). Compared with the blank serum group, Guizhi-plus-Gegen-decoction medicine serum at all doses could substantially improve the expression of CaM, CaMKII, CaMKIV and CREB in fibrous ring cells of cervical intervertebral disc of adult rats ($p < 0.01$), and with the increased doses of Chinese medicine, the capacity of improving the expression level was also increasing. Guizhi-plus-Gegen-decoction medicine serum could highly stimulate the development of fibrous ring cells of cervical intervertebral disc of adult rats. Clinically when it comes to the treatment by Guizhi plus Gegen decoction for cervical spondylosis, there is likely to be an important signal pathway called "CaM-CaMK-CREB".

Keywords: Guizhi plus Gegen decoction; fibrous ring cell; primary culture; signal pathway.

Introduction

Guizhi plus gegen decoction originally derives from "Treatise on Febrile Diseases" by Zhang Zhongjing, which says "When it comes to maladies in taiyang that leads to stiff neck, with aversion to wind but without sweating, can be resolved by guizhi plus gegen decoction." The original prescription is mainly used for wind-stroke syndrome and channel qi block of taiyang. Cervical spondylosis presents as a series of symptoms such as stiff neck, pain in neck and shoulders, where Taiyang channel runs through. Stiff movement of those areas is usually caused by the exterior tightened by pathogenic wind-cold, channel qi and body fluid block, which leads to the loss of nutrition. Guizhi acts as sovereign drug, improving defensive yang, and dredging the meridians, expelling patho-

genic factors from muscles and skin, as well as dispelling wind pathogen. Baishao enhances yin fluid and preserves nutrient qi, prevent them from loss by astringing. Guizhi and baishao mixed at equal doses can alter yingwei and yinyang from inside and expel pathogenic factors from muscles and skin from outside. Gegen dispels the wind and assists guizhi in the expelling as well as ventilating channel qi, which resolves qi and blood stasis. It also generates body fluid and relieves contraction of tendon, as a minister drug besides baishao. Ginger is pungent and warm-natured. It can assist guizhi in expelling exterior pathogenic factors and relieve gastric illness and vomiting. Chinese date is sweet and neutral-natured. It can invigorate spleen-stomach and replenish qi, as well as benefit spleen and produce body fluid. The mixture of ginger and Chinese date, is a common combination for invigorating spleen and stomach, also for harmonizing yingfen and weifen. They act as assistant drugs. Honey-broiled licorice harmonizes the nature of drugs, to be exact, generates yang with guizhi to enhance weifen, and generates yin with shaoyao to improve yingfen, which amounts to an assistant and envoy drug. The drugs mentioned above bears the function of expelling pathogenic factors from muscles, harmonizing yingfen and weifen, generating body fluid and relieving contraction of tendon. Documents by pei [1] shows that the top seven most frequently-used drugs are gegen, guizhi, huangqi, danggui, baishao, chuanqiong, qianghuo, whose occurrence is all above 30%, with gegen up to 75%. The most commonly used prescription are guizhi plus gegen decoction, huangqi guizhi decoction of five ingredients, buyang huanwu decoction, etc. According to the previous research [2] about the effects of Guizhi plus Gegen Decoction combined Tuina on later extensor group of cervical spondylosis, this combinative method was better than th single Tuina method, other scholars [3-5] have also proved clinically that some kinds of cervical spondylosis can also be resolved by guizhi plus gegen decoction, such as cervical type cervical spondylosis, vertebral artery type of cervical spondylosis and cervical spondylotic radiculopathy. However, the related fundamental study hasn't been reported in detail yet.

There is many causes leading to cervical spondylosis, where intervertebral disc degeneration is the most important one that attracts much attention for fibrous ring on the grounds that its main predisposing cause is considered to be fibrous ring rupture.[6] According to the previous study[7], there is a significant signal pathway called "CaM-CaMK-CREB" during the signal transduction of fibrous ring cells amongst CaM CaMK and CREB, the downstream products of secondary signaling molecules of Ca2+. Previously there was no systematic study on the coordination and controlling of this pathway, nor were there any integral documents on this subject. Therefore, the author is about to do the research from a brand-new perspective, or to be exact, the influence of decoction serum on the mechanism of signal transduction occurring in the fibrous ring cells of cervical intervertebral disc of adult rats. First, observe through experiment the impact of Chinese medicine serum on the expression and quantification of CaM, CaMK and CREB, as well as on the development of the fibrous ring cells of cervical intervertebral disc of adult rats. Second, detect the relationship between CaM－CaMK－CREB signal pathway and the mechanism of clinical treatment by guizhi plus gegen decoction for cervical spondylosis.

Material and Methods

Materials

Instruments and chemicals

Biological purification table (SW-CJ-IF, produced by Suzhou Purification Equipment Co., Ltd.); CO2 incubator(produced by SANYO, Inc, Japan) ;inverted microscope(produced by Leica, Inc, Germany); Centrifugal precipitator(80-2 CENTRIFUGE, produced by Shanghai Factory of Surgical Instruments); thermostat water bath(Shenzhen HH-4); 550-enzyme labeling instrument(produced by BIO-RAD, Inc, U.S.); stabilized electrophoresis apparatus(DYY-6B,produced by Beijing Liuyi Factory of Instruments); Gel imaging system(Gel Doc XR, produced by BIO-RAD, Inc, U.S.); All the Chinese medicine was purchased from the teaching hospital of BUCM; other chemicals are already prepared before purchase.

Animals

Experimental animal 1:80 healthy clean SD rats, weighing (250±10)g each, half males and half females; experimental animal 2: 2 healthy clean SD rats, 1 month old, weighing about 100g each, of either sex. Both experimental animals were purchased from Shanghai Slac Laboratory Animal Co., Ltd, whose licence No. is SCXX (Shanghai) 2008-0005. All rats were fed in the center of experimental animal (medical animal No. 23-016).

Methods

Chinese medicine preparation

Composition of the original guizhi plus gegen decoction: gegen 12 g, shaoyao 9 g, ginger 9g(cut-up), licorice 6 g (broiled), Chinese date 12, guizhi 9 g (peeled)(from "Treatise on Febrile Diseases") Provided by the center of pharmaceutical preparation, exactly following the original prescription.

Chinese medicine serum preparation [8,9]

Guizhi plus gegen decoction is provided by the center of pharmaceutical preparation, exactly following the original prescription. Convert the dosage to an equivalent crude drug dosage of 11.6 g•kg-1 on adult rats according to the body surface area. Select 80 adult SD rats, weighing

(250±10)g each, half males and half females, divided into four groups as follows by randomized block on computer:20 rats in low dosage group, 20 rats in moderate dosage group, 20 rats in high dosage group, and 20 rats in control group. Low dosage is 0.5-fold of equivalent dosage (1 ml each), moderate dosage is the equivalent dosage (2 ml each), high dosage is 2-fold (4 ml each, i.g.), and 2 ml of physiological saline for control group. All four groups treated by intragastric administration for 4 days (twice daily), and perform ether inhalation anesthesia 2 h after the last time. Collect blood from abdominal aorta and heart, then isolate serum from it, preserve at -20℃ for preparation.

Cells Culture

Primary culture, subculture and assay of fibrous ring cells of cervical intervertebral disc of adult rats has reached somewhere feasible and replicable through repetitive experiment on the basis of large numbers of documents [10-16], and now published on related journals[15].

MTT colorimetric assay of growth curve of fibrous ring cells

(1)for experimental procedure, see "About the Methodology of Optimization Study on Means of Cell Activity Assay" by Bo Huaben, Shao Hongwei, Hu Lingbo, etc.[16]
(2) analysis

Statistical analysis should be conducted with SPSS13.0, data should be noted as mean±standard deviation（X ±S）. Given this is a multiple comparison between groups, it might be prudent to do the analysis with one-way ANOVA, and consider the results to have statistical significance when P<0.05.

Western blotting for assay of expression levels of related proteins

1. For experimental procedure, see "About Western blotting assay of phospho-p38MAPK expression in nucleus pulposus during degeneration of cervical intervertebral disc" by Yang Zhigao, Zhang Fan, Chen Zhi, etc.[17]
2. Protein band analysis
Sample the film image with Gel imaging system by BIO-RAD, calculate the OD with Image-Pro Plus 6.0, and perform semi-quantitative analysis.
3. Data analysis

Statistical analysis should be conducted with SPSS13.0, data should be noted as mean±standard deviation（X ±S). Given this is a multiple comparison between groups, it might be prudent to do the analysis with one-way ANOVA, and consider the results to have statistical significance when P<0.05.

Results

Assay the influence of guizhi-plus-gegen-decoction serum on the activity of fibrous ring cells of cervical intervertebral disc of adult rats by MTT assaying the influence of guizhi-plus-gegen-decoction serum on the activity of fibrous ring cells of adult rats, it could be observed that(see Figure 1) the five groups showed no significant difference between each other during the first day after the intervention of Chinese medicine serum(p> 0.05); from the second to seventh day after the intervention, there were significant difference between the blank serum group and other groups(p <0.05), which shows both the medicine-serum and 10%-fetal-calf-serum group could substantially enhance the development of the fibrous ring cells(p <0.05); and with the increased doses of Chinese medicine, the capacity of improving the expression levels of CaM, CaMKII, CaMKIV and CREB was also increasing.

Western blotting for assaying the related proteins

The result from western blotting has shown that(see Figure 2 and 3) compared with the blank serum group, the protein expression level of three groups of serum with medicine at varying doses has significantly improved, as is the case with 10%-fetal-calf-serum group（p＜0.05); Compared with 10%-fetal-calf-serum group, groups of high and moderate dosage did not show significant difference in protein expression level（p＞0.05, while low-dosage and blank group did（p＜0.05; Compared with moderate-dosage group, high-dosage group showed significant difference in protein expression level.（p＜0.05） Note: Serum with medicine at all doses could improve the expression level of proteins related to fibrous ring cell of intervertebral disc of adult rats. However, with medicine at less than equivalent dose (tantamount to the moderate dose in this experiment), the serum showed significant difference with 10%-fetal-calf-serum in protein expression level, while at more than equivalent dose, there wasn't significant difference with 10%-fetal-calf-serum. Clearly, guizhi plus gegen decoction at all doses can improve the expression level of CaM CaMKII and CaMKIV, and the capacity of improving became highest at high dosage, and lowest at low dosage. See Table 2 below.

Table 1. *Growth curve of fibrous ring cells of cervical intervertebral disc of adult rats*

Group	First day	Second day	Third day	Fourth day	Fifth day	Sixth day	Seventh day
Blank	0.182±0.0016	0.244±0.0041	0.369±0.0010	0.456±0.0022	0.542±0.0033	0.619±0.0014	0.620±0.0009
low-dosage	0.183±0.0013	0.259±0.0015	0.442±0.0017	0.580±0.0030	0.635±0.0020	0.713±0.0022	0.775±0.0023
moderate-dosage	0.183±0.0015	0.284±0.0020	0.488±0.0017	0.658±0.0025	0.703±0.0026	0.821±0.0018	0.992±0.0013
high-dosage	0.184±0.0013	0.348±0.0019	0.583±0.0022	0.689±0.0012	0.792±0.0016	0.908±0.0016	1.080±0.0174
fetal-calf-serum	0.184±0.0023	0.392±0.0015	0.627±0.0018	0.748±0.0019	0.816±0.0036	0.958±0.0030	1.176±0.0151

Table 2. *Tthe expression level of related proteins by western blotting*

grey level	10% fetal calf serum+DMEM	10% high-dosage medicine serum+DMEM	10% moderate-dosage medicineserum+DMEM	10% low-dosage Medicine serum+DMEM	10% ddH_2O blank serum+DMEM
CaM	16.999%±0.089%	17.119%±0.085%	16.149%±0.056%	11.120%±0.154%	8.241%±1.169%
CaMKII	49.192%±1.405%	50.498%±1.359%	46.6420%±3.483%	20.431%±0.188%	11.450%±0.019%
CaMK IV	57.914%±0.100%	58.663%±1.058%	57.106%±0.052%	39.092%±0.522%	19.290%±0.156%
CREB	61.046%±0.483%	61.600%±0.413%	60.077%±0.493%	37.051%±0.084%	20.387%±0.431%

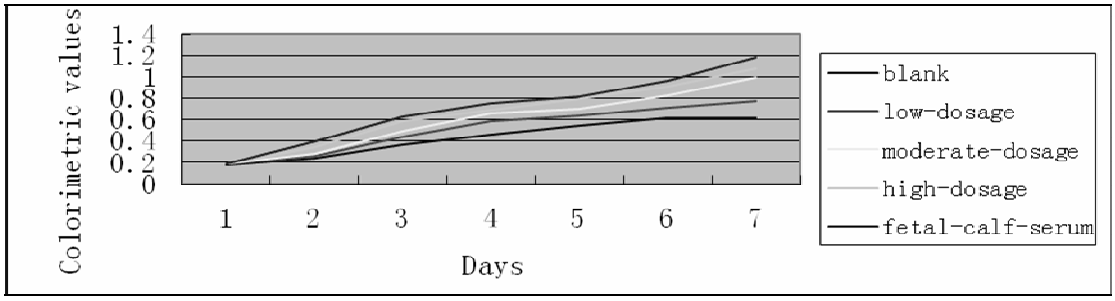

Figure 1. *Growth curve of fibrous ring cells of cervical intervertebral disc of adult rats*

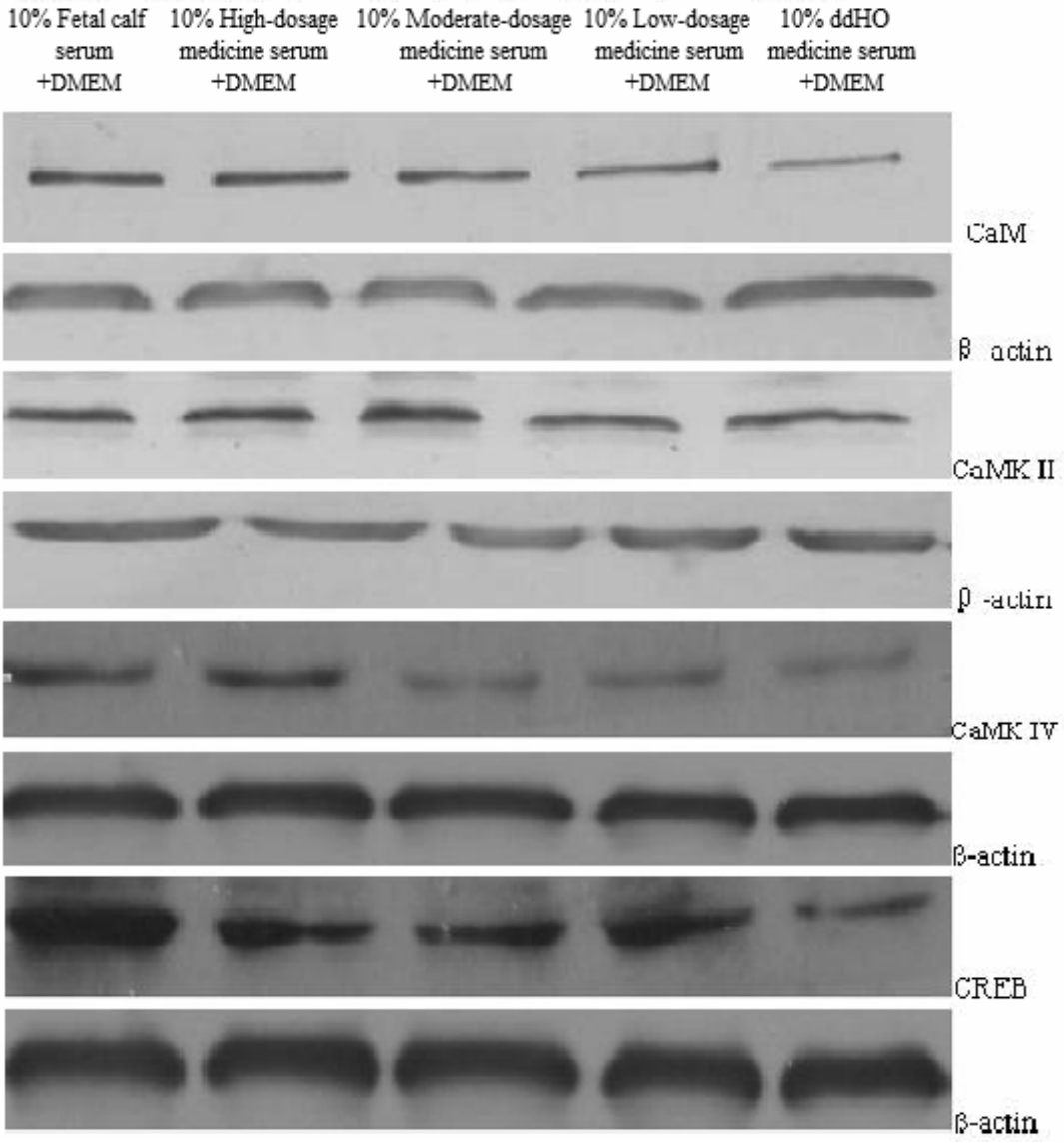

Figure 2. T*the results of related proteins by western blotting*

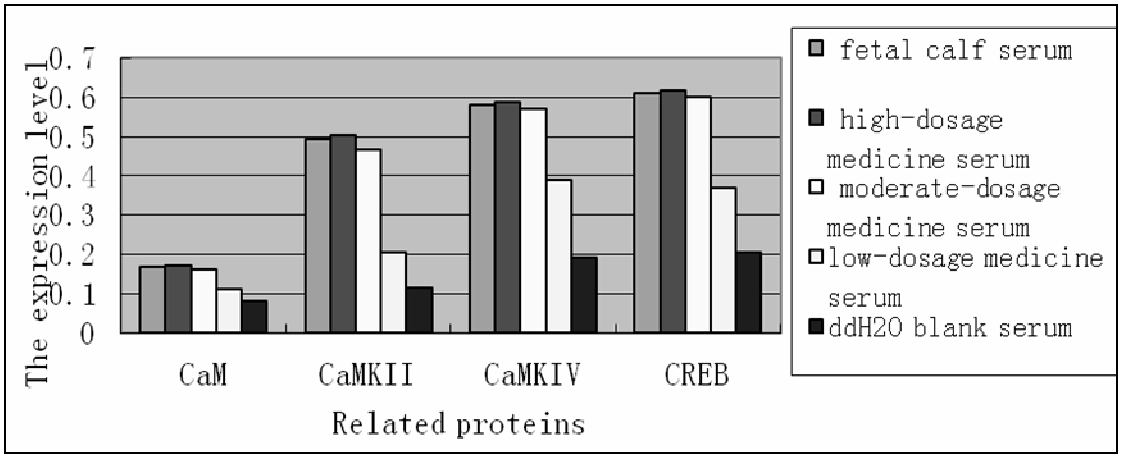

Figure 3. *the expression level of related proteins by western blotting*

Discussion

Assay the influence of decoction of guizhi and gegen serum on the activity of fibrous ring cells of cervical intervertebral disc of adult rats by MTT

There were no significant difference among the five groups during the first day after the intervention of Chinese medicine serum shown from results by MTT ($p > 0.05$); however, there were significant difference between the blank serum group and other groups from the second to seventh day after the intervention ($p < 0.05$). The point meant that decoction of guizhi and gegen could enhance the development of fibrous ring cells to a great extent, which is closely related to the time and dosage. The capacity of improving the development enhanced with the increased dosage of Chinese medicine. MTT method, with its simple, sensitive, good stability and repeatability, are widely used in the detection of culture cell growth and value-added, evaluating the cell toxicity of chemicals and malignant tumor susceptibility test in vitro[16]; However, MTT is somehow unpersuasive on the grounds that it can only be adopted for assaying the relative number and activity of cells rather than absolute number, which is why there is necessity of some profound discussion on how to assay the exact number of cells, and previous research might offer some reference to this issue[18].

Decoction of guizhi and gegen acts on cell development via CaM—CaMK—CREB signal pathway

Western blotting had shown that the protein expression level of three groups of serum with medicine at varying doses had significantly improved, compared with the blank serum group, as is the case with 10%-fetal-calf-serum group（$p < 0.05$); Compared with 10%-fetal-calf-serum group, groups of high and moderate dosage did not show significant difference in protein expression level,（$p > 0.05$）while low-dosage and blank group did（$p < 0.05$）; Compared with moderate-dosage group, high-dosage group showed significant difference in protein expression level.（$p < 0.05$）Note: Serum with medicine at all doses could improve the expression level of proteins related to fibrous ring cell of intervertebral disc of adult rats [19]. However, with medicine at less than equivalent dose (tantamount to the moderate dose in this experiment), the serum showed significant difference with 10%-fetal-calf-serum in protein expression level, while at more than equivalent dose, there wasn't significant difference with 10%-fetal-calf-serum. Combined with previous studies [20] and the results in this experiment, it can be illustrated that, from the molecular level, decoction of guizhi and gegen's effects on cervical spondylosis may be related to raise CaM/CaMK signal pathway, but the detailed mechanism needs further study.

Conclusion

Decoction of guizhi and gegen could improve the development of fibrous ring cells, and the capacity of improving cell development enhances as the dose of Chinese medicine in serum increases. Decoction of guizhi and gegen at all doses could improve the expression level of CaM CaMKII and CaMKIV, and the capacity of improving became highest at high dosage, and lowest at low dosage. Clinically there is likely to be an important signal pathway "CaM－CaMK－CREB" throughout the treatment for cervical spondylosis by decoction of guizhi and gegen[21]. Notwithstanding, there was no protein inhibitor in this project because of several limitations. The results from western blotting could only indicate that decoction of fuizhi and gegen serum was capable of altering the expression as well as qualification of CaM CaMK and CREB when acting on the fibrous ring cells of cervical intervertebral disc of adult rats. There is still some doubt that remains to be proved about the existence of CaM-CaMK-CREB, an important signal pathway, through the prevention and treatment of cervical spondylosis. Wheth-

er there are other signal pathways intervene during this process is also necessary for further discussion.

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