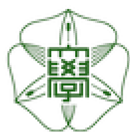
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Citation	Japanese Journal of Veterinary Research, 71(2), 35-47 https://doi.org/10.57494/jjvr.71.2_35
Issue Date	2023-11-27
Doc URL	http://hdl.handle.net/2115/90817
Type	bulletin (article)
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Japanese Journal of Veterinary Research 71(2): 35-47, 2023

JJVR

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Sulfate level relied effects of pentosan polysulfate sodium on inhibiting the proliferation of canine articular chondrocytes by targeting PI3K/Akt pathway

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Received for publication, January 4, 2023; accepted, April 4, 2023

Abstract

Articular chondrocytes experience a transient increase in proliferation and subsequent loss of chondrogenic phenotype in the early stage of osteoarthritis (OA). Pentosan polysulfate sodium (PPS) is a highly sulfated polysaccharide semi-synthetic from glucuronoxylan hemicelluloses, which has been applied for OA management in animals. To investigate the efficacy of different sulfate levels (5, 16%, and 19% as full sulfate level; w/w% of sulfur atoms) of PPS and unsulfured glucuronoxylan (as 0%) on cell cycle regulation and promotion of chondrogenic phenotype in canine articular chondrocytes. Canine chondrocytes were cultured for 24 hr, then incubated with PPS for 72 hr. Chondrocyte viability was measured by a 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cytotoxicity of PPS and cell cycle progression of chondrocytes were checked by flow cytometry. The mRNA levels of cell cycle regulators and chondrocyte phenotypic markers was quantitatively evaluated. Activity of PI3K/Akt pathway was detected by Western blotting. All sulfate levels PPS showed no cytotoxic effect in chondrocytes, while fully sulfated PPS suppressed chondrocyte cell cycle transition from the G1 to later phases at a high concentration. Chondrocytes cultured with full sulfate level PPS significantly reduced cyclin dependent kinase 4 (*CDK4*) and cyclin D1 (*CCND1*) mRNA levels, and upregulated type II collagen (*COL2A1*) and SRY-box 9 (*SOX9*) expression levels. Akt phosphorylation and PI3K catalytic subunit alpha (p110α) protein production were inhibited in "full" group. PPS suppressed the proliferation in canine articular chondrocytes by targeting PI3K/Akt pathway, and promoted chondrocyte specific gene expression. Full sulfate level maybe necessary for PPS to achieve these effects in chondrocytes.

Key Words: articular chondrocyte, cell cycle, pentosan polysulfate sodium, PI3K/Akt, sulfate level

Introduction

Osteoarthritis (OA) is a multi-factorial degenerative disease process characterized by pathological changes of the synovial joint structures, including progressive degradation of articular cartilages in humans, dogs, and horses[7,22,42]. OA is considered to be one of the most common forms of joint diseases worldwide, which could lead to loss of mobility and reduces the quality of live in humans, especially in older population[22]. A study in dogs also reported up to 20% of dogs over 1-year old in the U.S.A suffered from OA[21]. Despite many efforts have been made

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doi: 10.57494/jjvr.71.2_35

to unravel its mechanism, there is still much to be understood about the pathogenesis of OA.

Articular cartilages consist of a single resident cell type called chondrocyte, which is enclosed in their extracellular matrix (ECM) composed of collagen II, IX, XI and proteoglycans, and plays critical roles in maintaining ECM components[2]. Physiologically, articular chondrocytes maintain a chondrogenic phenotype and present very low replicate ability[2,9,20]. However, during early stage of OA, the quiescent chondrocytes could rapidly regain the proliferative activity and form clusters to adjust the changed microenvironments, which has been found to affect their phenotype, and changed the amount and composition of ECM produced by the cells[9,17,29]. Although hypertrophic changes are the most recorded phenotype shift of chondrocytes in OA pathogenesis, recent studies have recognized various degenerated phenotypes of chondrocytes during OA progression, including de-differentiated-like phenotypes[8,39]. Similarly, de-differentiative changes have been observed in monolayer-cultured chondrocytes, characterized by rapid proliferation and increased metabolic activity, as well as impaired chondrogenic phenotypes[27]. Thus, investigating the proliferative activity and phenotype change of chondrocytes using monolayer culture models could provide information for understanding the disease progression and provides potential therapeutic targets for OA treatment.

Pentosan polysulfate sodium (PPS) is a highly sulfated heparin-like polysaccharide derived from European beech wood[3,7]. While clinical trials have proved its therapeutic effects on osteoarthritic joints in humans and horses[15,23,42], *in-vitro* studies suggested that PPS reduced degeneration of cartilage compositions, inhibited inflammatory-related signaling pathway in chondrocytes, and promoted chondrogenic properties of chondrocytes and mesenchymal stem cells (MSCs)[1,7,16]. Nevertheless, the underlying mechanism between PPS and chondrocytes is still not fully understood, including the regulation on cell proliferation. PPS is semi-synthesized by an exhaustive and non-selective sulfonation process on a β-(1→4)-linked glucuronoxylan backbone, which allows its sulfate level reach to approximately 2.0 per monosaccharide[3,26]. Despite the lower average molecular weight of PPS (4,000 to 6,000 Da), several papers have compared its properties with heparin or heparinoid[4,40]. Biological properties of heparin class largely involve with the electrostatic effects between positive charge of protein residues and negative charged groups of polysaccharides[31,37]. In fact, previous studies have determined that high sulfation of the 2-O and 6-O positions on heparin and heparan sulfate is crucial for their effects[5,31]. However, the impact of sulfate level on the treatment effects of PPS, particularly in chondrocytes has not been explored.

This study carried two purposes: to look into the efficacies and mechanisms of PPS in chondrocyte proliferation and cartilage specific gene markers in canine articular chondrocyte monolayer cultures; and to investigate how the different sulfate levels of PPS would change these effects.

Materials and Methods

Collection of canine chondrocytes

Canine articular chondrocytes from humeral heads of five dogs cryopreserved in liquid nitrogen were used for the experiments after recovery. Four dogs were experimental beagles (all females; 3 to 4 years old) that were euthanized at the end of unrelated studies. One sample was from a castrated male 13-year-old mixed-breed dog which received front limb amputation in Hokkaido University Veterinary Teaching Hospital due to the growth of tumor. Collection of samples from experimental dogs and clinical samples was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval #: 12-0059). Cartilage samples were dissected by a scalpel, followed with overnight digestion at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL, Grand Island, NY, U.S.A.) containing 0.3% type I collagenase (Wako, Osaka, Japan). After digestion, the cell suspension was passed through a 40 µm membrane into a 50 mL FALCON® polypropylene

tube (Corning, Lowell, MA, U.S.A.) to remove cartilage fragments. Primary chondrocytes (Passage 0; P0) were then pelleted by centrifuging at 300×g for 5 min, resuspended, and subsequently seeded into polystyrene culture plates (Corning) containing DMEM supplemented with 10% of fetal bovine serum (FBS; Batch #: BCBZ5443, Sigma-Aldrich, St. Louis, MO, U.S.A.), 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo, Kumamoto, Japan), 25 mM of sodium hydrogen carbonate (Wako), 73 Unit/mL streptomycin sulphate (Wako) and 100 Unit/mL of penicillin G potassium (Wako) and incubated at 37°C in 5% CO_2. When reaching to 80-90% confluency, P0 chondrocytes were detached from culture plates using 0.05% trypsin (Wako) and 0.02% ethylenediaminetetraacetic acid (Dojindo) in phosphate buffered saline (PBS), then cryopreserved in liquid nitrogen for future use. Characterization of the chondrocytes was performed described in Suppl. 1.

Chondrocytes culture and treatment

Four types of therapeutic reagents (Oji Pharma Co., Tokyo, Japan) used for treating the chondrocytes were marked as sulfate level (w/w% of sulfur atoms in the molecule) of PPS in this study, including 0% (OJ119A-06), 5% (OJ119A-07), 16% (OJ119A-08), and full (OJ119A-04). "Full" indicated the commercial type of PPS with sulfate level approximate 19%, and "0%" referred to glucuronoxylan without sulfation. For cell cultures, chondrocytes at passage 2 (P2) were plated at a number of 5.0×10^4 cells/cm^2 in culture plates containing DMEM with 10% FBS and cultured for 24 hr to allow adherence. The medium was then replaced by fresh culture medium, and chondrocytes were treated with PPS for 72 hr. The concentration of PPS was various between experiments due to different purposes of the tests.

Cell viability analysis

The cell viability of canine chondrocytes was analyzed at different concentrations and at different times. Firstly, chondrocytes were cultured in 96-well plates for 24 hr to allow attachment. For the assay at different concentration, chondrocytes were incubated 0, 5, 10, 20, 40, 80, and 120 μg/mL of PPS for 72 hr. For the assay at different time point, chondrocytes were treated with 80 μg/mL PPS for 24, 48, and 72 hr before analysis. The cell viability assay was performed with 3-(4,5-dimehylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Dojindo) method. Briefly, chondrocytes were washed by PBS, then incubated with MTT solution (0.5 mg/mL in DMEM) at 37°C. After 4 hr, the solution was removed, and 100 μL dimethyl sulfoxide (Wako) was added to dissolve the formazan crystals. The absorbance at 570 nm was determined by a microplate reader (Multiskan FC; Thermo Scientific, Vantaa, Finland).

Analysis of cytotoxic effect of PPS

After 24 hr incubation in 6-well plates, the chondrocytes were cultured with or without 80 μg/mL of PPS for 72 hr, then harvested by trypsin digestion. The cytotoxic effect of PPS in canine chondrocytes was measured on a flow cytometer (FACS Verse; BD Biosciences, Heidelberg, Germany) using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to manufacturer's protocol. The chondrocytes (main population) were gated from the total population depending on the forward scatter and side scatter to exclude the non-cell fragments, then divided into four populations based on the FITC and propidium iodide (PI) fluorescence.

Cell cycle analysis

Chondrocytes were incubated in 6-well plates for 72 hr in the presence (20 and 80 μg/mL) or absence of PPS with different sulfate level. After collected in 1.5 mL polypropylene tubes, the cells were stained with Cell Cycle Assay Solution Deep Red (Dojindo) according to manufacturer's protocol and analyzed by flow cytometry. The chondrocytes were gated as described in the previous section, then divided in to G1, S, and G2 phase depending on DNA contents. The data was processed by FlowJo software (Version 10.8.1; Treestar, Ashland, Oregon, U.S.A.) using Watson Pragmatic model.

Table. 1. The primers used for real-time quantitative PCR.

Gene	Primer sequence	Amplicon (bp)	Accession
GAPDH	Forward: 5′-CTGAACGGGAAGCTCACTGG-3′	129	NM_001003142.2
	Reverse: 5′-CGATGCCTGCTTCACTACCT-3′		
CCND1	Forward: 5′-AGTGTGATGCGGACTGTCTC-3′	184	NM_001005757.1
	Reverse: 5′-GCGCACCCTCAAATGTTCAC-3′		
CDK4	Forward: 5′-TAGCTTGCGGCCTGTCTATG-3′	145	XM_038679138.1
	Reverse: 5′-CAGAGAAGACCCTCACTCGG-3′		
CDK6	Forward: 5′-AGCCAAACGTCCCTAGAAGC-3′	121	XM_038436151.1
	Reverse: 5′-GAGAGATGCCTGGTAGACGC-3′		
COL1A2	Forward: 5′-GTGGATACGCGGACTTTGTT-3′	164	NM_001003187.1
	Reverse: 5′-GGGATACCATCGTCACCATC-3′		
COL2A1	Forward: 5′-CACTGCCAACGTCCAGATGA-3′	215	NM_001006951.1
	Reverse: 5′-GTTTCGTGCAGCCATCCTTC-3′		
SOX9	Forward: 5′-GCCGAGGAGGCCACCGAACA-3′	179	NM_001002978.1
	Reverse: 5′-CCCGGCTGCACGTCGGTTTT-3′		

GAPDH = glyceraldehyde-3-phosphate dehydrogenase, *CCND1* = cyclin D1, *CDK4* = cyclin dependent kinase 4, *CDK6* = cyclin dependent kinase 6, *COL1A2* = type I collagen alpha 2 chain, *COL2A1* = type II collagen alpha 1 chain, *SOX9* = SRY-box 9.

RNA extraction and quantitative real-time polymerase chain reaction

After cultured in 6-well plates for 24 hr, canine chondrocytes were incubated in fresh culture medium supplemented with 0, 5, 20, and 80 μg/mL of PPS for 72 hr. Total RNA was extracted using NucleoSpin RNA Purification Kit (Macherey-Nagel, Dürren, Germany). RNA concentration was quantified by a spectrophotometry at 260 nm. Total RNA of 1 μg was reverse-transcribed into cDNA using M-MLV RT Kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instruction. Quantitative real-time polymerase chain reaction (qPCR) was performed by two step method using KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA, U.S.A.). The relative expression levels of target genes were calculated by delta-delta Ct method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the gene expression. Cyclin D1 (*CCND1*), cyclin dependent kinase 4 (*CDK4*),and *CDK6* genes were used to evaluate the effect of PPS on cell cycle regulation. Type I collagen alpha 2 chain (*COL1A2*), type II collagen alpha 1 chain (*COL2A1*), and SRY-box 9 (*SOX9*) genes were used to confirm the phenotype change of chondrocytes. The primer sequences are shown in Table. 1.

Protein extraction and Western blotting

Chondrocytes received same treatment as described in RNA extraction. After the treatment, chondrocytes were wash with cold PBS and lysed with chill a radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with 1% of protease inhibitor cocktail (Sigma-Aldrich). Insoluble materials were then removed by centrifugation at 13,000×g for 20 min at 4°C. Total protein concentration was determined by a microplate reader using a Protein Quantification Assay Kit (Macherey-Nagel). After which, 4 μg protein for each sample was separated on NuPAGE™ 4 to 12% Bis-Tris gels (Invitrogen) by electrophoresis and electroblotted to polyvinylidene difluoride membranes. After blocked with 0.1% tween 20 in PBS containing 3% bovine serum albumin (Sigma-Aldrich) for 1 hr, membranes were incubated with primary antibodies overnight at 4°C, followed by an incubation with horseradish peroxidase (HRP)-linked secondary antibodies for 1 hr. The blots were developed by Western Blot Ultra-Sensitive HRP Substrate (Takara Bio Inc., Otsu, Japan) and visualized using Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, U.K.). Density of the bands was quantified by ImageJ software (NIH, Bethesda, MD, U.S.A.). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.): anti-PI3K catalytic subunit alpha (p110α) antibody (Cat. #4249), anti-Akt antibody (Cat. #9272), anti-p-Akt antibody (Cat. #9271), anti-β-actin antibody (Cat. #4970), and

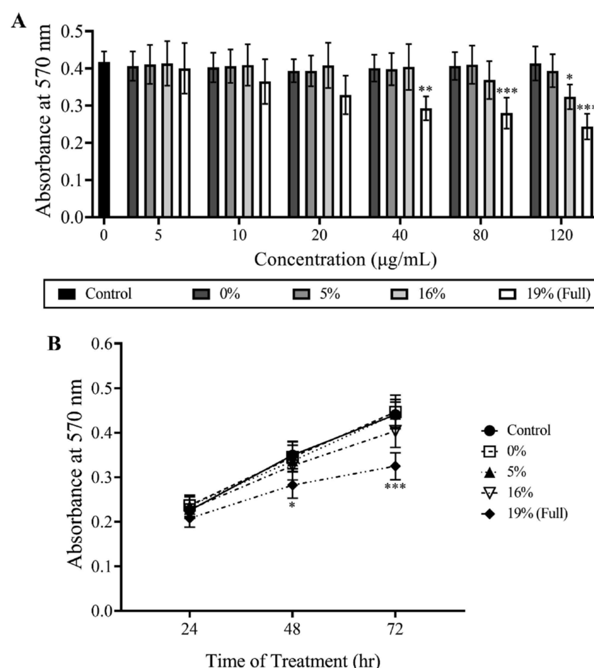

Fig. 1. Fully sulfated PPS decreased the cell viability of canine chondrocytes.
The viability of canine chondrocytes was determined by MTT assay. Chondrocytes were cultured in 96-well plates for 24 hr, (A) then treated with different concentrations (0, 5, 10, 20, 40, 80, and 120 μg/mL) of pentosan polysulfate sodium (PPS; sulfate level: 0, 5, 16%, and full sulfate) for 72 hr, (B) or treated with 80 μg/mL of PPS for 24, 48, and 72 hr. (A) Full sulfate level PPS treatment significantly reduced the cell viability in a concentration-dependent manner at 72 hr. (B) Reduced cell viability was also observes at different time point after incubated with fully sulfated PPS. Data are expressed as mean ± SD (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001, compared with the control).

anti-rabbit IgG HRP-linked antibody (Cat. #7074). Protein level was normalized by β-actin, and the value of p-Akt was normalized by Akt.

Statistical analysis

Quantitative analysis was performed using GraphPad Prism (version 9.2.0; GraphPad Software Inc., La Jolla, CA, U.S.A.). All quantitative results are presented as mean ± standard deviation (SD). Statistical comparisons were performed using analysis of variance (ANOVA), with Dunnett's test to compare between PPS-treated groups and the control group. A $P < 0.05$ was considered statistically significant.

Results

Fully sulfated PPS reduced chondrocyte viability in MTT assay

After 72 hr incubation with different concentrations of PPS, the viability of canine chondrocytes was reduced in a concentration-dependent manner with fully sulfated PPS, which statistical significance was found at 40 ($P = 0.001$), 80 ($P < 0.001$), and 120 ($P < 0.001$) μg/mL (Fig. 1A). A weaker reduction was noticed in 16% group at higher concentrations, and a significant difference was observed at 120 μg/mL ($P = 0.006$). Sulfate levels of 0 and 5% showed no significant effect in chondrocyte viability. The growth curves showed the cell viability of chondrocytes incubated with 80 μg/mL PPS for different times (Fig. 1B). The cell viability was significantly reduced in fully sulfated group at 48 ($P = 0.024$) and 72 hr ($P < 0.001$) compared to the control. Lower sulfate levels of PPS did not exert significant effects on cell viability at all time points.

All sulfate levels of PPS showed no cytotoxicity in canine chondrocytes

The flow cytometry with annexin V-FITC and PI double staining divided the chondrocytes into live cells and non-viable (including necrotic, early apoptotic and late apoptotic) cells. The chondrocytes were treated with 80 μg/mL of PPS for 72 hr before harvesting. Similar distribution of cells was observed between treatments and the control at the end point (Fig. 2A). No significant difference was detected in the percentage of live cells in 0, 5, 16%, and full sulfate level PPS groups compared with the control (Fig. 2B).

Full sulfate level PPS inhibited the cell cycle progression from G1 to subsequent phases in canine chondrocytes

To determine the proliferative activity of chondrocytes treated with PPS, distribution of cells in the G1, S and G2 phase of cell cycle was determined by flow cytometry based on DNA content in the cells (Fig. 3). Compared with the control group, the proportion of chondrocytes in the G1 phase ($P = 0.002$) of cell cycle was

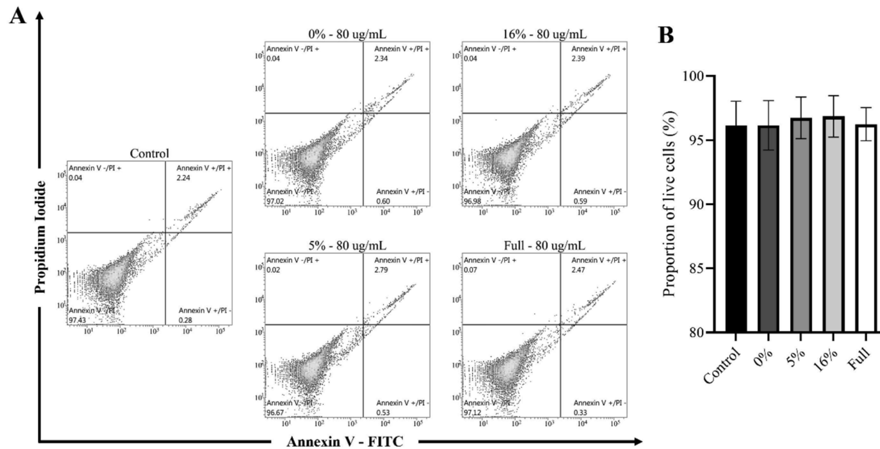

Fig. 2. Different sulfate levels of PPS showed no cytotoxic effects in canine chondrocytes. Apoptosis of canine chondrocytes was detected by flow cytometry with Annexin V and propidium iodide (PI) staining. Chondrocytes were cultured for 24 hr then treated with various sulfate level of pentosan polysulfate sodium (PPS; 0, 5, 16%, and full sulfate level) for 72 hr. (A) The distribution of cells stained with annexin V-FITC and PI was shown (Upper left: necrotic cells; upper right: late apoptotic cells; lower left: live cells; and lower right: early apoptotic cells). (B) The column graph expresses the proportion of live cells from five independent experiments. There was no significant difference between all treatment groups and the control. Data represent mean ± SD.

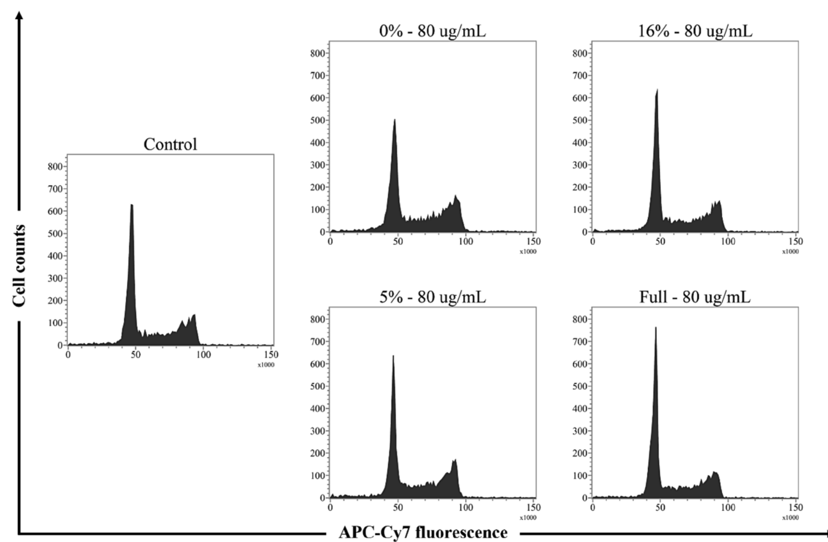

Fig. 3. Chondrocytes distributed in the G1 phase of cell cycle was increased after treated with fully sulfated PPS.
Chondrocytes were cultured for 24 hr prior to the incubation with various concentrations (0, 20, and 80 μg/ml) and sulfate levels (0, 5, 16%, and full) of pentosan polysulfate sodium (PPS) for 72 hr. The histograms showed the chondrocytes distributed in different phases of cell cycle in the control and 80 μg/mL PPS-treated groups. The first peak refers to the G1 phase, and the second peak indicates the G2 phase.

significantly increased after 72 hr incubation with 80 μg/mL of full sulfate PPS, while the cells in the S ($P > 0.05$) and G2 ($P = 0.037$) phases were reduced (Table. 2). No significant different was found between the control and other treatments.

Fully sulfated PPS decreased the gene expression of cell cycle regulators for G1/S transition and upregulated the expression of chondrogenic phenotype related genes

The relative gene expression of *CCND1*, *CDK4* and *6*, the regulators for G1/S transition

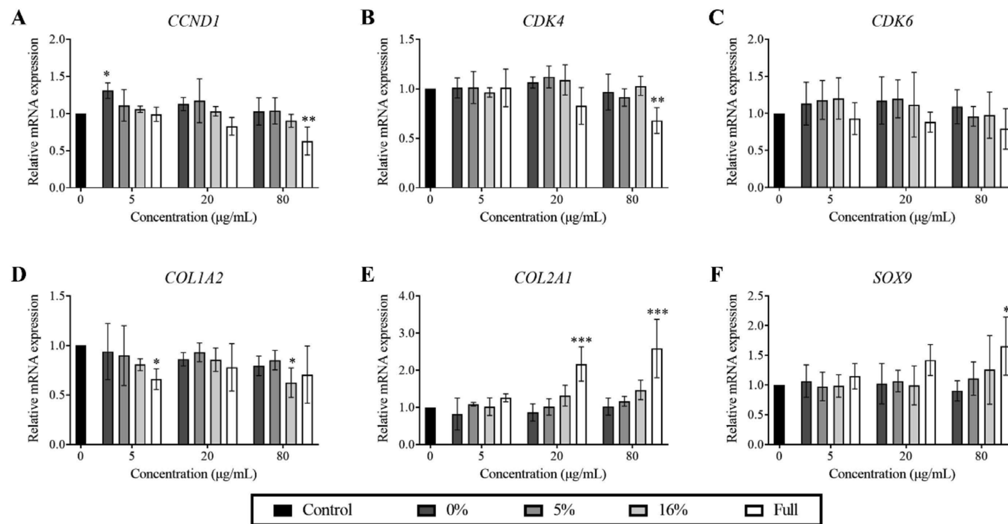

Fig. 4. Fully sulfated PPS reduced *CCND1* and *CDK4* gene expression, while upregulated *COL2A1* and *SOX9* genes.
After cultured with different types of pentosan polysulfate sodium (PPS; sulfate level: 0, 5, 16%, and full) for 72 hr, mRNA expression in chondrocytes was evaluated by real-time quantitative PCR (qPCR). Three concentrations of PPS (5, 20, and 80 μg/mL) were used. Expression level was normalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for each target gene. Relative mRNA expression of (A) cyclin D1 (*CCND1*) and (B) cyclin dependent kinase 4 (*CDK4*) 4 were significantly decreased with fully sulfated PPS at 80 μg/mL, while the level of (B) type II collagen alpha 1 chain (*COL2A1*) and SRY-box 9 (*SOX9*) were on the rise in a concentration-dependent manner. Data are expressed as mean ± SD (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with 0 μg/mL PPS).

Table. 2. Quantitative results of chondrocyte distribution in each phase of the cell cycle.

Phase	Control	0%	5%	16%	Full
	0 μg/mL	20 μg/mL			
G1 (%)	40.00 ± 2.87	38.90 ± 2.13	37.68 ± 2.68	39.68 ± 3.33	43.20 ± 2.27
S (%)	38.41 ± 2.13	38.76 ± 3.60	39.96 ± 3.16	37.73 ± 3.52	32.88 ± 2.71
G2 (%)	21.59 ± 2.28	22.34 ± 1.87	22.35 ± 2.56	22.41 ± 1.91	23.92 ± 2.72
		80 μg/mL			
G1 (%)		35.61 ± 4.33	35.80 ± 3.49	39.12 ± 4.20	48.14 ± 2.27(a)
S (%)		41.42 ± 4.61	41.65 ± 2.69	39.74 ± 3.90	34.38 ± 1.11
G2 (%)		22.97 ± 2.44	22.55 ± 2.56	21.13 ± 2.04	17.48 ± 1.25(b)

The results of cell cycle analysis from five independent experiments were quantified by the FlowJo software using Watson Pragmatic model. Percentage of chondrocytes in each phase of cell cycle was shown in the table. Pentosan polysulfate sodium (PPS) with full sulfate level significantly increased the proportion of chondrocytes in G1 phase of cell cycle at 80 μg/mL, while reduced the proportion of cells distributed in S and G2 phase. Data represent mean ± SD.
(a) $P = 0.002$ and (b) $P = 0.037$, compared with 0 μg/mL group.

of cell cycle, in chondrocytes was evaluated by qPCR at 72 hr of the treatment. Notably, the expression levels of *CCND1* and *CDK4* genes were downregulated in full sulfate level group in a concentration-dependent manner, in which the significant reductions were observed in *CCND1* ($P = 0.003$) and *CDK4* ($P = 0.002$) at 80 μg/mL compared to control (Fig. 4A and 4B). There was no significant difference in the gene expression of *CDK6* between PPS groups and the control at all three concentrations (Fig. 4C). The expression of phenotype related genes in chondrocytes, *COL1A2*, *COL2A1* and *SOX9*, was also measured by qPCR. The expression of *COL1A2* gene was

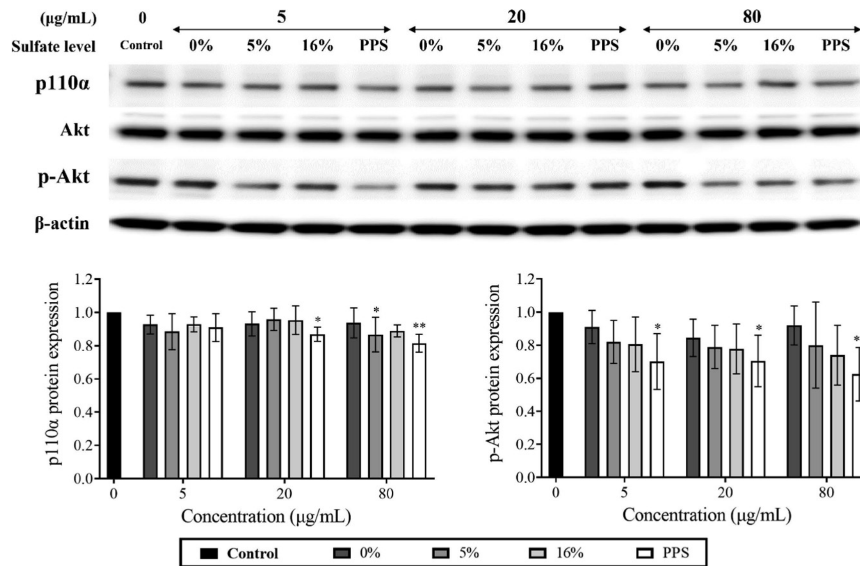

Fig. 5. Full sulfate level PPS suppressed p110α protein production and Akt phosphorylation in canine chondrocytes.
Protein levels of p110α, Akt, and p-Akt was analyzed by (A) Western blot. The β-actin was used as a reference protein, and expression of p-Akt was presented after normalized by Akt value. Chondrocytes were treated with four sulfate level of pentosan polysulfate sodium (PPS; 0, 5, 16%, and full sulfate) at concentration of 5, 20, and 80 μg/mL for 72 hr. (B) Protein level of p110α was significantly reduced with fully sulfated PPS at 20 and 80 μg/mL compared with control. (C) Akt phosphorylation in the full sulfate group was inhibited in a concentration-dependent manner. Data are expressed as mean ± SD (*$P < 0.05$ and **$P < 0.01$, compared with 0 μg/mL PPS).

downregulated in full sulfate level group (Fig. 4D), yet the significant difference was found only at the lowest concentration ($P = 0.029$) when compared with the control. In contrast with *COL1A2*, the results revealed a significant increase in *COL2A1* mRNA expression with the treatment of full sulfate of PPS at 20 ($P < 0.001$) and 80 ($P < 0.001$) μg/mL compared to control (Fig. 4E). Gene expression of *SOX9* was upregulated in full sulfate level group, in which the statistical significance was observed at 80 μg/mL (Fig. 4F).

PI3K/Akt signaling pathway was suppressed by PPS with full sulfate level

The protein expression of p110α subunit in PI3K and the phosphorylation of Akt were confirmed by Western blotting (Fig. 5A). Significant reductions of p110α protein level were noticed in chondrocytes treated with 20 ($P = 0.044$) and 80 ($P = 0.001$) μg/mL of fully sulfated PPS at 72 hr, respectively compared to the control (Fig. 5B). In constant with p110α, Akt phosphorylation was significantly inhibited by fully sulfated PPS at 5 ($P = 0.026$), 20 ($P = 0.029$), and 80 ($P = 0.003$) μg/mL when compared with the control group, respectively (Fig. 5C). Besides a significant reduced expression of p110α in 5% PPS-treated group at 80 μg/mL ($P = 0.040$) (Fig. 5B), no other statistical significance was found either in p110α level or Akt phosphorylation in lower sulfate level groups.

Discussion

Articular chondrocytes are considered as post-mitotic cells under physiological condition[9,20], whereas upregulation of proliferative activity and loss of chondrogenic phenotype in chondrocytes are observed in the early stage of OA[9,17]. Similarly, chondrocytes actively proliferate and de-differentiate into fibroblast-like shape during monolayer cultures, due to the sudden change of the microenvironment by isolating from cartilage[27]. This study investigated the properties of PPS in regulating the proliferation

and differentiation of chondrocytes in monolayer cultures model.

The results of MTT assay indicated a dose-dependent reduction of chondrocyte viability from 10 μg/mL at 72 hr in the fully sulfated PPS group. In addition, growth curves showed that 80 μg/mL of fully sulfated PPS reduced cell viability at 48 and 72 hr compared to the control. However, MTT assay measures the accumulation of chromogenic substances by produced live cells to reflect the cell number, both cell proliferation and cell death can affect the results[24)]. Therefore, the cytotoxic effect of PPS at 80 μg/mL was determined by flow cytometry with annexin V-FITC and PI staining. The results of flow cytometry showed that no significant change in the proportion of live cell in all treatment groups compared to the control, which demonstrated the safety of PPS with different sulfate level in chondrocytes. This finding was consistent with previous studies[1,14,16)]. Meanwhile, the results suggested that the reduced MTT assay results might be related to reduced chondrocyte proliferative activities.

Cell cycle analysis confirmed that the percentage of chondrocytes in the G1 phase of cell cycle was significantly increased with the treatment of 80 μg/mL fully sulfated PPS for 72 hr, while the proportion of chondrocytes in the S and G2 phase was reduced compared with control. Unlike a previous study indicated that higher concentration of PPS significantly increased chondrocyte proportion in the G1 phase at 24 and 48 but not 72 hr[1)], our results showed a higher percentage of cells in the G1 phase until 72 hr. An explanation is the chondrocytes used in the previous study were harvested from joints with clinical symptoms, which led to an early cluster formation of cells in cultures[17,29)], which consequently triggered the contact inhibition in the chondrocytes and downregulated the proliferation[13)]. Besides this difference, our finding was in agreement with the previous study that PPS with full sulfate level could inhibit chondrocyte proliferation and the progression of cell cycle from G1 to later phases.

The results of qPCR indicated significant downregulation of *CDK4* and *CCND1* mRNA levels in chondrocytes treated with 80 μg/mL fully sulfated PPS for 72 hr, which corresponded to the increased proportion of cells in the G1 phase and reduced proportion of cells in S and G2 phase. The cell cycle of eukaryotic cells was controlled by several checkpoints. In the G1 phase, CDK4 and 6 combine with cyclin D to form a catalytic complex, which phosphorylates the retinoblastoma protein critical for G1/S transition[6)]. In order to explore how PPS involves in regulating the cell cycle, we evaluated the gene expression of *CDK4* and *6* and *CCND1*. Although no statistical significance was found between treatment groups and the control in the mRNA level of *CDK6*, reduction of *CDK4* and *CCND1* was shown in full sulfate group. On the other hand, previous studies have found that promotion of cell proliferation in chondrocytes was associated with activation of PI3K/Akt signaling pathway[18,41)], while downregulation of this pathway inhibited chondrocyte proliferative activities[25)]. In the present study, Western blotting results showed that Akt phosphorylation and the protein level of p110α in chondrocytes were both significantly inhibited in full sulfate level group after 72 hr treatment. Judging from all these findings, we could infer that fully sulfated PPS may regulate the proliferation and cell cycle in chondrocytes through suppressing *CDK4* and *CCND1*, together with PI3K/Akt pathway. Similar regulation of cell proliferation through *CCND1* and PI3K/Akt pathway were observed in other type of mammalian somatic cells[10, 34)].

Although no clear pattern was found in *COL1A2* mRNA level, qPCR results revealed that expression of *COL2A1* and *SOX9* genes in chondrocytes were upregulated in a concentration-dependent manner when treated with fully sulfated PPS for 72 hr[12,30,36)]. Notably, the increased trend of *COL2A1* and *SOX9* mRNA expression corresponded with the downregulation of cell cycle regulator genes. This finding was coincident with previous studies that the expression of *COL2A1* and *SOX9* was negatively correlated with chondrocyte proliferation and expression of *CDK6* and *CCND1*[1,20,35)]. On the other hand, the *COL2A1* and *SOX9* expression levels were negatively associated with p110α protein production and Akt

phosphorylation in our study. While some previous finding indicated a positive regulatory effect of Akt pathway on *COL2A1* expression[11,19], other studies reported that downregulation of PI3K/Akt activity related to the upregulation of type II collagen, aggrecan and SOX9 production and attenuation of hypertrophy and fibrosis in chondrocytes[28]. Although the exact role of PI3K/Akt signaling pathway in chondrocyte differentiation needs to be further explored, the upregulated *COL2A1* and *SOX9* gene expression levels suggested that PPS with full sulfate level may be beneficial for preserving chondrogenic phenotype of canine chondrocytes. However, further investigation is needed to confirm this effect.

One major objective of this study is to explore the connection between the sulfate level of PPS and its therapeutic effects in chondrocytes. Practically, all hydroxyl groups on 2-O and 3-O position of each monosaccharide are sulfated replaced in the common structure of PPS during the synthesis[3,26]. Due to its highly sulfated degree and structural similarity, PPS has been considered to be one of the heparinoid substances[14]. In our study, glucuronoxylan (as 0% sulfate level) and PPS with three sulfate levels (5, 16%, and full sulfate level ≈ 19%) were tested. Interestingly, only the "full" PPS significantly increased the proportion of chondrocytes in G1 the phase of cell cycle, regulated the expression level of target genes and inhibited PI3K/Akt pathway, while other sulfate level of PPS showed weaker (16%) or no (0 and 5%) effect. Similarly, previous studies discovered that non-sulfated forms of heparan sulfate and other GAGs (dextran sulfate and dermatan sulfate) showed less chondrogenic effects in MSCs[33,38]. Moreover, a recent article reported that over sulfate modification of a polysaccharide, GY785 DR, enhanced the effects in stimulating of MSCs chondrogenesis, which indicated the importance of sulfo groups in the bioactivities of polysaccharide[32]. Combined with our data, it seems that an exhaustive sulfation of the hydroxyl groups is important for PPS to exert therapeutic effects in chondrocytes.

Although three-dimensional culture methods might be more conducive to preserving the phenotype of chondrocytes *in vitro*, it cannot be denied that monolayer culture is a more suitable model for studying cell proliferation. In conclusion, the data of our study confirms that PPS suppresses cell proliferation of canine articular chondrocytes through inhibiting the transition of cell cycle from G1 to S phase. The inhibitory effects on cell cycle progression appears to be through the downregulation of the checkpoint regulatory genes, *CDK4/6* and *CCND1* in chondrocytes. Furthermore, the reduced p110α production and Akt phosphorylation indicates that the suppression of cell proliferation in chondrocytes by PPS might be achieved by targeting PI3K/Akt pathway. The upregulated the expression levels of *COL2A1* and *SOX9* indicates that PPS may be beneficial in regulating chondrocyte phenotype. However, further study is needed to explore the underlying mechanism. The findings of this study could deepen our understanding of chondrocyte physiology, and provides potential therapeutic targets for the development of OA treatments. Of notice, the significant changes in either cell proliferation or the expression level of phenotype related genes can be only observed with fully sulfated PPS. Thus, full sulfate level might be necessary for PPS to achieve its treatment effects.

Acknowledgments

This study was supported by JST SPRING, Grant Number JPMJSP2119.

Conflict of interest

None of the authors have a conflict of interest to declare.

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