Parathyroid hormone 1 receptor as a potential therapeutic target for intervertebral disc degeneration

Ningbo City First Hospital
Kui Xu
Anhui University of Chinese Medicine
Jianle Wang
Zhejiang University School of Medicine, Translational Research of Zhejiang Province
Xiongxiong Cai
Ningbo City First Hospital
Jialiang Lin
Peking University Third Hospital
Baiwen Hu
Ningbo City First Hospital
Ting Liu
Ningbo City First Hospital
Hongyu Xu
Ningbo City First Hospital
Qinghua Song
Ningbo City First Hospital
Qi Yao
Ningbo City First Hospital
Dongdong Xia (✉ drdongdongxia@163.com)
Ningbo City First Hospital

Research Article

Keywords: Intervertebral disc degeneration, parathyroid hormone 1 receptor, therapeutic target, nucleus pulposus cells, extracellular matrix metabolism

Posted Date: March 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2692280/v1
Abstract

It is reported that parathyroid hormone 1 receptor (PTH1R) is crucial for intervertebral disc homeostasis maintenance. Annular tear was widely accepted as a common condition to destroy the immune privilege of the disk. To explore whether PTH1R is related to the pathogenesis of annular tear induced-intervertebral disc degeneration (IVDD) in this study, we analyzed the protein content of PTH1R in deteriorated people nucleus pulposus (NP) structure. Moreover, PTH1R activity and extracellular matrix (ECM) metabolism-related factors in the rat nucleus pulposus cells (NPCs) under oxidative stress conditions were evaluated by quantitative real-time polymerase chain reaction (RT-qPCR) in vitro. In addition, a rat IVDD model was constructed by a customized annulus needle puncture (ANP) device to evaluate IVDD grades in vivo. Immunohistochemical staining was used to detect the performance of type II collagen (Col II) and PTH1R. The results displayed that the expression of PTH1R declined in degenerated human NP tissue. The increased PTH1R activity were observed in rat NPCs with low concentration Tert-Butyl hydroperoxide (TBHP) treatment in vitro. In the rat IVDD model, the disc height had progressively narrowed and the disc structure was apparently disrupted in the ANP punctured discs. The protein expression of Col II and PTH1R was significantly down-regulated in ANP-punctured disc. This research demonstrated that our previous rat annulus needle puncture model could provide a reliable guide to the study of biologic processes in degenerating disks. Besides PTH1R has an inevitable connection with IVDD disease.

Introduction

Intervertebral disc is composed by internal nucleus pulposus (NP), as well as external annulus fibrosus (AF), besides it also holds upper and lower cartilage endplates.\(^1\) Its main function is to distribute pressure. Intervertebral disc degeneration (IVDD) is considered as a fundamental factor of the low back sick, it is thought to be the common drivers of musculoskeletal system-related disability.\(^2\) By the way, IVDD is found in the early stage of teenage, as well as 60% of 70 years old-human generate serious disc deterioration.\(^3\) However, limited knowledge about definite aetiology and pathophysiology of IVDD is not clear. In the past, many researchers thought that the abnormal biomechanics or nutritional dysfunction caused cell metabolism disorder, in turn resulting in IVDD.\(^4,5\) Nevertheless, in recent years, some scholars have proposed that some alterations are controlled by the changes in the number as well as function of cells existed in the intervertebral disc.\(^6\) Nucleus pulposus cells (NPCs) can regulate the homeostasis of intervertebral disc environment and also maintain the normal function of intervertebral disc by secreting extracellular matrix (ECM) and corresponding regulatory factors.\(^7,8\) Progressive breakdown of ECM is highly probable to be one of the most important IVDD causative factors. Therefore, intervening ECM metabolism of NPCs is usually thought to be a useful therapeutic method for IVDD.

ECM of nucleus pulposus belongs to a type of hydrated tissue to large extend that is composed primarily of the network type II collagen (Col II) and proteoglycan.\(^9\) Several proteins have shown protective effects on NPCs viability and ECM anabolism in treating disc degeneration.\(^10,12\) In our previous work, we found
that polydatin (PD) is able to ameliorate IVDD in rat model by promoting the up-regulation of nuclear factor erythroid-2 related factor 2, preserving ECM and inhibiting senescence in NPCs.\textsuperscript{13} Kim \textit{et al.} found bone morphogenetic protein-7 (BMP-7) can stimulate the expression of aggrecan and Col II via the Smad1/5/8 signaling pathways.\textsuperscript{14} In addition, osteogenic protein-1, also called as the BMP-1, was confirmed the potential to stimulate ECM production of NPCs and retard disc degeneration in animal model.\textsuperscript{15} These researches implied the correlation between skeletal development and intervertebral disc homeostasis.

Parathyroid hormone 1 receptor (PTH1R), which is a type of the G-protein-coupled receptor kind, serves as a specific receptor for the parathyroid hormone (PTH) as well as the parathyroid hormone-related peptide (PTHrP). Most of researches suggest that PTH1R is a fundamental controller for the bone metabolism and skeletal development. For example, PTH/PTH1R pathway was identified to regulate the process of longitudinal bone development by the way of mediating the chondrocyte differentiation in the epiphyseal development of the cartilage.\textsuperscript{16} The studies of Lanske \textit{et al.} showed that PTH/PTHrP receptor are downstream effectors of isolated hypogonadotropic hypogonadism pathway that regulates the correct spatial and temporal progression of chondrocyte differentiation.\textsuperscript{17} In recent years, a few studies also support the significant role of PTH1R in the joint degenerative disease. Becher \textit{et al.} found that the expression of parathyroid hormone receptor in osteoarthritis cartilage is less than that in normal cartilage, and only a few cells express the receptor.\textsuperscript{18} And according to the study of Yang \textit{et al.}, activated PTH1R signaling could cause inhibition of Ihh and then reverses temporomandibular joint osteoarthritis.\textsuperscript{19} Importantly, in Zheng and his colleagues’ study,\textsuperscript{20} mechanical stress induced PTH1R translocation to the primary cilia of NP cells. Mechanistically, they revealed that PTH1R could promote the expression of $\beta6$ integrin to active the extracellular TGF-$\beta$, hence maintaining intervertebral disc homeostasis. They concluded cilia in NP cells have a critical role in intervertebral disc function, particularly during aging. However, the function of PTH1R in NPCs during annulus needle puncture (ANP)-induced IVDD remains incompletely understood.

Annular tear was widely accepted as a common condition to destroy the immune privilege of the disk, as well as annular tear animal IVD could present a similar mechanical and biochemical environment to human IVD. Based on these, we established a novel minimally invasive ANP rat model using customized precise puncture devices by three-dimensional (3D) printing technique. Under the standardized protocols of puncture point and depth, we can induce a progressive IDD model in a few-minute procedure without extra bleeding. Based on this rat ANP model, we further research on the expression of PTH1R in degenerating disks in this study. Schematic flow chart of this study was in Fig. 1.

In this study, we hypothesized that PTH1R may play a key role in the treatment of IVDD disease. We firstly detected the expression of PTH1R in degenerated human disc tissues. Then, in the process of oxidative stress induced by TBHP, we measured the gene expression of anabolism and catabolism. Moreover, we detected the expression of PTH1R in an annulus needle puncture (ANP)-induced rat IVDD model.
Materials And Methods

Ethics statement

In this study, all surgical interventions, treatments and the corresponding nursing process for animals after the operation were complied with the standards of the Animal Care and Use Committee of xx. In addition, the collection of human nucleus pulposus and related tests involving human nucleus pulposus have been approved by the Ethics Committee of xx, and it is also carried out under the guiding principle of Helsinki Declaration.

Reagents and antibodies

TBHP was obtained from sigma-aldridge company (located in St. Louis, USA). Collagenase type II was purchased from Solarbio Science & Technology Co. (Beijing, China). Goat monoclonal antibodies against PTH1R and Col II, as well as horseradish peroxidase-labeled secondary antibodies were purchased from Abcam Plc (Cambridge, UK). In addition, TRizol™ reagent was acquired from Invitrogen Biotech. Co. (Carlsbad, USA). PrimeScript™ RT kit and SYBR premixed dimer Eraser™ were obtained from Takara (Dalian, China). β-actin antibody and RNAeasy Plus animal RNA isolation kit (with rotating column), BCA protein assay kit and phenylmethylsulfonyl fluoride (PMSF) were bought from Beyotime Biotech. Inc. (Shanghai, China). Cell culture reagents include fetal bovine serum, phosphate buffered saline, DMEM/F12, 0.25% trypsin-ethylenediaminetetraacetic acid, penicillin and streptomycin, all of which were obtained from Gibco Co. (Carlsbad, USA). EXPOSE rabbit-specific HRP/DAB detection IHC kit was acquired from Zhongshan Golden Bridge Biotech. Co., Ltd. (Beijing, China). Other chemicals were acquired from Sinopharm Chemical Reagent Co. (Shanghai, China).

Cell culture

Gel-like NP tissue was obtained from the tail of Sprague Dawley rats (male, 2-week-old). This process was operated in the basis of the previously reported research. Briefly, rats were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 minutes. Vertebrae were dissected from the tails and stored on ice in PBS supplemented with 100 U/mL penicillin as well as the 100 µg/mL streptomycin. The NP tissue was acquired by cutting through the annulus fibrosus. After that, the NP tissue was digested in 0.2% collagenase type II for 3 hours in the environment of 37°C and terminated with DMEM/F12 containing 15% FBS. After 5 minutes of centrifugation at 1500 rpm, the precipitated digestive tissues were suspended again, and then washed twice by PBS. Subsequently, the tissues were added to DMEM/F12 containing 15% FBS and 1% streptomycin/penicillin in an incubator with 37°C and 5% CO₂. When the cells reached about 90% fusion, they were passaged with 0.25% trypsin-ethylenediaminetetraacetic acid, and then reculture in a 10 cm culture plate with proper density. NPCs at the 3rd - 5th passage were used for the next experiments.

Quantitative real-time polymerase chain reaction (qPCR)
NPCs were treated with different concentration of TBHP (0, 15, 30 and 75 µM) and RNA extracted from NPCs after incubation of 4 h. RNA concentration and purity were detected by Thermo Scientific Multiscan GO (Thermo Scientific, USA). Briefly, the first strand cDNA was synthesized by primer reverse transcription kit. The RT-PCR was carried out to assess mRNA expression by applying SYBR premixed dimer Eraser™. During the whole experiment, the expression levels of β-actin can be regarded as a control. Furthermore, the relative gene expression level was calculated by the $2^{-\Delta \Delta CT}$ method. The qPCR primers are shown in Table 1.

### Table 1
Primer sequences for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
</table>
| Sox9      | Forward: AGGTGAAGGTGGAGTAGAGCC  
Reverse: GCACATCAAGACGGAGCAA |
| CoL2A1    | Forward: TCTGGACGTTAGCGGTGTGT  
Reverse: GAGCGGAGACTACTGGATTT |
| ADAMTS-5  | Forward: GTTAGGTGGGCAGGGTAT  
Reverse: GGTCAGTGTTCTCCTCCTT |
| MMP-13    | Forward: AAGCCAAAGAAAGACTGC  
Reverse: CCCCTTCCCTATGGTGAT |
| PTHRP     | Forward: GACCAGGTCCTTCGCTT    
Reverse: AGACGACGAGGGCAGATA |
| PTH1R     | Forward: GCAGGAGGCGTTAAGGAAT  
Reverse: AGCAGAAAGTGGAGTAGCA |
| β-actin   | Forward: CCTAGACTCTCGAGCAAGAGA  
Reverse: GGAAGGAAGGCTGGAAGA |

### Western blotting

NPCs were lysed in ice-cold RIPA with 1 mm PMSF and BCA protein assay kit was used to measure the protein concentration of the samples. The obtained protein was first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride membrane (Millipore, USA). After sealing with 5% skim milk, the bands were detected with primary specific antibodies of PTH1R (1: 1000) and β-actin (1: 1000) at 4°C overnight, and then incubated with the respective secondary antibodies. Finally, the intensity of the bands was quantified by Image Lab 3.0 software (Bio-Rad).

### Human nucleus pulposus tissues collection
To study the relation between PTH1R and intervertebral disc degeneration, NP tissues from IVDD patients (aged from 40 to 50 years, grade III (n = 4) and grade IV (n = 5)) were applied for further experiments based on the Pfirrmann grading scale. All NP tissue donors had no other complications related to IVDD, like the diabetes. The collected NP tissue was lysed and subsequently used for western blotting.

**Animal ANP puncture device**

In the present study, a new device for ANP was made by 3D printing technique. With the dimensions measured from rat tail, 3D image of the tail was reconstructed by UG 8.5 (Siemens, Germany). Accordingly, we designed the size and shape of the ANP device which looks like a ring and can be attached to the experimental level of the rat tail. The device was consisted of two parts: one is the "body", like a ring and have 8 holes which were used for puncture and localization; another one is the minimally invasive puncture needle which can puncture the RTD through the puncture holes of the device, at the same time, the needle's head can't be pull through which can control the depth of the puncture.

**Animal model**

Eight-week-old male Sprague-Dawley rats (n = 20) were bought from the Laboratory Animals Center of xx. Before the experiments, rats were randomly divided into two groups, including control group and IVDD group. After anesthesia with 2% (w/v) pentobarbital sodium (40 mg/kg), the specific part of rat caudal intervertebral disc (Co7/8) was located by palpation of caudal vertebrae, and the location of intervertebral disc was determined by X-ray. The skins corresponding to the disc and 4 main vessels in the experimental level were noted with a permanent marker. ANP device were used to puncture the AF in a perpendicular way through the tail skin and the needles remained in the disc for one minute. All rats were sacrificed at 4 and 8 weeks post-puncture. The animal manipulation was performed in compliance with Chinese legislation concerning the safeguard as well as the use of the laboratory animals and permitted by the Animal Care and Use Committee of xx.

**Histopathologic analysis**

The experimental rat tail discs with adjacent vertebral bodies were harvested and fixed with 10% formalin at 4°C overnight. The samples were rinsed twice with PBS and decalcified with 10% (w/v) sodium citrate/22.5% (v/v) formic acid for 7 days. And then they were neutralized with 5% sodium sulfate for 1 day. After washing with water, the samples were dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed as the standard procedure for histological analysis.

**Immunohistochemistry staining**

Immunohistochemistry (IHC) staining was used to detect the protein levels expression and distribution of Col II and PTH1R. In this study, IHC staining was conducted with EXPOSE rabbit-specific HRP/DAB detection IHC kit according to the instructions. Primary antibodies of Col II (1: 100) and PTH1R (1: 200) were diluted and incubated overnight at 4°C.

**Statistical analysis**
Results

Expression of PTH1R in human degenerated NP tissue

To determine whether the PTH1R protein was correlated with IVDD, we collected NP tissue from patients with different degrees of disc degradation to measure the PTH1R level by western blotting. We classified the samples according to Pfirrmann grade system and collected them of 4 grade III and 5 grade IV. As shown in Fig. 2, we found that the protein level of PTH1R in NP samples decreased with the degree of disc degeneration, which might be associated with NPCs degeneration in NP tissue.20

TBHP-induced ECM degeneration and PTH1R up-regulation

To study the effect of TBHP on rat NPCs, cells were stimulated by different concentrations of TBHP (0, 15, 30 and 75 µM). Sox9 is an important nucleus pulposus development marker at early-stage. Col II represents the ability of ECM synthesis. qPCR results showed that slight oxidative stress (0–30 µM TBHP) caused a protective increase of Col II and Sox9 expression in NPCs, while this phenomenon was significantly inhibited by severe oxidative stress (75 µM of TBHP) (Fig. 3, A and B). What happened then was the expression of ADAMTS5 and MMP-13 was observed because they were admittedly reported as ECM degradation markers in IVDD. A little bit like Col II and Sox9 expression in NPCs treated by TBHP, their levels were maximized at this concentration a relative mild TBHP concentration (15 µM) (Fig. 3, C and D). Besides, the same thing happened to the mRNA expression levels of PTHrP and PTH1R (Fig. 3, E and F). These results indicate that TBHP-treated NPCs showed ECM damage and PTH1R activation.

ANP-induced rat IVD degeneration

On the basis of the results obtained in vitro, we subsequently developed a rat model of disc degeneration using an ANP device to detect PTH1R expression in vivo. The rat’s caudal vertebra (Co7/8) was punctured to cause IVD degeneration (Fig. 4).

We retrieved the caudal disc for X-ray analysis to assess disc height. At 2 weeks after surgery, it was obvious that a loss of disc height of punctured discs in IVDD group. Whereas under the same conditions, the control group showed no disc height changes. And the difference between IVDD group and control group became more obvious after operation for 4 weeks (Fig. 5). In a word, these results suggest that ANP device successfully induced disc degeneration in the rat model of caudal discs.

Morphological changes of rat NP tissues were observed by HE staining. As shown in Fig. 6, NP tissues of the control group were distributed evenly in the extracellular matrix. Moreover, NP displayed a clear AF structure and had continuous NP tissue. However, disc (Co7/8) demonstrated apparent lamellar
disorganization or fragmentation in AF and gradually diminished normal NP cell number at 2 weeks in the ANP-punctured group. After 4 weeks, the punctured disc became apparent fibrosis of NP tissue and disruption of the AF structure.

**Reduced PTH1R expression in degenerative intervertebral disc rats**

ECM destruction is considered as one of the important characters representing degeneration in NPCs. In this study, the expression of the ECM component Col II was analyzed to evaluate the disc degeneration at the molecular level. As displayed in Fig. 7, a significant reduction of Col II level in the punctured group compared with that of the control group at 2 and 4 weeks after surgery. Moreover, according to the experimental results, the expression of PTH1R protein showed a significant down-regulation in the rat NP tissues of IVDD, which suggests that PTH1R could be involved in the pathogenesis of IVDD *in vivo*.

**Discussion**

Nowadays, intervertebral disc degeneration is one of the most common degenerative diseases in clinical medicine. During degeneration, NPCs exhibit dramatic molecular changes in ECM metabolism. In this study, we discovered that the changes in PTH1R expression during the IVDD, and thus inferred the important effect of PTH1R deficiency on NPCs. The PTH1R level showed a significant decreasing trend with the development of human IVDD. In vitro IVDD model, PTH1R expression of NPCs treated with TBHP (a recognized oxidative stress inducer) increased significantly under mild oxidative stress (0–30 µM) compared with the untreated control group. However, excessive oxidative stress (induced by 75 µM TBHP) aggravated NPCs degeneration and declined PTH1R expression. As ANP animal model could simulate the radioactive tear injury of human AF, our developed rat ANP model was used for further *in vivo* research. Our *in vivo* experiments showed a reduced PTH1R expression in the NP tissue of disc degeneration rats induced by ANP device. To our knowledge, this is the first study to describe the function of PTH1R in ANP-induced IVDD.

Many previous studies focused on the bone development regulation of PTH1R. For instance, PTHrP signaling could regulate bone cell survival through PTH1R, which has been notably approved for the treatment of post-menopausal osteoporosis to promote bone synthesis metabolism. In recent years, PTHrP-PTH1R signaling has been discovered to associate with the osteochondral defects repair. Besides, Kamal *et al.* found recombinant human PTH as a new treatment for osteoarthritis and activation of PTH1R signaling could maintain articular chondrocytes in a non-hypertrophic state and prevent the cartilage from degeneration. Whereas the role of PTH1R in IVD is still unclear. In the present study, PTH1R activity was evaluated in human NP tissue and ANP-induced rat IVDD model. Our data showed that pathological condition could significantly reduce the expression of PTH1R. We also found that the PTH1R contents were changed with the degree of disc degeneration, which indicates a potential link between PTH1R and IVDD progression.
Previous research reported the pathophysiology in IVDD is caused by multiple factors involvement, including metabolic stressors, mechanical and inflammatory, which all induced the increased level of reactive oxygen species (ROS). It is widely accepted that disturbance between the excessive ROS and insufficient antioxidant, defined as oxidative stress, contributes to the progression of IVDD. In this process, NPCs play a key role to maintain the physiological function of intervertebral disc.\textsuperscript{7,10,11}

NPCs mainly produce Col II and proteoglycan, which are the main ECM protein compositions of the gelatinous tissue and enable the intervertebral disc to swell against overloaded force. Sox9, as a critical transcription factor, plays an essential role in the growth and development of cartilage tissues.\textsuperscript{27} In this study, NPCs were treated with a relative mild concentration of TBHP (0–30 µM). The results showed an up-regulation of Col II and PTH1R expression in TBHP-treated NPCs, but there was a contrary finding that transcription factor EB was reduced by ROS in TBHP treated NPCs and in rat NP tissue of IVDD. This contradictory tendency may be attributed to the variation of ROS release. In the study of Zheng et al., NPCs were stimulated with TBHP for total 24 h duration, while we used TBHP for a duration of 4 h. The mild concentration of TBHP (0–30 µM) treatment may cause a self-protective responding to combat pathological conditions, which may drive an increased PTH1R and NPCs-derived extracellular matrix proteins. MMP-13 and ADAMTS5 are the representative catabolic factors in ECM metabolism of NPCs and contribute to IVDD.\textsuperscript{7} Herein, we demonstrated that MMP-13 and ADAMTS-5 were activated by transient oxidative stress (15 µM of TBHP). Their activity would be significantly inhibited when the Col II and Sox9 were increased compensatorily under the TBHP stimulation (30 µM). Nevertheless, a much higher concentration of TBHP (75 µM) decreased the PTH1R and NPCs matrix components (\textit{i.e.}, Col II and Sox9), and promoted the expression of matrix degrading enzymes (\textit{i.e.}, MMP-13 and ADAMTS5), which might indicate the NPCs degeneration.

In conclusion, we found that the expression of PTH1R is significantly decreased in human NP tissue and rat IVDD model. Therefore, we largely hypothesized that PTH1R may play a potential role in the progression of IVDD. However, this work only preliminarily demonstrated that PTH1R has an inevitable connection with IVDD disease. In order to elaborate whether PTH1R plays a protective role in IVDD, more experiments we will be infected NPCs (or rat IVDD model) with lentivirus to infecte NPCs (or rat IVDD model) should be done \textit{in vivo} and \textit{in vitro}.

\section*{Conclusion}

In summary, we observed an increased PTH1R activity in rat NPCs treated with low concentration TBHP (0–30 µM). The high concentration of TBHP (75 µM) decreased the PTH1R and NPCs matrix components (\textit{i.e.}, Col II and Sox9), and contributed to the up-regulation of matrix degrading enzymes (MMP-13 and ADAMTS 5), which indicates that excessive oxidation stress caused by TBHP promoted the NPC degeneration. In the rat IVDD model, the disc height had progressively narrowed and the disc structure was apparently disrupted in the ANP punctured discs. The protein expression of Col II and PTH1R was significantly down-regulated in ANP-punctured disc. In addition, the expression and activation of PTH1R in human NP tissue decreased with the aggravation of disc degeneration. The study indicates that
PTH1R has an inevitable connection with IVDD disease, and there is a great possibility of positive influence.

**Declarations**

**CONFLICT OF INTEREST:**

The authors declare no potential conflicts of interest.

**AUTHOR CONTRIBUTIONS**

CZ, KX and JL: intellectual ideas, experimental design, and manuscript writing. CZ, KX, JW, XC, HB, LT, XH: experimental procedures. SQ: statistical analysis. QY and DX: manuscript writing, supervision, project administration, funding acquisition. All authors contributed to manuscript editing and revisions, and approved the final version of the manuscript.

**ACKNOWLEDGMENTS**

This work was financially sponsored by the National Natural Science Foundation of China (No. 82002272), the China Postdoctoral Science Foundation (No. 2021M701434), the Medical Health Science and Technology Project of Zhejiang Province (Nos. 2020KY812 and 2021KY979 and 2021KY985 and 2022KY311 and 2022KY1112), the Ningbo University Medical College - Medical United Fund (No. 201911), and the Natural Science Foundation of Zhejiang Province (No. LQ20H060001).

**DATA AVAILABILITY STATEMENT**

The [DATA TYPE] data used to support the findings of this study are included within the article.

**References**


Figures
Figure 1

Schematic flow chart of this study.
Figure 2

The decreased expression of PTH1R in degenerated human disc tissues. (A) Representative MRI images of two different degrees of IVDD patients: (a) Pfirrmann grade III patient, male, 47-years old, lumbar disc herniation; (b) Pfirrmann grade IV patient, female, 49-years old, lumbar disc herniation. (B) and (C) Representative western blots and bar diagram of PTH1R levels in each human NP tissue group (Pfirrmann grades III and IV). All experiments were performed in duplicates, and the data were reported as the mean ± SD. ***p < 0.001.
Figure 3

The mRNA expression of PTH1R and apoptosis-related genes in rat NPCs after TBHP treatment. The NPCs were incubated with 0, 15, 30 and 75 μM TBHP for 24 h. Total RNA was isolated to evaluate expression of NPCs apoptosis associated genes by qPCR. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4

The fabrication of ANP device by 3D printing technique. (A) and (B) The device was consisted of two parts: one is the “body”, like a ring. Another is the minimally invasive puncture needle which can puncture the RTD through the puncture holes of the device. Legends: 1-Needle plug, 2-Needle handle, 3-Lantern ring, 4-Rat tail, 5-Blood vessel, 6-Annulus fibrosus, 7-Nucleus pulposus. (C) A product of ANP device. (D) Under digital palpation guidance, ANP device body was attached to the rat tail and 27G needles were used to puncture the AF though four localization holes.
Figure 5

Radiographic images of rat tails with a needle-punctured disc at 2 and 4 weeks after surgery (Black circles). The results showed that the disc height had progressively narrowed in the disc of punctured IVDDs and no disc height changes were observed in the control groups.

Figure 6
The histological observation of the disc of punctured IVDDs by using H&E staining. In control groups, (a, c) disc (Co7/8) demonstrated well-organized, intact AF with concentric lamellae and normal nucleus cells with large vacuoles at 2 and 4 weeks after surgery. In ANP-punctured groups, (b) disc (Co7/8) demonstrated apparent lamellar disorganization or fragmentation in AF and gradually diminished normal cell number in NP at 2 weeks after surgery. (d) After 4 weeks, disc (Co7/8) demonstrated apparent disruption of the disc structure and fibrosis of NP tissue. Scale bar: 1 mM.

**Figure 7**

**Immunohistochemical staining of collagen II and PTH1R in the rat disc samples.** The expression of collagen II n and PTH1R significantly decreased in ANP-punctured disc. Scale bar: 1 mM.