

Icariin Attenuates Serum Deprived-Induced Premature Senescence in Rat Annulus Fibrosus Cells and Ameliorates Disc Degeneration *In Vivo*

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ABSTRACT

Purpose: To explore the effect of icariin, a traditional chinese medicine, on serum deprived-induced premature senescence in rat annulus fibrosus cells *in vitro* as well as it's possible mechanism and IDD *in vivo*.

Methods and Results: Rat annulus fibrosus cells were separated from intervertebral disc of 200g rat with sterile surgical instruments and they were used for all experiments after 3 passages. The rat annulus fibrosus cells were divided into three groups: A (blank control: 10% fetal bovine serum of F12 DMEM); B (2% fetal bovine serum of F12 DMEM); C (2% fetal bovine serum of F12 DMEM + 20 μ M icariin). We detected cell proliferation, SA- β -Gal activity, cell cycle, and the expression of matrix macromolecules (aggrecan and collagen I) and senescence markers (p16 and p53). All results supported each other and showed a remarkable protect effect of icariin which can attenuate serum deprived-induced premature senescence in rat annulus fibrosus cells *in vitro*. We also found the variation of ROS/NF- κ B pathway which indicated that ROS/NF- κ B pathway was at least partly included in this protect process. Meanwhile, in a rat model, we observed protect effect when rat accept the intervention of icariin intraperitoneal infection every other day. These protect effect have been verified by HE stains and immunohistochemistry stain.

Conclusion: These results reveal that icariin could protect against serum deprived-induced premature senescence of rat NP cells through ROS/NF- κ B pathway and ameliorate disc degeneration *in vivo* and suggest that icariin can be a new therapeutic candidate for treatment of IDD.

KEYWORDS: Icariin; Annulus fibrosus cells; ROS/NF- κ B pathway; Premature senescence; Serum deprived

ABBREVIATIONS: IDD: Intervertebral Disc Degeneration; ROS: Reactive Oxygen Species; NF- κ B: Nuclear factor kappa-B; NP: Nucleus Pulposus; AF: Annulus Fibrosus; SA- β -Gal: Senescence-associated β -galactosidase

INTRODUCTION

Degeneration of intervertebral disc is a worldwide degenerative disease and brings us large load of economic burden [1,2]. Regeneration and repair of intervertebral disc is vital. Annulus fibrosus senescence is important in this pathological process [3]. Research showed annulus fibrosus is different in young

and old sample using intralamellar matrix of single lamellae [4], Quantitative proteomic analysis [5] and medical imaging methods [6]. Although death and reduction of extracellular matrix in nucleus pulposus cells contribute to the degeneration of intervertebral disc [7], the senescence and fracture of annulus fibrosus is cannot be neglected.

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Icariin is a traditional Chinese medicine and a kind of natural flavonol glycoside. Plentiful literature report icariin have effect of prevent apoptosis [8-10], attenuate inflammation [11], prolong life [12] and resistance senescence [13-18]. Nevertheless, the mechanism of resistance senescence is poorly understanding. We know intervertebral disc cells including nucleus pulposus cells (NP) and annulus fibrosus cells (AF) are nutritional deficiency *in vivo* for the special inter-vertebra location and blood supply deficiency. We chose serum-deprived model *in vitro* for simulation of nutritional deficiency *in vivo*. Serum-deprived have the effect of activating NF- κ B pathway have been verified [19-21] in some research and the activation of NF- κ B is associated with senescence [22-24]. So, we explored if icariin has protected effect when AF cells are exposed to nutritional deficiency and the effect of IVDD repair *in vivo*.

MATERIALS AND METHODS

Cell culture and synchronization. Annulus fibrosus cells were isolated using about 200 g NP tissue of rats. Briefly, annulus fibrosus tissue was aseptically removed in a petri dish containing 0.25% (w/v) type I collagenase and cut into pieces 0.1 mm \times 0.1 mm. Then samples were digested with 0.25% (w/v) type I collagenase for 3-4h and serum was used to stop the reaction. After centrifugation at 1200 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in F12-Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin. Cell cultures were maintained at 37 °C and 5% CO₂. Medium was changed 3-5 days later when the cells had attached, and then changed every other day. When AF cells reached approximately 80% confluence, each primary culture was subculture at a 1:3 ratio with a 0.25% (w/v) trypsin solution.

Grouping. A (blank control: 10% fetal bovine serum of F12 DMED); B (2% fetal bovine serum of F12 DMEM for 48h); C (2% fetal bovine serum of F12 DMEM + 20 μ M icariin). *In vivo* experiment Rats were randomly divided into three groups: the control group (A), the IVDD group (B) and the icariin treated group (C). Intervertebral disc degeneration and premature senescence model was applied to the latter two groups and referred to needle puncture model [25]: Briefly, rat tail disc (Co7/8) was chosen as the experimental level. Needles (27G, 4mm long) were used to puncture the whole layer of annulus fibrosus through the tail skin and kept in the disc for 1 min. After the procedure, the rats were placed back in their cages and given food and water. And the rats in group C were intraperitoneal injected with icariin (50 mg/kg) every other day. After 8 weeks puncture, the rats was killed and tail discs (Co7/8) were analyzed. SA- β -Gal activity detection. AF cells were seeded in 6-well plates and incubated with different test compounds for 24 hours. Then, SA- β -Gal staining was performed using a Senescence β -Galactosidase Staining Kit (Beyotime, China) and the SA- β -Gal staining-positive NP cells were observed under a light microscope (Olympus BX51). SA- β -Gal activity was expressed as the percentage of SA- β -Gal positive AF cells.

Cell cycle analysis. AF cells were seeded in six well plates and grown to 60-70% confluence and with different test compounds for 24 hours. Thereafter, the AF cells were digested with trypsin (0.25% without EDTA, Gibco) and centrifuged to collect the cell pellets. After fixation with 75% ethanol overnight, treatment with RNase for 30min in 37 °C and staining with propidium iodide dye (50 μ g/ml, Beyotime, China) for 30 minutes. AF cells were subjected to flow cytometry analysis. The cell cycle of each group was analyzed using multicycle software (Japan PHENIX Company).

Immunocytochemistry staining. Expression of matrix macromolecules (collagen I) and senescence related protein was analyzed by immunocytochemistry. Briefly, AF cells were first fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin. After incubation with primary antibodies (collagen I: NOVUS, NB600-450, diluted 1:1000; p53: abcam, ab26, diluted 1:500) at 4 °C overnight and incubation with the corresponding secondary antibodies conjugated with green or red fluorescence (ZSGB-BIO, China, diluted 1:2000) at 37 °C for 2 hours. Finally, the AF cells were observed under a light microscopy (Olympus BX51, Japan) and immunostaining intensity was analyzed using Image-Pro Plus software (Version 5.1, Media Cybernetics, Inc.).

Detection of intracellular ROS levels by flow cytometry: Cells were treated differently according to the experiment grouping design. Then 200 μ L of culture medium from each group were gathered to detect intracellular ROS levels. Experimental steps were strictly executed according to the manufacturer's instructions.

Expression of p16, p53, NF- κ B (p-P65) by Western blot analysis: Proteins were extracted according to the instructions of the Total Extraction Sample Kit. Equal amounts of proteins (10 μ g) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels, electrophoresed, and then transferred to polyvinylidene fluoride membranes. The membranes were incubated with 5% nonfat milk for 2 h followed by incubation with a primary antibody overnight at 4 °C (0.5 μ g/mL p16, p53, NF- κ B (p-P65); 1:1000). After washing in TBST, membranes were incubated with the secondary antibody for 1.5 h at room temperature (rabbit anti-mouse or goat anti-rabbit, 1:5000). Bands were visualized by incubating with enhanced chemiluminescence reagent for 2 min after membranes were washed with TBST. Densitometry of p16, p53 as well as NF- κ B (p-P65) levels was performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis: Data are presented as means \pm standard deviation. For group-wise comparisons, a one-way ANOVA with the LSD or Dunnett's T3 test was performed using SPSS 19.0 (IBM, Chicago, IL, USA). Values were considered significantly different for $P < 0.05$.

RESULTS

Icariin reduced SA- β -Gal activity of serum-deprived AF cells. SA- β -Gal activity is an examinational mean based on increased activity of SA- β -Gal when cells get into senescence phase. SA- β -Gal can be detecting with a reaction substrate of X-Gal when pH is 6.0. Compared with control group (Figure 1A), serum-deprived AF cells change into larger volume and more SA- β -Gal activity stain (Figure 1B). Icariin attenuate this senescence change (Figure 1C). We simulate a premature senescence model using serum-deprive method and there are reasons to believe that icariin has significant protective effect when rat annulus fibrosis cells exposed in an environment of nutritional deficiency.

G0-G1 cell cycle arrest is another important characteristic of senescence cell. Cell in G0-G1 period stand for block of cell. Cell cycle was delayed in G1 period when serum was deprived compared with blank group. Icariin have apparent effect on reversing this phenomenon (Figure 2). There has statistic difference between group B and group C with three times repetition (* $p < 0.01$).

P53 and P16 are two classic markers of cell senescence, their protein expression was investigated to support our conclusion

that icariin can protect against serum-deprived-induced AF cell senescence proteins P53 and P16 increased (Figure 3). Immunofluorescence in the cells of nucleus proteins P53 and P16 (Figure 3A), same as western blot support each other to come to a

conclusion that icariin have the ability to decrease the expression level of senescence relative proteins (Figure 3B). This data has a significant statistic difference (Figure 3C). (* $p < 0.05$, ** $p < 0.01$).

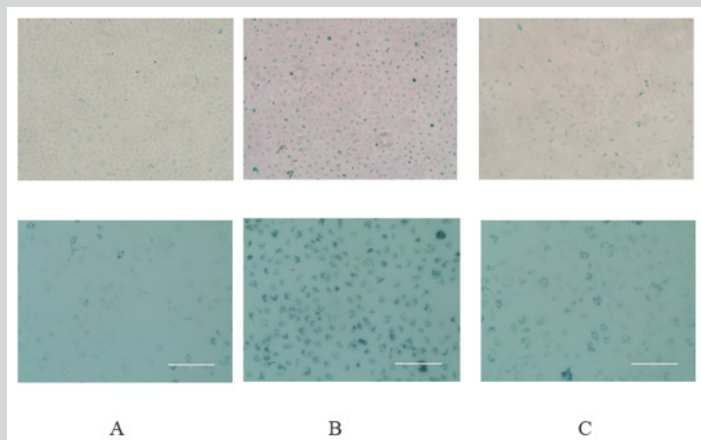


Figure 1: SA-β-Gal activity stain in four groups: A (blank control: 10% fetal bovine serum of F12 DMED); B (2% fetal bovine serum of F12 DMEM); C (2% fetal bovine serum of F12 DMEM + 20 μM icariin). We observed more SA-β-Gal activity stain in group B and this activity stain have been attenuate.

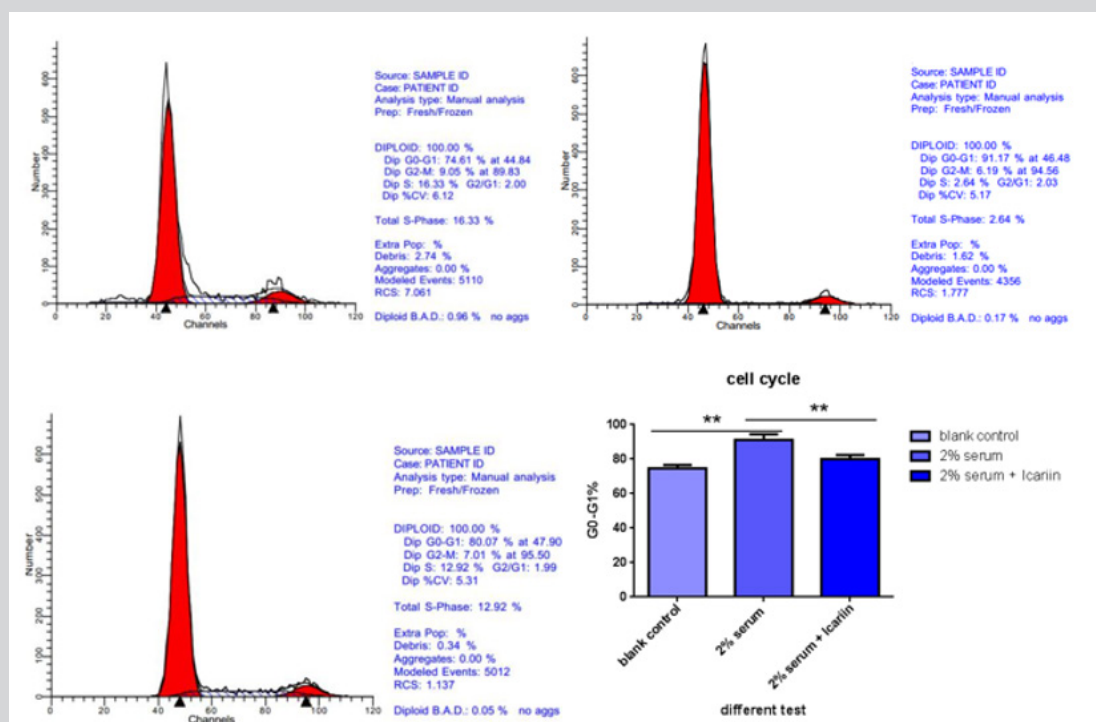


Figure 2: Icariin decreased the percentage of G₁ phase serum-deprived-treated annulus fibrosus (AF) cells. AF cells were incubated with different test compounds for 48 hours. The percentage of AF cells in the G₀/G₁, G₂/M and S phases in each group was shown in the respective images. There was statistic different in blank group and 2% serum group (* $p < 0.05$) but no different in 2% serum group and icariin group. Icariin only have little effect on cell cycle under this situation.

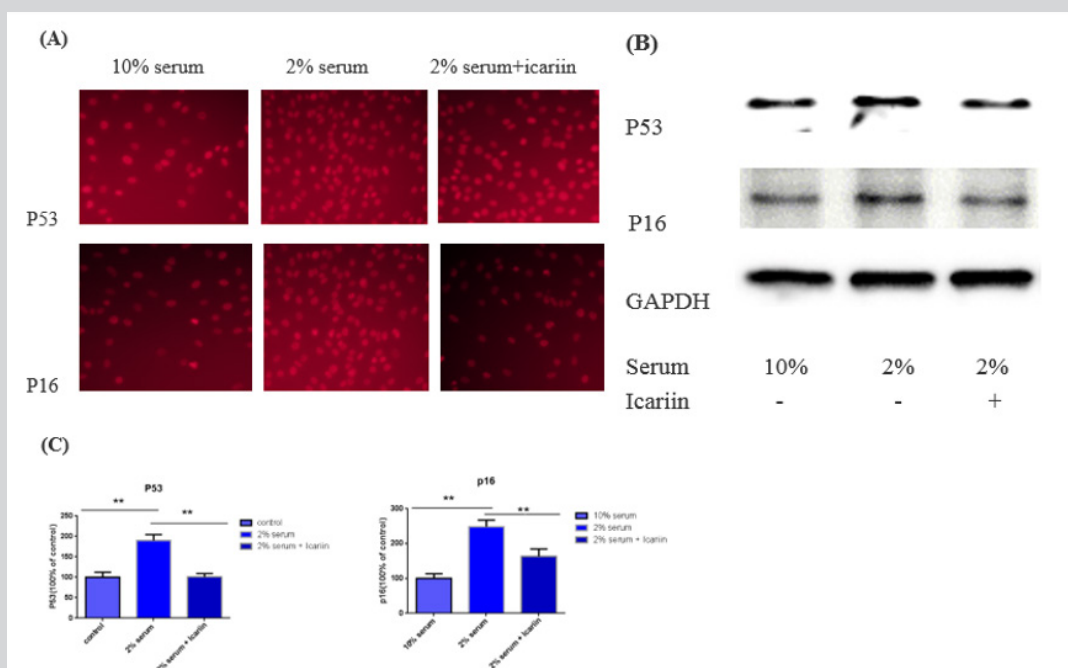


Figure 3: Compared with 10% serum group (blank group), 2% serum group showed up a significant increase in the expression of P53 and P16 (* $p < 0.01$), the two classic markers of cell enescence. These were supported by immunofluorescence in the cell (A) and western blot (B). Icariin decrease this protein expression and this data have significant statistic different (* $p < 0.01$) (C).

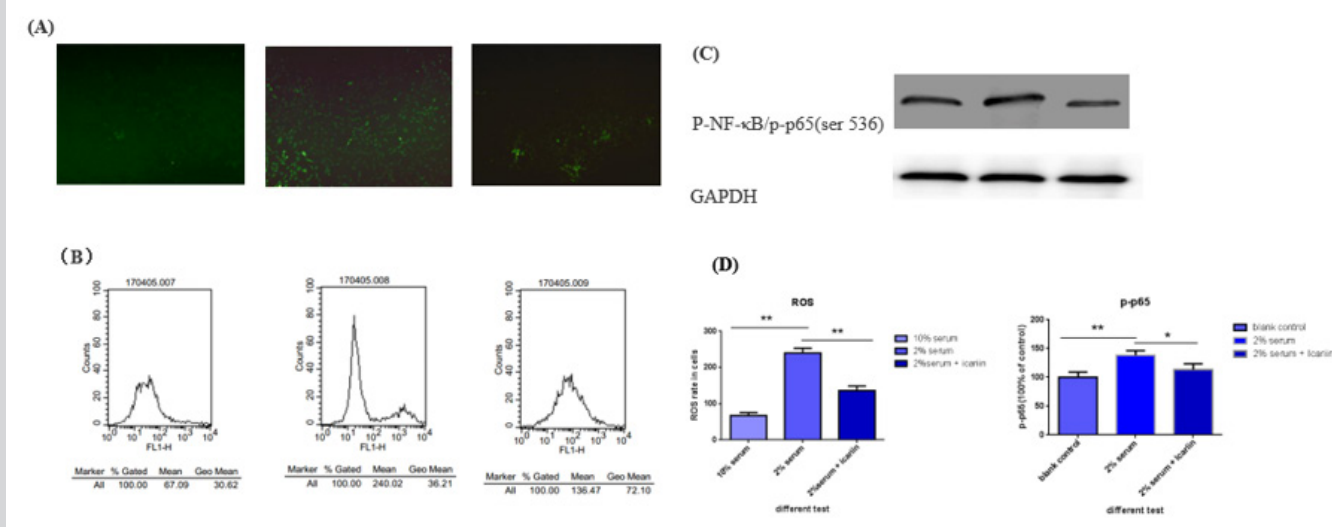


Figure 4: ROS rate rise remarkably when 10% serum became 2% serum indicate starvation induced oxidative stress in cells and NF-κB was activated and these were observed by intracellular immunofluorescence (A), flow cytometry (B) and western blot (C). Icariin can attenuate this effect a certain extent. This data has a significant statistic difference (D). (* $p < 0.05$, ** $p < 0.01$).

As showed in Figure 4, ROS/NF-κB pathway have a change in this serum-deprived model. ROS/NF-κB pathway is responsible for cell proliferate, apoptosis and survive. Continuously activated of ROS/NF-κB pathway have ability to induce senescence [22-24]. Here, we observed activation of ROS/NF-κB pathway when serum-deprived include the intercellular ROS rate rise by the methods of intracellular immunofluorescence (Figure 4A), flow cytometry (Figure 4B) and western blot (Figure 4C). The ascent of p-NF-κB

(p-p65) and this activation of pathway was inhibition by icariin. This data has a significant statistic difference (Figure 4D). (* $p < 0.05$, ** $p < 0.01$)

Icariin ameliorates disc degeneration in rat *in vivo*. As shown in Figure 5, the disc section was normal with round shape of the NP and well-organized collagen lamellae in the control group. However, in the IDD group, the disc appeared degenerative changes, and the

histologic score was significantly higher than that of the control. Icariin treatment group improved the degenerative changes and decreased the histologic score. Furthermore, immunohistochemical

staining showed that icariin could decreased expression levels of senescence markers p53 and up-regulated collagen I in AF cells as well as collagen II in NP cells, which confirmed our results *in vitro*.

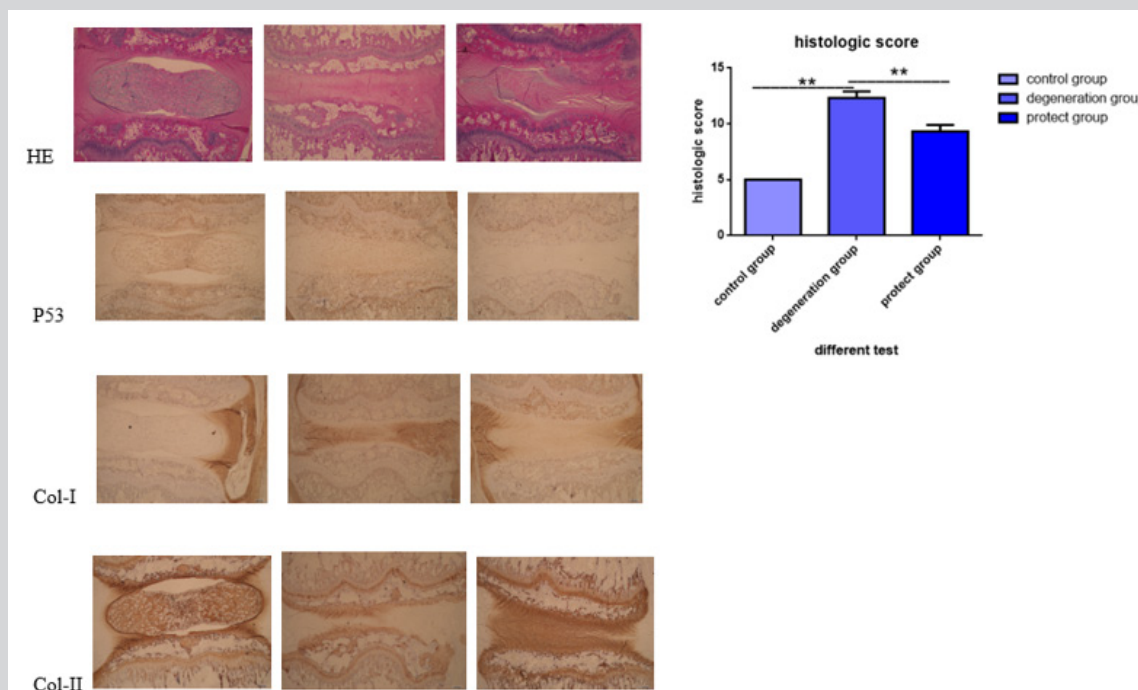


Figure 5: HE stains of intervertebral disc show we made the IVDD model successfully, histologic score demonstrated icariin have protect effect when rat nucleus pulposus tissue exposed to impaired fibrous rings tissue. Immunohistochemical of P53 showed fibrous rings aged when punctured by needle and icariin can protect against this aging process. Col-I and col-II are the symbolic extracellular matrix (ECM) of intervertebral disc. We found icariin have active ability of deposition ECM, maintain the basic location of col-I and col-II, which can prevent the aging process of intervertebral disc.

DISCUSSION

Cellular senescence, which features irreversible growth arrest, can be induced by continuous replication and various stresses [26,27]. Under the adverse microenvironment, including pressure, hypoxia, nutrient deprivation, or reactive oxygen species, IVD cells senescence and apoptosis can accumulate in degenerative IVD [28]. Delaying IVD cell senescence may contribute to the IVDD repairment.

Our previous research indicated that icariin could protect against H_2O_2 -induced apoptosis in rat nucleus pulposus cells, and it was reported that icariin is relative to aging [29,30]. However, there is no research discussing the effect of icariin on delaying senescence in intervertebral disc. We chose serum-deprived model to simulate the microenvironment of intervertebral disc for alimentary deficiency is an important factor for intervertebral disc degeneration [31]. After treatment of serum-deprived for 48h, we observed significant difference of cell premature senescence and these results are reflected by SA- β -gal stain, cell cycling detection, senescence relative proteins. Among these, SA- β -gal stain is used most wide for it is convenient and visual. Our results support that β -galactosidase expressed more when serum-deprived and icariin rescue this change. cell cycling detection had significant statistical significance in group B and C. As previous literature reported [32], in normal growth environment, annulus fibrosis cells have approximate 70%-80% G0-G1 cells. In our work, we got similar

conclusion. And after 48h serum-deprived treatment, almost all cells got into "period of dormancy" to adapt this unfavorable environment. We are amazed that icariin turns the scale to almost normal status. Our date gave the evidence that icariin has potential effect to restraint senescence. What's more, senescence relative protein p53 and p16 had same results. Nevertheless, target spot of icariin to play this role required more research.

For explore possible mechanism in this protect effect, we detect ROS/NF- κ B pathway for icariin have strong effect to anti oxidative stress in previous research [33-37] and icariin may have effect on suppressing NF- κ B [38-41]. When NF- κ B pathway been activated, it's subunit p65 phosphorylate and get into cell nucleus to regulate transcription. So, detection of p-p65 is accurate to reflect state of NF- κ B pathway. In group B, we observed remarkable change in intracellular ROS, an activating factor of NF- κ B, accompanied by increase of p-p65. When icariin pre-treatment, ROS and p-p65 decreased together. We believed that ROS/NF- κ B pathway is at least partly involved in this protect effect of icariin. Certainly, more research is required to find more precise target spot, for icariin may be a potential medicine to treat IDD and delay senescence.

To evaluate the effect of icariin *in vivo*, a disc degeneration rat model was established and assessed. According to our histological analyses, icariin can prevent intervertebral disc from aging. The AF cells showed decreased expression levels of senescence markers (p53), up-regulated matrix protein expression and maintain the

location distribution of collagen in intervertebral disc tissue in icariin treated group. All these results were in line with those from the AF cell culture *in vitro*.

CONCLUSION

In conclusion, Cell senescence induced by serum deprivation may contribute to AF degeneration and, thereby, disrupts IVD homeostasis. The results of this study indicated that icariin attenuate serum deprived-induced premature senescence in rat annulus fibrosus cells via ROS/NF- κ B pathway and ameliorate disc degeneration *in vivo*. Icariin may be developed as a new therapeutic candidate for treatment of IVDD.

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