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Original Research Article

Chondrocyte proliferation is promoted by 7-H-pyrrolo[2,3-d]pyrimidine via up-regulation of type II collagen matrix

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Abstract

Purpose: *To investigate the effect of 7-H-pyrrolo[2,3-d]pyrimidine derivative (7-HPPD) on the viability of chondrocytes in vitro, and to elucidate the associated mechanisms.*
Methods: *Chondrocyte proliferation was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay while cell cycle was assessed by flow cytometry. Western blot and immunohistochemical staining assays were used to determine protein levels.*
Results: *The results show that 7-HPPD significantly increased the proliferation rate of chondrocytes in a concentration-dependent manner ($p < 0.05$). The proportion of chondrocytes in S phase increased significantly with subsequent reduction in G0/G1 phase of cell cycle on treatment with 7-HPPD ($p < 0.05$). Thus, 7-HPPD increased the rate of chondrocyte proliferation by promoting transition through G1/S phase of cell cycle. Reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis revealed that treatment of chondrocytes with 7-HPPD caused a marked increase in the level of cyclin D1, cyclin dependent kinase (CDK)-4 and CDK6 proteins ($p < 0.05$). Western blot and immunohistochemical staining assays showed that 7-HPPD treatment caused a significant increase in the levels of type II collagen matrix in the chondrocytes.*
Conclusion: *Proliferation of chondrocytes is increased by 7-HPPD and this occurs by facilitating G1/S phase transition and increasing expression of cyclin D1, CDK4 and CDK6 proteins. Therefore, 7-HPPD may be developed as a chemotherapeutic agent for the treatment of osteoarthritis.*

Keywords: *Immunohistochemistry, Type II collagen, G1/S phase, Cyclin D1, Osteoarthritis*

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INTRODUCTION

Osteoarthritis is the disease of joints associated with the degradation of articular cartilage and bones [1,2]. The disorder is characterised by acute pain and stiffness of the joints with problems in movement [1,2]. There is a large number of non-proliferating chondrocytes present in the articular cartilage of the adult human beings. The chondrocytes secrete extracellular matrix which is comprised mainly of various collagen molecules like types II, IX & XI and proteoglycans. The homeostasis of bone tissues is regulated by the chondrocytes through preparation and decomposition of the components of extracellular matrix [3]. Taking

into account the importance of chondrocytes their proliferation can be of immense importance for the treatment of osteoarthritis.

The reproduction of cells in eukaryotic organisms is regulated by a sequence of events which constitute the cell cycle. The initial stage of cell cycle involves replication of DNA followed by the doubling of chromosomes during S phase transition. The opening of DNA double helical structure and synthesis of DNA by the replication machinery takes place at the replication origins [4]. The gap between G1 and S phases which represents transition from one to the next cell cycle is regulated by various intracellular and extracellular signals [5,6]. M-phase is very important in cell cycle because during this phase cell either remains committed or escape the cell cycle [7,8]. Some factors have been found to control the progression of cell cycle through different phases. These factors include protein kinases CDK4 and CDK6 which regulate progression through G1 phase. Another factor is cyclin D1 which positively regulates G1/S transition and on binding to CDK4 or CDK6 controls G1 to the S phase cell cycle progression [9].

Natural products have been found to act as great treasures for the development of treatment for various diseases [10]. In the present study the effect of 7-*H*-pyrrolo[2,3-*d*]pyrimidine derivative (7-HPPD) on chondrocyte proliferation and cell cycle progression was investigated.

The study demonstrated that 7-HPPD increased chondrocyte proliferation and promoted cell cycle progression by up-regulation of cyclin protein and type II collagen matrix level in chondrocytes.

EXPERIMENTAL

Animals

Fifty 4 - 5 week-old male Sprague-Dawley rats (weight, 95 - 115 g) were supplied by the Animal Laboratory of Shandong University (Jinan, China). The rats were placed in 12 h light/dark cycles at 23 - 24 ˚C temperature and 58 - 65 % humidity. All the rats were provided free access to a standard laboratory chow diet and fresh drinking water. Experimental protocols for the rats were carried out in accordance with the guidelines from the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology, People's Republic of China [11]. The study was approved by the Ethics Committee, The Second Affiliated Hospital, Xián Medical University, China (approval no. XMU/0017/2015).

Chondrocyte isolation and culture

The rats were anesthetized using halothane anaesthesia and right knee joint was exposed carefully after shaving and disinfecting the skin. The articular cartilage was excised and then washed in PBS and DMEM. Thin 1 mm^3 sections of the cartilage were cut which were subsequently digested in type II collagenase (0.2 %). After digestion, the sections were placed at 37 ˚C in an incubator for isolation of the chondrocytes. The supernatant formed was isolated after every 1.5 h duration and then centrifuged for 10 min at 4000 xg to get the cell pellets. A 210 mesh size stainless steel filters was used for filtration of the cells. The cells were distributed at 2 x 10^5 cells/mL density in 6-well plates containing DMEM and 10 % FBS. The cells were cultured for 24 h under 5 % CO_2 atmosphere at 37 ˚C and 60 % humidity in an incubator [12]. The cells were monitored using an inverted microscope till achieving 80 % confluence and then passaged.

Identification of chondrocytes

The cells of second generation were put and cultured for 72 h on the cover slips. Then the cells were PBS washed twice and subsequently fixed for 45 min with formalin (4 %). Immunohistochemical staining was used for the assessment of type II collagen expression in the chondrocytes.

Determination of chondrocyte viability

MTT assay was used for determination of the effect of 7-HPPD (Sigma-Aldrich) on viability of chondrocytes. The chondrocytes at 1.5 x 10^4 cells per well density were put into the 96-well plates containing DMEM mixed with 10 % FBS. Following 24 h of culture the medium in plates was replaced by fresh medium mixed with 20, 40, 60, 80, 100, 150 and 200 µM concentrations of 7-HPPD. The cells were incubated with 7-HPPD for 72 h following which medium was removed and 30 µL MTT solution (0.5 %) was added to the wells. Incubation of the cells with MTT solution was performed for 4 h at 37 ˚C. Then 150 µL of DMSO was added to the wells for dissolving purple coloured formazan precipitate formed. The ELISA reader (ELx800™; BioTek Instruments, Inc., Winooski, VT, USA) was used for the measurement of absorbance for the wells to determine cell viability.

Cell cycle analysis

The chondrocytes at 3 x 10^5 cells/mL density were put into the 35 m petri dishes in DMEM containing 12 % FBS. Following 12 h of culture, medium was changed with fresh medium mixed with 80, 100, 150 and 200 μM concentrations of 7-HPPD. The chondrocytes were incubated for 72 h with 7-HPPD followed by digestion with type II collagenase. Then chondrocytes were washed, harvested and subsequently fixed with 70 % ethyl alcohol at 4 ˚C overnight. The chondrocytes were treated with Tris-hydrochloride buffer (pH 7.5) containing 1 % RNase A and then stained with propidium iodide (PI, 5 mg/mL). Flow cytometry was used for determination of difference in DNA content of the chondrocytes.

Western blot analysis

The chondrocytes after 72 h of treatment with 80, 100, 150 and 200 μM concentrations of 7-HPPD were harvested using cold PBS. The chondrocyte pellets were re-suspended in the lysis buffer [Tris pH 7.9 (48 mM), sodium chloride (145 mM), EDTA (4 mM), sodium dodecyl sulphate (0.08 %), nonyl phenoxypolyethoxylethanol-40 (0.4 %)] mixed with phenylmethylsulfonyl fluoride (9 mm) and aprotinin (1.8 mg/mL). In the lysates concentration of proteins was determined by the bicinchoninic acid (BCA) protein assay. Then 20 μg protein samples were resolved by electrophoresis using 10 - 12 % SDS-polyacrylamide gels. The proteins were transferred to PVDF membranes which were blocked on incubation with TBST solution of 5 % skimmed milk.

The membrane incubation with primary antibodies was performed overnight at 4 ˚C. After washing with TBST the membranes were incubated for 3 h with peroxidase-conjugated secondary antibody at room temperature. The bands were developed using an electrochemiluminescence detection system (cat. no. WBKLS0500; Merck Millipore), and quantification was performed by gel documentation. The data were analysed by the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

RT-PCR

The chondrocytes at 2.5 x 10^5 cell density in 3 mL of medium were put into the 6-well plates and incubated for 72 h with 80, 100, 150 and 200 μM concentrations of 7-HPPD. TRIzol reagent was used for the isolation of total RNA from the chondrocytes. Then 1 μg RNA samples were subjected to reverse transcription to synthesize cDNA using a HiFi-MMLV cDNA kit in accordance with the manual protocol. Quantitative PCR was carried out using the Ultra SYBR Mixture (CWBio Co. Ltd.) and GoldStar Taq DNA Polymerase according to the instructions on manual.

The PCR reactions were carried out in a 20 μL system consisting of 2X Ultra SYBR mixture (8 μL), forward primer (0.5 μL), reverse primer (0.5 μL), cDNA (2 μl) and 7.2 μL ultrapure water. The PCR reactions were carried out at 93 ˚C for 3 min, then 38 cycles for 20 sec at 93 ˚C, for 15 s at 52 ˚C and for 25 s at 65 ˚C. The internal loading control used was β-actin. The following primer sequences (Table 1) were used for amplification.

Table 1: Sequence of primers used.

Gene	Forward	Reverse
Cyclin D1	5'-AAT GCC AGA GGC GGA TGA GA-3'	5'-GCT TGT GCG GTA GCA GGA GA-3'
CDK4	5'-GAA GAC GAC TGG CCT CGA GA-3'	5'-ACT GCG CTC CAG ATT CCT CC-3'
CDK6	5'-TTG TGA CAG ACA TCG ACG AG-3'	5'-GAC AGG TGA GAA TGC AGG TT-3'
β-actin	5'-CGT TGA CAT CCG TAA AGA CC-3'	5'-GGA GCC AGG GCA GTA ATC T-3'

Statistical analysis

The presented values are mean ± standard deviation (SD) of three experiments performed independently. Analysis of the results was performed using one-way ANOVA followed by Student's *t*-test. The SPSS 16.0 software was used for analysis of the data. Differences were considered statistically significant at $p < 0.05$.

RESULTS

7-HPPD increases chondrocyte viability

Changes in chondrocyte viability by 7-HPPD was assessed on treatment with 20, 40, 60, 80, 100, 150 and 200 μM concentrations (Figure 1). 7-HPPD treatment enhanced chondrocyte viability at 72 h in concentration based manner. Treatment with 20, 40, 60, 80, 100, 150 and 200 μM concentrations of 7-HPPD increased viability of chondrocytes by 7.32 ± 1.55, 13.67 ± 2.65, 18.24 ± 2.89, 21.74 ± 3.33, 24.72 ± 3.34, 27.45 ± 3.67 and 31.08 ± 3.87 %, respectively.

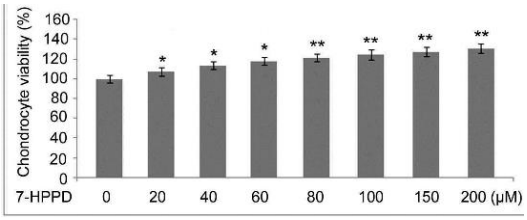

Figure 1: Changes in chondrocyte viability caused by 7-HPPD. Chondrocytes after 72 h of exposure to 20, 40, 60, 80, 100, 150 and 200 μM concentrations of 7-HPPD were analysed by MTT assay; *p < 0.05 and **p < 0.01 vs. control cells

Morphological changes in chondrocytes by 7-HPPD

Morphological changes in chondrocytes on treatment with 100, 150 and 200 μM concentrations of 7-HPPD were examined using phase contrast microscopy (**Figure 2**). The shape of 7-HPPD treated chondrocytes was regular and the number are markedly higher in comparison to the control cultures.

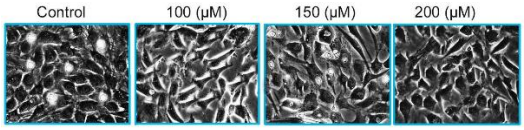

Figure 2: 7-HPPD regulates morphology of chondrocytes. Phase contrast microscopy was used for determination of morphological changes in chondrocytes at 72 h of treatment with 100, 150 and 200 μM concentrations of 7-HPPD

7-HPPD promoted chondrocyte cell cycle progression

The changes in cell cycle progression in chondrocytes by 7-HPPD was determined on treatment with 100, 150 and 200 μM concentrations at 72 h (Figure 3). Treatment of the chondrocytes with 7-HPPD caused a significant increase in S phase population and decrease in G0/G1 phase proportion. The percentage of chondrocytes in G0/G1 phase decreased to 62.12, 54.53 and 49.67 %, respectively on treatment with 100, 150 and 200 μM concentrations of 7-HPPD in comparison to 65.72 % in control. In S phase chondrocyte percentage increased on treatment with 100, 150 and 200 μM concentrations of 7-HPPD to 10.34, 13.91 and 17.44 %, respectively in comparison to 8.29 % in control. The population of chondrocytes in G2/M phase was 26.46, 28.89 and 29.64 %, respectively in cultures treated with 100, 150 and 200 μM concentrations of 7-HPPD in comparison to 24.87 % in control.

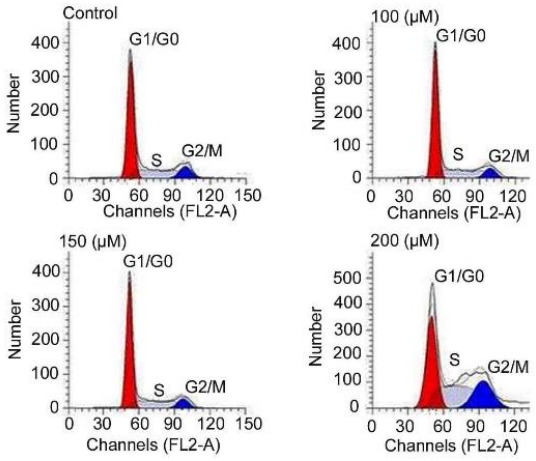

Figure 3: 7-HPPD increases chondrocyte percentage in S phase of cell cycle. Chondrocytes after exposure to 100, 150 and 200 μM concentrations of 7-HPPD were analysed by flow cytometry. *p < 0.05 and **p < 0.02 vs. control cells

7-HPPD increases cyclin protein expression in chondrocytes

Effect of 80, 100, 150 and 200 μM concentrations of 7-HPPD on cyclin expression at 72 h was assessed using western blot and RT-PCR assays (Figure 4). The expression of cyclin D1, CDK4 and CDK6 proteins in chondrocytes was increased markedly on treatment with 7-HPPD in concentration based manner (Figure 4 A). 7-HPPD treatment also caused a significant increase of mRNA corresponding to cyclin D1, CDK4 and CDK6 in the chondrocytes (Figure 4 B). The level of cyclin D1, CDK4 and CDK6 was increased to maximum on treatment with 200 μM concentration of 7-HPPD at 72 h.

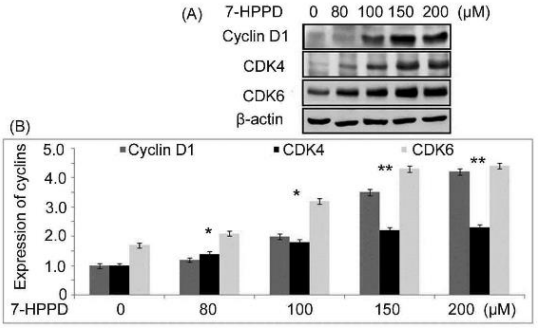

Figure 4: Effect of 7-HPPD on cell cycle proteins in chondrocytes. The chondrocytes were treated with 80, 100, 150 and 200 μM concentrations of 7-HPPD for 72 h. (A) The level of cyclin D1, CDK4 and CDK6 proteins was determined by western blotting and (A) mRNA level by RT-PCR; *p < 0.05 and **p < 0.02 vs. control cells

7-HPPD increases type II collagen matrix in chondrocyte

The level of type II collagen matrix in the chondrocytes was determined using western blot and immunohistochemical staining assays (Figure 5). 7-HPPD treatment markedly increased the level of type II collagen matrix in chondrocytes in concentration based manner. Increase in concentration of 7-HPPD from 80 to 200 µM caused a marked enhancement in type II collagen matrix level in chondrocytes.

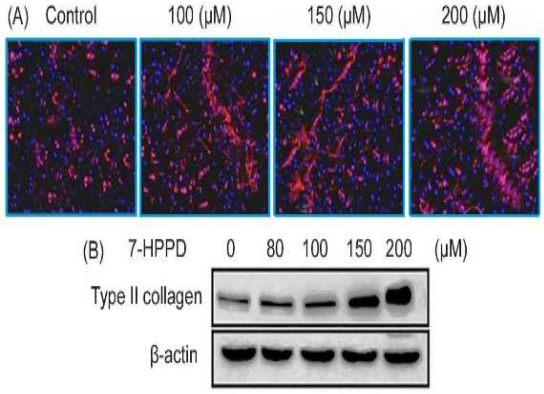

Figure 5: Effect of 7-HPPD on type II collagen matrix in chondrocytes. Effect of 80, 100, 150 and 200 µM concentrations of 7-HPPD on expression of type II collagen matrix was determined using (A) immunohistochemical staining and (B) by western blotting. Magnification x200

DISCUSSION

Osteoarthritis is the disorder of joints caused by the ECM degradation and apoptosis of bone cells leading to the cartilage damage [13]. Chondrocytes possess the potential to repair the cartilage damage in adult human beings. The rate of proliferation of osteoarthritic chondrocytes is very low and thus promotion of proliferation may be of vital importance for the treatment of osteoarthritis. The present study was aimed to explore the effect of 7-HPPD on chondrocyte proliferation and investigate the associated mechanism. The study demonstrated that 7-HPPD treatment increased chondrocyte proliferation by promotion of type II collagen matrix expression and up-regulation of cell cycle proteins.

The articular cartilage strength is determined by chondrocytes through the synthesis of ECM molecules which regulate cartilage homeostasis [14,15]. There is depletion of matrix synthesis by apoptosis of chondrocyte which results in cartilage damage and subsequently to osteoarthritis [16]. The main molecules which regulate the functioning of chondrocytes through facilitation of interaction between cell and matrix are type II collagen and sulfated proteoglycan [17]. In the present study effect of various concentrations of 7-HPPD on proliferation of chondrocytes was determined. The study demonstrated that 7-HPPD treatment caused a significant increase in the proliferation of chondrocytes in concentration dependent manner. These findings suggest that 7-HPPD plays an important role in the up-regulation of chondrocyte proliferation and may be of therapeutic value for osteoarthritis treatment.

There are four phases of cell cycle namely, G1, S, G2 and M phases. The G1-phase is concerned with the preparations for DNA synthesis and S-phase is associated with DNA synthesis. The preparations for cell mitosis takes place in G2-phase whereas the mitosis is completed in M-phase. After the DNA is synthesised in S-phase the content of DNA in G2 and M phases becomes 4N [18]. In the present study treatment of chondrocytes with 7-HPPD caused a marked increase in the cell population in S-phase in comparison to the control cells. 7-HPPD treatment leads to a significant decrease in population of chondrocytes in G0/G1 phase in concentration based manner. These findings suggest that 7-HPPD treatment enhances progression of cell cycle by catalysing transition of cells through G1/S phase.

The transition of cells through various phases of cell cycle is regulated by the interaction between cyclin-CDK and CDK inhibitor. The activity of cyclins is regulated positively on interaction with CDKs [19,20]. Some of the molecules which play important role in the G1/S transition include cyclin D1, CDK4 and CDK6 [19,20]. In the present study treatment of chondrocytes with 7-HPPD markedly increased the expression of cyclin D1, CDK4 and CDK6. Increase in expression of cyclin proteins confirmed that 7-HPPD promoted chondrocyte proliferation through promotion of cell cycle progression. The specific protein present in chondrocytes and used for their characterisation is type II collagen matrix [21,22]. It is reported that type II collagen matrix plays an important role in maintaining the strength of cartilages [21,22]. The results from the present study showed that 7-HPPD treatment lead to increased expression of type II collagen matrix in the chondrocytes.

CONCLUSION

The findings of the present study demonstrate that 7-HPPD increases proliferation of

chondrocytes, and also promotes cell cycle progression via up-regulation of cyclin and type II collagen matrix expression. Therefore, 7-HPPD can potentially be developed as therapeutic agent for the treatment of osteoarthritis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Junwu Zhang designed the study and wrote the paper. Jinsong chen and Lin Zhang performed the experimental work. All three authors performed the literature study, analyzed and compiled the data. The research article was thoroughly read by all the authors before communication for consideration of publication.

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