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Original Article

Effect of calcitonin pretreatment on naturally occurring intervertebral disc degeneration in guinea pig

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Abstract: Introduction: Our previous study suggested protective effects of calcitonin (CT) on experimental osteoarthritis. The aim of the present study was to provide evidence of whether CT pretreatment could prevent naturally occurring intervertebral disc degeneration in guinea pigs. Methods: Forty-two 3 months old female guinea pigs were randomly assigned into 2 groups as follows: Twenty-four were treated by normal saline as control group and sacrificed at 3, 6, 9 and 12 months of age (6 animals at each time point), the other 18 were received salmon CT (8 ug/kg/day, everyday) treatment at 3 months of age and sacrificed at the age of 6, 9 and 12 months respectively. Van Gieson stain and the histological score were used to identify the histological changes of the lumbar intervertebral discs. The disc height and vertebral body height were measured. Immunohistochemistry measurements for glycosaminoglycan, type II collagen, and matrix metalloprotease (MMP)-1 expressions were performed. Bone quality and microstructural changes in the L3-6 lumbar vertebral bodies were assessed by bone mineral density (BMD), micro-CT analysis and biomechanical testing. Results: Histological analysis indicated significantly higher disc degeneration scores in 9-month-old guinea pigs in comparison with younger animals, and grew higher with increasing age. CT treatment significantly reduced the histological score, and increased the disc height and the ratio to vertebral body height in 12 months old animals, as well as upregulated the glycosaminoglycan, type II collagen and inhibited the MMP-1 expression. Micro-CT analysis showed decreased percent bone volume (BV/TV) and increased trabecular separation (Tb.Sp), structural model index (SMI) in 12 months old animals in comparison with the younger animals. Markedly increased BV/TV and decreased Tb.Sp were observed in CT treated animals when compared with control animals. The biomechanical properties including maximum load, maximum stress, yield stress and elastic modulus increased from 3 to 6 months old and thereafter maintained in a stable level, which were enhanced by CT treatment. Conclusion: Pretreatment with CT could prevent naturally occurring intervertebral disc degeneration in guinea pigs, which might be related to the modulation of extracellular matrix metabolism and the integrity and biomechanical properties in adjacent vertebral body.

Keywords: Calcitonin, guinea pig, intervertebral disc degeneration, Micro-CT, biomechanics

Introduction

Intervertebral discs (IVDs) are complex anatomical structures that are essential for the mobility of intervertebral joints. Back pain is closely associated with intervertebral disc (IVD) degeneration [1, 2], and is an almost universal symptom, with its prevalence by lifetime, month and time-point being 84% [3], 23.2% and 11.9% [4], respectively. The economic impact of managing this condition and compensating for its associated losses and disabilities accounts for 12 billion pound annually in the United Kingdom [5] and 85.9 billion dollars in the USA [6]. Over 90% of surgical spine operations are performed due to consequences of IVD degeneration [7]. There is no definitive cure for IVD degeneration and current surgical treatments for IVD degeneration-associated conditions (such as herniation, stenosis or deformity) rely on discectomy, spinal fusion and disc replacement. These treatments, however, are associated with important

complications, such as degeneration of the spinal levels adjacent to the fused/replaced one [8] and prosthesis-related complications, such as migration into the adjacent vertebral body, extrusion and failure [9]. More importantly, these treatments address the disease symptoms and not disease itself, failing to repair or regenerate the IVD. Thus, there is growing consensus that the efficacy of currently used therapeutic methods is inadequate, and new treatment strategies aimed at the prevention or even full restoration of the IVD are being investigated.

Studies for treatment on spontaneous IVD degeneration in humans are difficult because of slow disease progression, and symptoms are often only present in the later stages of the disease, hence animal models are important for the study of IVD degeneration. Basic science studies of IVD degeneration are complicated because in most species, disc degeneration must be induced with either surgical injury such as needle puncture [10, 11] or chemonucleolysis [12, 13]. The limitation of these models are quickly developing IVD degeneration and thus cannot mimic slower changes in the IVD associated with onset and progression of disc degeneration. It also is not appropriate as a model to test therapies that focus on early degeneration. Dunkin Hartley (DH) strain guinea pigs are a well established and widely used naturally occurring osteoarthritis model [14, 15]. In light of the somewhat similarity in tissue composition of articular cartilage and intervertebral disc, DH guinea pigs may also be prone to IVD degeneration.

Calcitonin (CT) is a 32 amino acid peptide hormone, produced by the parafollicular cells in the thyroid gland, which possesses antiresorptive effects by binding to its receptor on osteoclasts [16]. As a commonly used bone resorption inhibitor, a new pharmacological characteristic of CT strongly supports its therapeutic benefit in the treatment of intervertebral disc degeneration. A recent in vitro study [17] revealed that CT exerted great potential for clinical therapy for disc regeneration as being able to induce the chondrogenesis in human nucleus pulposus cells by elevating chondrogenic specific-mRNA and protein expressions. However, up to present, there is no study focus on the effect of CT treatment on intervertebral disc degeneration.

The specific aims of the present study are (1) to determine whether and when IVD degeneration would occur in DH guinea pig; and (2) to further investigate the effect and mechanism of CT on intervertebral disc integrity in DH guinea pig.

Materials and methods

Animal handling

All experiments were approved by Hebei United University Animal Care and Use Committee. Forty-two 3-month-old female guinea pigs (Vital River Experimental Animal Technical Co., Ltd, Beijing, China) were fed a standard rodent diet and housed in the Center of Experimental Care. They were randomly assigned into 2 groups as follows: Twenty-four were treated by normal saline as control group and sacrificed at 3, 6, 9 and 12 months of age (6 animals at each time point), the other 18 were received salmon CT (Novartis AG, Switzerland) (8 ug/kg/day) treatment at 3 months of age and sacrificed at the age of 6, 9 and 12 months respectively.

Disc height measurement and histological evaluation

The L4-5 segments of the lumbar spine (including the intervertebral disc) were fixed in neutral-buffered 10% formalin, decalcified and embedded in paraffin. Samples were then cut into 4-m-thick sections and stained with van Gieson (VG) for light microscopic examination.

Disc height measurements were taken from endplate to endplate on histological samples from the L4-5 segments, for each image, the height was determined by an average of three measurements made in three areas of the disc space: on the left side, center and right side. The ratio of disc height to L6 vertebral body height was calculated.

Degenerative changes in the intervertebral discs were observed and the degrees of change in the stained sections were scored independently by three individuals blinded to the experimental protocol, using the disc degeneration assessment scoring system (**Table 1**) described by Wang *et al*. [18].

Porosity of bony endplate and the ratio of bony endplate area to total endplate area were measured on the image field amplified by 40-fold. The porosity of bony endplate indicated the ratio of the volume of the pores in the bony end-

Table 1. Lumbar intervertebral disc degeneration assessment scoring system

Score	Nucleus pulposus	Annulus fibrosus	Osteophyte
0	Bulging gel with abundant notochordal cells	Compact fibrous lamellas	Absence
1	Notochordal cells loss; chondrocyte-like cells emergence	Proliferation of fibrocartilaginous tissue and loss of nuclear-annular border	Appearance
2	Focal mucoid degeneration; clefts	Fissures in annulus fibrosis	Overgrowth
3	Diffuse mucoid degeneration and clefts throughout nucleus		

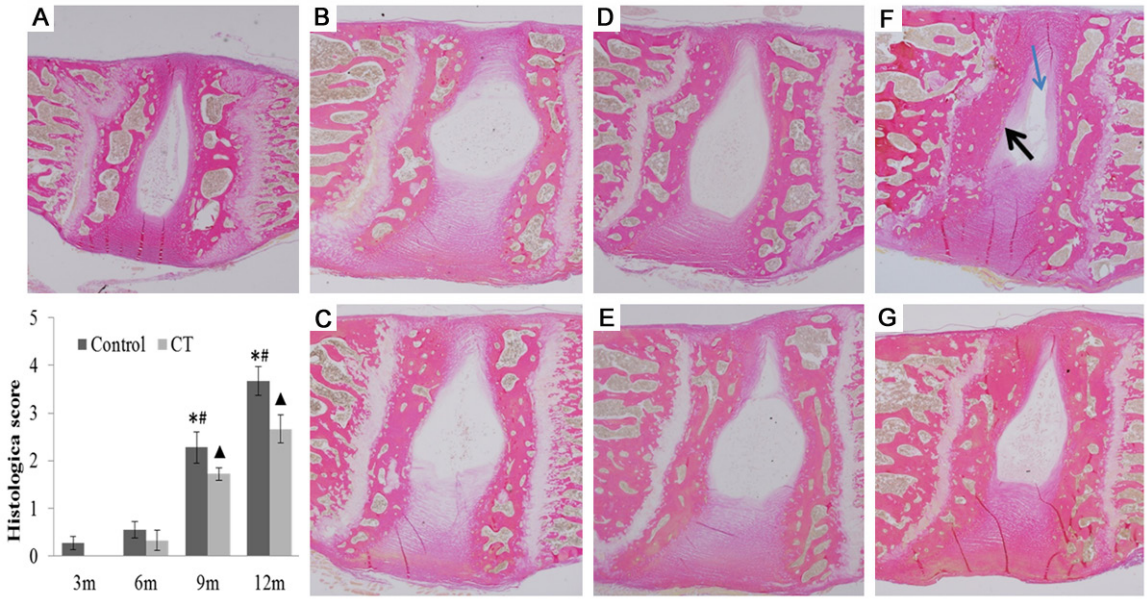

Figure 1. Histological assay of the L4-5 segments of the lumbar spine by van Gieson staining. (40×). The degenerative changes occurred at 9 months of age, including the disc narrowing, loss of cellularity in notochordal cells, and increased bony tissues in cartilage endplate, which were more severe in 12 months old animals, as obvious disappear of notochordal cells (blue arrow) and calcification (black arrow) were observed . CT treatment could partially retard these changes though increase the bony tissues in cartilage endplate, which were confirmed by histological scores. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group .*$P < 0.05$, compared with 3 months group; $^{\#}P < 0.05$, compared with 6 months group; $^{\&}P < 0.05$, compared with 9 months group; $^{\blacktriangle}P < 0.05$, CT group compared with control group of same age.

plate over the total volume of the bony endplate. The bony endplate area and total endplate area were also measured and the ratio of bone endplate area to total endplate area was calculated.

Immunohistochemistry analysis of glycosaminoglycan, type II collagen and matrix metalloprotease (MMP)-1

Tissue sections were deparaffinized in xylene and rehydrated in a reverse-graded series of ethanol. After antigen retrieval, quenching of endogenous peroxidase and blocking of nonspecific binding, sections were incubated overnight at 4°C with polyclonal antibodies glycosaminoglycan, type II collagen and matrix metalloprotease (MMP)-1, (1:100; all from Bioss Inc., Beijing, China). The remaining procedures were performed according to the PV-6001 Two-Step IHC Detection Kit (ZSGB-BIO Corporation. Beijing, China) and the color (brown) was developed by incubation in DAB (ZSGB-BIO Corporation. Beijing, China). Sections were counter-stained with hematoxylin. The sample appearing yellow or brownish yellow was considered as positive staining. All sections were semi-quantitatively analyzed by Image Pro Plus (IPP) version 6.0 software, and the integrated optical density (IOD) was measured by the staining in 6 fields in each section on the images at 400× magnification, the average IOD from 3 observers was the final observation result and used for statistical analysis.

Bone mineral density assessment

Segments L3-5 of each animal were removed, dissected free of muscles, and bone mineral density (BMD) measurements were performed on the anteroposterior plane by dual energy X-ray absorptiometry. This was performed using a densitometer (QDR Discovery, Hologic, Bedford, MA, USA) operating in high-resolution mode and specialized software for small animals supplied by the equipment's manufacturer.

Micro-CT measurements

Three-dimensional (3D) analysis was performed on the trabeculae of the cancellous tis-

Table 2. The ratio of disc height to vertebral body height

	Disc height(mm)	Vertebral body hight(mm)	Disc height/Vertebral body hight
3M	0.8368 ± 0.0961	7.9123 ± 0.2917	0.1055 ± 0.0085
6M	0.9264 ± 0.1039	8.7917 ± 0.3059*	0.1052 ± 0.0083
9M	0.8372 ± 0.0894	9.6333 ± 0.2493*,#	0.0868 ± 0.0072*,#
12M	0.4821 ± 0.0695*,#,&	9.4784 ± 0.1904*,#	0.0508 ± 0.0065*,#,&
6M-CT	0.9278 ± 0.0860	8.9703 ± 0.2839	0.1033 ± 0.0066
9M-CT	0.8905 ± 0.0424	9.7213 ± 0.1843	0.0916 ± 0.0035
12M-CT	0.6282 ± 0.0539▲	9.6769 ± 0.1446	0.0649 ± 0.0065▲

Note: *$P < 0.05$ *vs.* 3M group; #$P < 0.05$ *vs.*6M group; &$P < 0.05$ *vs.*9M group; ▲$P < 0.05$ *vs.* same-age control group Data from CT treated groups compared only with same-age control groups.

sue of the L6 vertebra, using a SkyScan 1076 micro-CT (SkyScan, Aartselaar, Belgium). The micro-CT equipment comprised a microfocus X-ray tube with a voxel size of 9 μm to produce a fan beam detected by a charge-couple device array, and a turntable that could be shifted automatically in the axial direction. The energy and intensity were equal to 40 kVp and 250 μA, respectively. On the original 3D images, morphometric indices were directly determined from the volume of interest (VOI), which was restricted to an inner cylinder with 1.5 mm diameter and the entire vertebra, excluding the region 1 mm from the proximal and distal growth plate. The following 3D morphometric parameters were calculated to describe the bone mass and microstructure: percent bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and structural model index (SMI).

Biomechanical testing

Using a mechanical strength analyzer (AG-IS, SHIMADZU, Japan), the mechanical strength of the lumbar vertebra (L2) was measured by compression test [19]. In this test, the planoparallel surfaces were obtained by removing the cranial and caudal ends of the vertebral specimen, thereby allowing for a uniform compression test to be performed on individual rodent vertebrae. From the vertebral body, a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. All compression tests were performed in the displacement-control mode at a crosshead speed of 0.5 mm/min to eliminate any strain rate effects. Maximum load, yield stress, maximum stress, and elastic modulus were obtained from compression tests of the vertebral bodies.

Statistical analysis

All data were analyzed using SPSS 17.0 software and results were expressed as mean ± SD. The statistical significance between groups was estimated using one-way analysis of variance (ANOVA) and Fisher's protected least significant difference test. *P* values less than 0.05 were considered statistically significant.

Results

Histological findings

Normal histological appearance was observed in 6 months old or younger animals, the evidence of disc degeneration was present in 9 months old animals as the nucleus pulposus became narrowed, coupled with loss of notochordal cells, and these changes were aggravated as age increased to 12 months old.

Loss of collagen, proliferation of fibrocartilaginous tissue and disruption of the nuclear-annular border were observed at the annulus fibrosus. In contrast to the control animals, CT treated animals showed wider disc and more notochordal cells in nucleus pulposus, coupled with more clear nuclear-annular border.

Accordingly, the histological score reflected pathological changes to the IVD. For the Control groups, the scores increased with age, significant differences were observed between older (9 and 12 months) and younger (3 and 6 months) animals. CT treatment markedly reduced the histological score at 12 months of age, in contrast to the control group (**Figure 1**).

As **Table 2** showed, the disc height in guinea pig was increased from 3 months to 6 months of age, thereafter decreased with age, with significant difference between 12 and 9 months old animals. At 12 months of age, CT treated animals showed significantly higher disc height when compared with control animals. In consideration of inter-animal size differences as the animals we used in the present study differ, measurements for disc height were normalized to adjacent vertebral body heights. In addition to the changes observed by assessing the disc height, the ratio of disc height to vertebral body height showed more pronounced difference

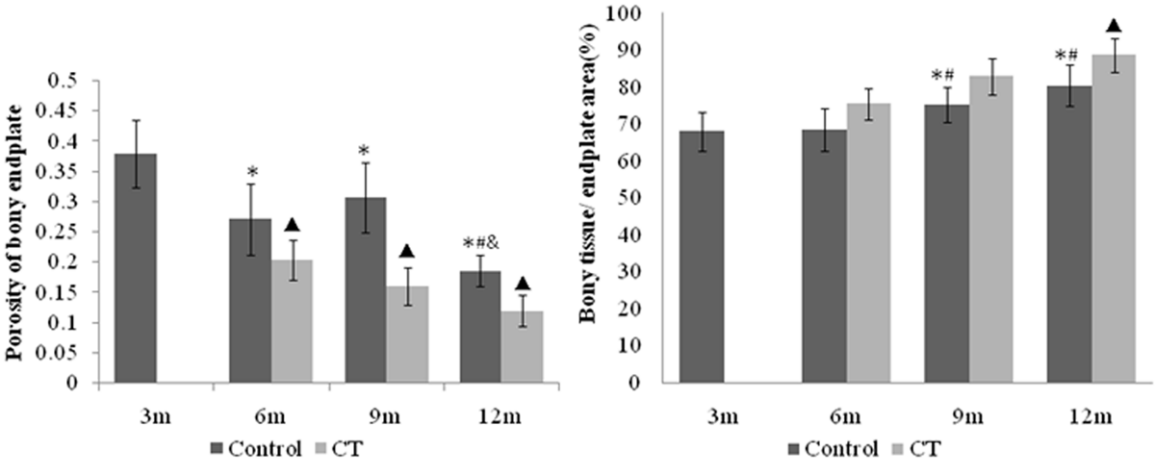

Figure 2. Porosity of bony cartilage and the percentage of bony area/total endplate area. *$P < 0.05$, compared with 3 months group; #$P < 0.05$, compared with 6 months group; &$P < 0.05$, compared with 9 months group; ▲$P < 0.05$, CT group compared with control group of same age.

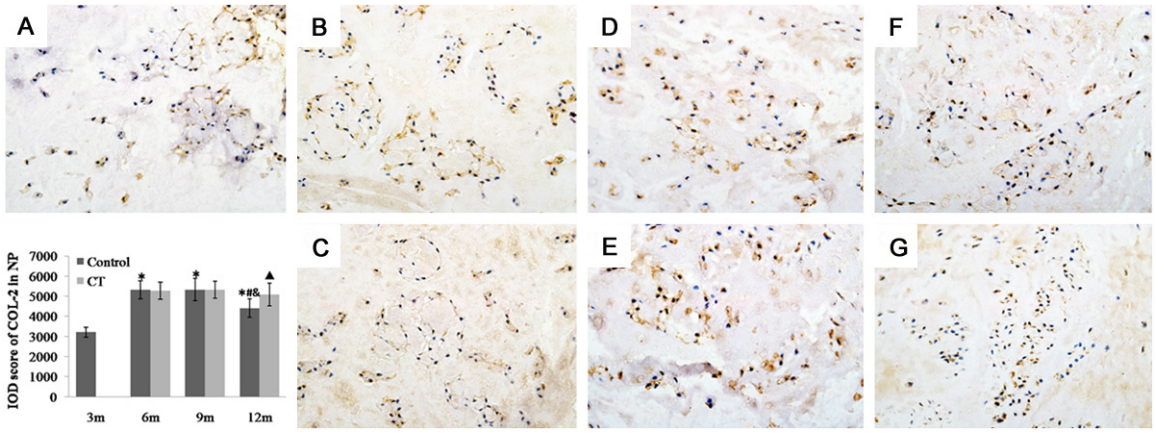

Figure 3. Immunohistochemistry assay for type II collagen in the nucleus pulposus. (400×). Immunohistochemical analysis showed that the protein levels of type II collagen increased from 3 to 9 months of age and decreased at 12 months of age, CT treatment dramatically retarded the decrease. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group. *$P < 0.05$, compared with 3 months group; #$P < 0.05$, compared with 6 months group; &$P < 0.05$, compared with 9 months group; ▲$P < 0.05$, CT group compared with control group of same age.

between 9 and 6 months old animals, which was not significant when assessed the disc height only.

The endplate of guinea pig consists of a robust bony endplate and thin cartilage endplate containing less than 5 chondrocyte cell layers, with progressive aging, the chondrocyte cell layers in cartilage endplate became thinner, CT treated animals showed more chondrocyte layers in comparison with control animals. In addition, porosity of bony endplate and the ratio of bony endplate area to total endplate area were also assessed in this study. As **Figure 2** showed, the porosity of bony endplate was decreased with age, CT treatment markedly decreased the porosity.

The ratio of bony endplate area to total endplate area were much higher in older (9 and 12 months) animals than those of younger (3 and 6 months) animals. CT treatment could increase this ratio in 12 months old animals.

Immunohistochemical staining

The protein levels of glycosaminoglycan, type II collagen and MMP-1 expression in the nucleus

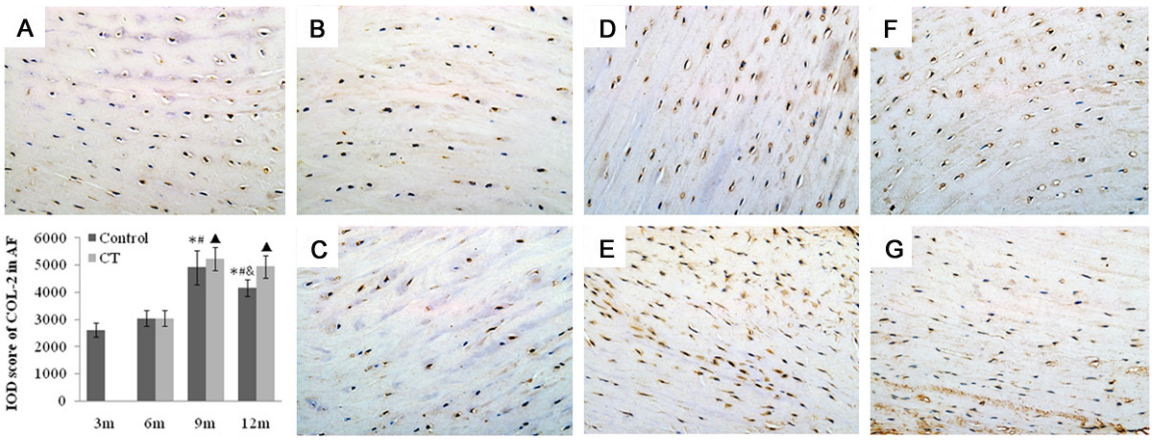

Figure 4. Immunohistochemistry assay for type II collagen in the annulus fibrosus. (400×). Immunohistochemical analysis showed the peak protein level of type II collagen at 6 months of age and thereafter decreased with age, CT treatment dramatically retarded the decrease. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group. $^{*}P < 0.05$, compared with 3 months group; $^{\#}P < 0.05$, compared with 6 months group; $^{\&}P < 0.05$, compared with 9 months group; $^{\blacktriangle}P < 0.05$, CT group compared with control group of same age.

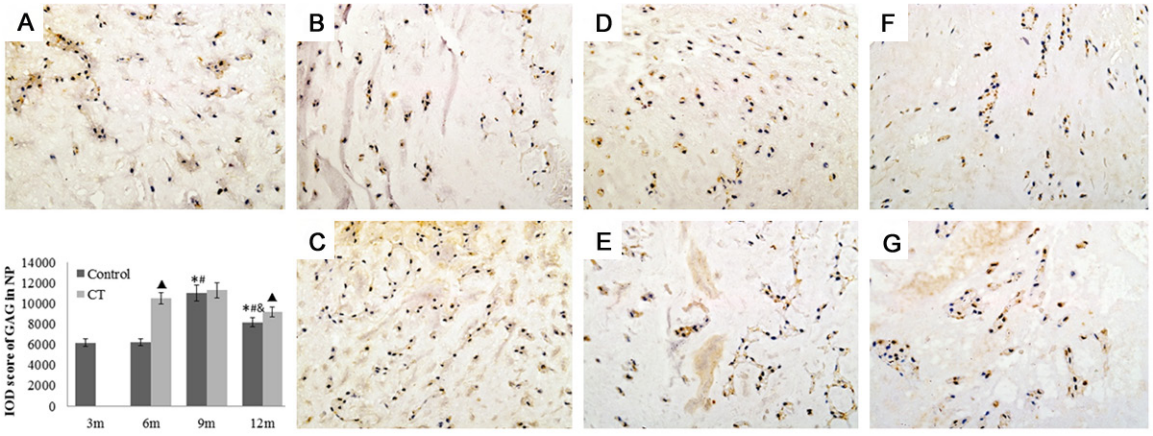

Figure 5. Immunohistochemistry assay for GAG in the nucleus pulposus. (400×). Immunohistochemical analysis showed that the protein levels of GAG increased from 3 to 9 months of age and decreased at 12 months of age, CT treatment dramatically retarded the decrease. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group. $^{*}P < 0.05$, compared with 3 months group; $^{\#}P < 0.05$, compared with 6 months group; $^{\&}P < 0.05$, compared with 9 months group; $^{\blacktriangle}P < 0.05$, CT group compared with control group of same age.

pulposus and annulus fibrosus were detected by immunohistochemical staining. Positive staining was confirmed as the intensity from faint yellow to brownish yellow. The IOD value in nucleus pulposus annulus fibrosus was statistically analyzed separately.

For type II collagen in the nucleus pulposus, the protein levels increased from 3 to 9 months of age and decreased at 12 months of age. In annulus fibrosus, the peak protein level of type II collagen was also observed at 9 months of age and thereafter decreased with age, CT treatment dramatically retarded the decrease in both nucleus pulposus and annulus fibrosus. (**Figures 3**, **4**).

For glycosaminoglycan in the nucleus pulposus, the protein levels increased from 3 to 9 months of age and decreased at 12 months of age, while in annulus fibrosus, the peak protein level of type II collagen at 9 months of age and thereafter decreased with age, CT treatment dramatically increased GAG expression at 6 and 12 months of age in the nucleus pulposus and at 6 and 9 months in annulus fibrosus. (**Figures 5**, **6**).

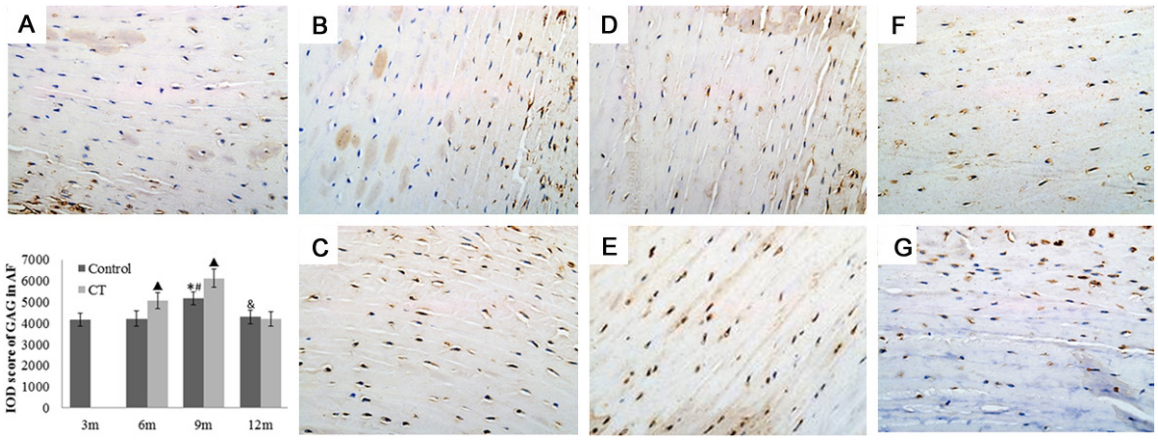

Figure 6. Immunohistochemistry assay for GAG in the annulus fibrosus. (400×). Immunohistochemical analysis showed the peak protein level of type II collagen at 9 months of age and thereafter decreased with age, CT treatment dramatically increased GAG expression at 6 and 9 months of age. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group . *$P < 0.05$, compared with 3 months group; #$P < 0.05$, compared with 6 months group; &$P < 0.05$, compared with 9 months group; ▲$P < 0.05$, CT group compared with control group of same age.

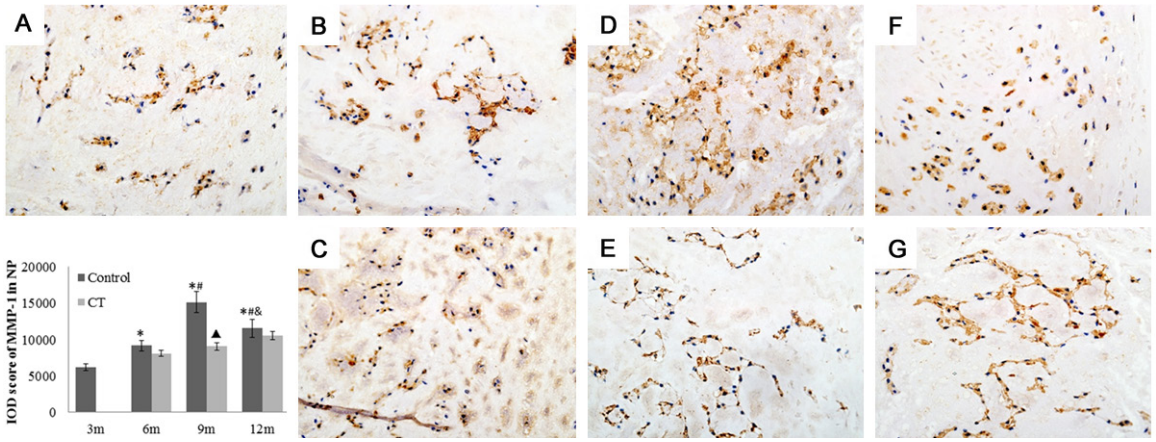

Figure 7. Immunohistochemistry assay for MMP-1 in the nucleus pulposus. (400×). Immunohistochemical analysis showed that the protein levels of MMP-1 increased from 3 to 9 months of age and decreased at 12 months of age, CT treatment dramatically decreased the MMP-1 expression at 9 months of age. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group . *$P < 0.05$, compared with 3 months group; #$P < 0.05$, compared with 6 months group; &$P < 0.05$, compared with 9 months group; ▲$P < 0.05$, CT group compared with control group of same age.

For MMP-1, the protein levels increased from 3 to 9 months of age and decreased at 12 months of age in both nucleus pulposus and annulus fibrosus, CT suppressed the MMP-1 expression in nucleus pulposus at 9 months of age and in annulus fibrosus at 6, 9 and 12 months of age (**Figures 7**, **8**).

Bone mineral density of L3-5

The bone mineral density of L3-5 were increased from 3 to 6 months of age, and maintain at stable levels until 12 months old, CT treatment notably increased the bone mineral density in 12 months old animals (**Figure 9**).

Micro-CT parameters of L6 vertebral

Trabecular bone loss was indicated in 12 months old animals as BV/TV was markedly lower than younger animals, while the Tb.Sp and SMI were markedly higher in 12 months old animals than those of younger animals. Compared with control groups, CT treatment

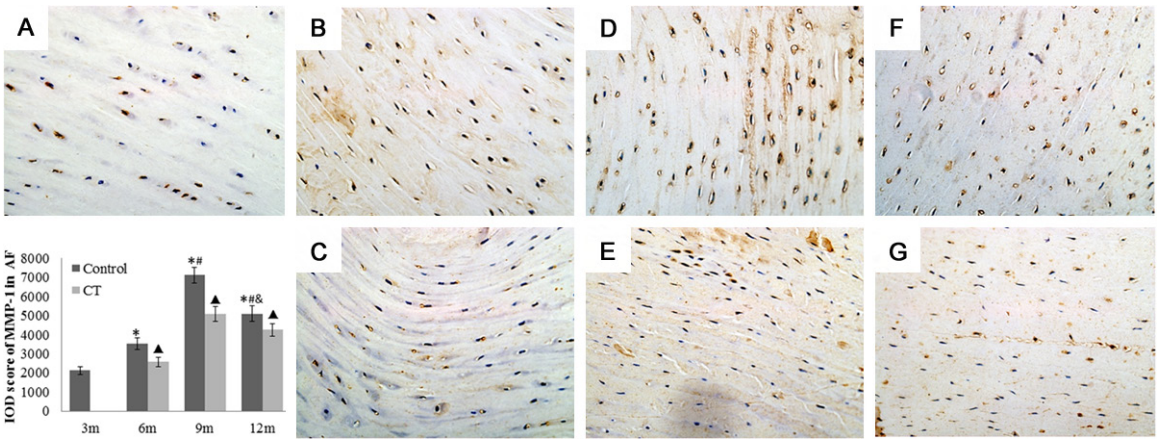

Figure 8. Immunohistochemistry assay for MMP-1 in the annulus fibrosus. (400×). Immunohistochemical analysis showed that the protein levels of MMP-1 increased from 3 to 9 months of age and decreased at 12 months of age, CT treatment dramatically decreased the MMP-1 expressions. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group. $^{*}P < 0.05$, compared with 3 months group; $^{\#}P < 0.05$, compared with 6 months group; $^{\&}P < 0.05$, compared with 9 months group; $^{\blacktriangle}P < 0.05$, CT group compared with control group of same age.

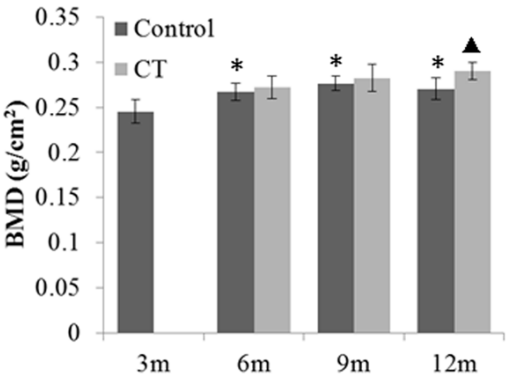

Figure 9. Bone mineral density of L3-5. $^{*}P < 0.05$, compared with 3 months group; $^{\blacktriangle}P < 0.05$, CT group compared with control group of same age.

notably increased the BV/TV and markedly suppressed the Tb.sp in 12 months old animals (**Table 3**; **Figure 10**).

Biomechanical testing results of L2 vertebral

Maximum load, yield stress, maximum stress, and elastic modulus were all increased from 3 months to 6 months of age, thereafter maintained in stable levels untill 12 months old, with no significant difference between any two groups from 6, 9 and 12 months old control animals.

CT treatment markedly increased maximum load, yield stress, maximum stress in all time points, but for elastic modulus, only 6 months old animals showed significant difference between CT treated and control animals (**Table 4**).

Discussion

To the best of our knowledge, we are the first to report the age related changes in IVD in guinea pig. In the present study, we provided a series of morphological and histological evidences supported that the degenerative appearance in guinea pig IVD occurred at 9 months of age, and more pronounced at 12 months old. In addition, we also collected the data about the age related changes in quantity and quality of adjacent vertebral bone.

Though significant difference exists, guinea pig IVD degeneration shares similarities with human in some histological characteristics, such as the disc narrowing and the loss of notochordal cells [20], which were observed in the 9 months and older animals in the present study, in regard of different age of the animals we used in the present study, we not only measured the disc height, but also the ratio of disc height to the vertebral body height, which confirmed the marked disc narrowing occurred at 9 months old animals and more severe in 12 months old animals. Moreover, with age increasing, disruption of the nuclear-annular border was observed at the annulus fibrosus, especially in 12 months old animals.

Table 3. Microarchitecture parameters of vertebral body by micro-CT analysis

	BV/TV	Tb.Th	Tb.Sp	SMI
3M	30.135 ± 4.272	83.855 ± 5.027	175.288 ± 15.437	0.989 ± 0.275
6M	32.330 ± 2.589	92.761 ± 11.135	153.509 ± 20.336	1.009 ± 0.147
9M	32.676 ± 4.337	93.432 ± 5.477	154.023 ± 11.582	1.139 ± 0.235
12M	25.127 ± 1.147$^{*,\#,\&}$	80.293 ± 10.914	180.288 ± 15.953$^{\#,\&}$	1.410 ± 0.162$^{*,\#,\&}$
6M-CT	35.806 ± 0.946	99.155 ± 6.957	131.683 ± 13.494	0.992 ± 0.040
9M-CT	37.016 ± 4.442	97.406 ± 14.979	134.902 ± 25.532	0.981 ± 0.110
12M-CT	30.955 ± 1.740$^{▲}$	83.571 ± 8.175	155.730 ± 7.468$^{▲}$	1.351 ± 0.054

Note: $^{*}P < 0.05$ *vs.*3M group; $^{\#}P < 0.05$ *vs.*6M group; $^{\&}P < 0.05$ *vs.*9M group; $^{▲}P < 0.05$ *vs.* same-age control group Data from CT treated groups compared only with same-age control groups.

In addition to the intervertebral disc, the endplate also plays important roles in IVD degeneration as it serves important physiological functions, such as dispersing the compressive load experienced by the vertebral body and providing nutrition to the disc via diffusion [21-24]. The human endplate undergoes calcification in response to aging and disc degeneration, which may further decrease the nutrient diffusion into the disc degeneration. The present study demonstrated that the tissue components of endplate in guinea pig is much more likely to rabbit and goat than to human, with robust bony endplate and much thinner cartilage endplate [20]. In parallel with the loss of chondrocyte layer as age increasing, the ratio of bony endplate to total endplate was increased, whereas the porosity of bony endplate was decreased, this process may lead to the development of disc degeneration.

Accordingly, the histological score confirmed these changes quantitatively. In light of the data in previously studies, the age when degeneration in IVD occurred is older than that of osteoarthritis, which appeared at 6 months of age or even younger [14, 15, 25-27], and grew more severe with increasing age.

ECM metabolism plays a key role in the integrity and function of the IVD. The collagen fibers of the IVD provide a strong durable framework which supports the cells and confines the highly hydrated PG aggregates. Approximately 90% of the collagen in the IVD is made up of fibrillar collagen types I and II [28]. The NP is a gelatinous structure composed primarily of aggrecan and collagen type II [29]. One of the changes that occurs during IVD degeneration development is the loss of matrix proteoglycans [30], most of the biological activity of proteoglycans is mediated by their GAG chains. The present study found notably decreased expressions of GAG and collagen type II in IVD in 12 months old animals, in regard of the age of histological degeneration occurring, the delayed activated matrix catabolic metabolism might be due to the time points we chose that are not detailed enough to thoroughly reflect the pathological process in relation to the matrix metablism, the possible peak value might be appeared at 7 or 8 months old animals. MMPs, including MMP-1, are thought to be important catabolic enzymes responsible for ECM turnover, such as degrading collagen type II [31, 32]. However, the unexpected result that MMP-1 expressed lower in 12 months old animals than that of 9 months old animals is difficult to explain, though feedback may be attributed to this phenomenon, further study was needed to confirm this hypothesis.

As an anti-resorptive agent, CT has been used in the treatment of OP [33, 34]. Recent evidence suggests that CT induces the chondrogenesis in human nucleus pulposus cells by elevating chondrogenic specific-mRNA and protein expressions [17]. Our previous study described the beneficial effects of another antiresorptive drugs on IVD degeneration in ovariectomized rat [35], but the IVD degeneration developed based on estrogen deficiency enlarges the window for antiresorptive drugs in IVD degeneration treatment. The realistic is that the most popular IVD degeneration is naturally developed as age increasing. We therefore performed the present study to investigate whether calcitonin pretreatment could prevent naturally occurring intervertebral disc degeneration in guinea pig. The present study provides a new insight into the potential benefits of CT on joint degenerative disease, as the histological results indicated, CT treatment notably decreased the histological score in 12 months

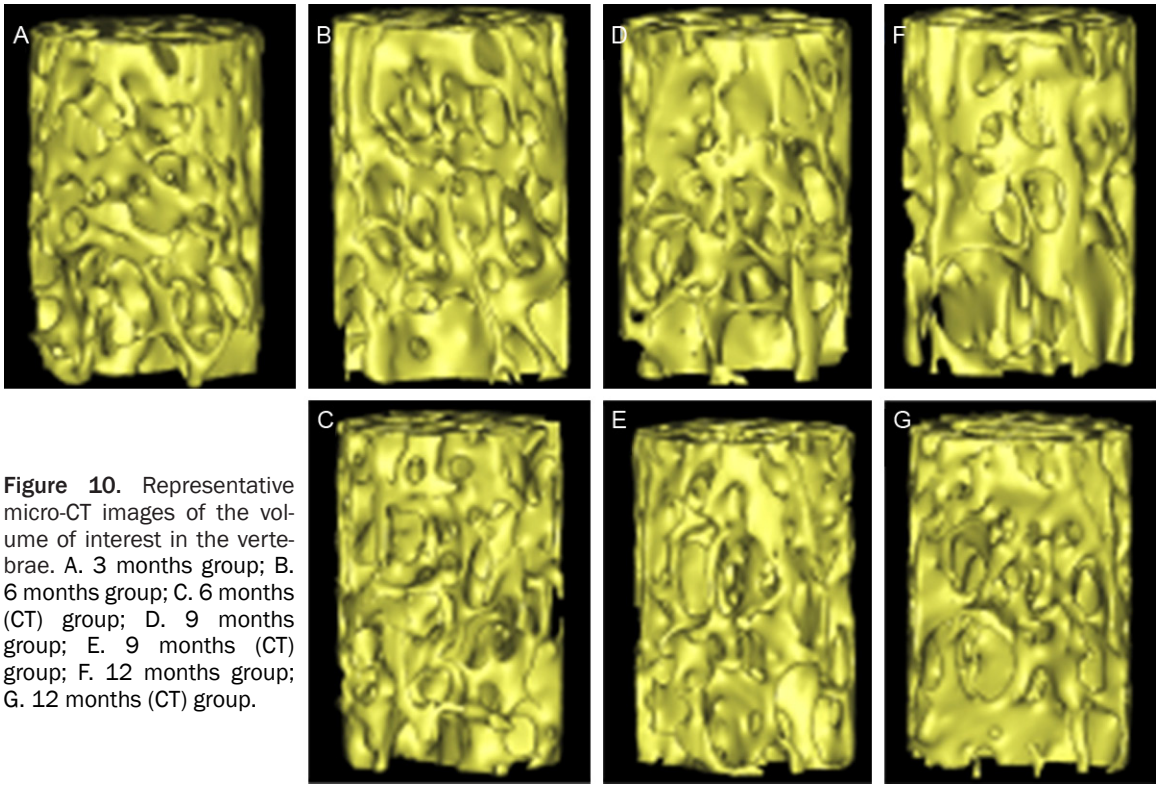

Figure 10. Representative micro-CT images of the volume of interest in the vertebrae. A. 3 months group; B. 6 months group; C. 6 months (CT) group; D. 9 months group; E. 9 months (CT) group; F. 12 months group; G. 12 months (CT) group.

Table 4. Mechanical properties of vertebral body

	Maximum load (N)	Maximum stress (MPa)	Yield stress (MPa)	Elastic modulus (GPa)
3M	117.625 ± 16.694	10.297 ± 0.860	8.517 ± 0.380	443.876 ± 55.926
6M	195.469 ± 19.415*	14.3976 ± 2.210*	12.164 ± 1.967*	502.063 ± 82.062*
9M	197.552 ± 33.674*	15.048 ± 1.882*	11.979 ± 1.690*	599.909 ± 60.870*
12M	210.938 ± 30.055*	16.205 ± 1.401*	11.950 ± 1.526*	571.444 ± 54.163*
6M-CT	278.802 ± 35.339▲	17.420 ± 0.995▲	14.734 ± 1.338▲	611.028 ± 58.470▲
9M-CT	262.709 ± 28.569▲	17.921 ± 2.564▲	14.819 ± 1.981▲	594.214 ± 69.376
12M-CT	313.231 ± 26.468▲	20.070 ± 1.668▲	17.611 ± 1.384▲	595.838 ± 82.917

Note: *$P < 0.05$ *vs.*3M group; ▲$P < 0.05$ *vs.* same-age control group. Data from CT treated groups compared only with same-age control groups.

old animals. Furthermore, immunohistochemistry findings suggest that the beneficial effect of CT treatment on degenerated IVD is at least partially due to promoting synthesis of collagen type II and GAG, together with inhibiting MMP-1 expression, thereby maintaining integrity of the ECM during the development of disc degeneration in guinea pig.

However, the increased bony endplate ratio and decreased porosity were also observed in CT treated guinea pigs, which may increase the occlusion of nutrition transport channel, thereby promote the degeneration of IVD, these results seems contradicted with the beneficial effects we observed in IVD histological analysis. However, the effects of CT on bony tissues in the present are far more than that observed in endplate. The bone mass and quality of adjacent vertebral body were also affected by CT treatment. The bone mineral density in L3-5 was increased after 6 months intervention of CT. CT treatment notably increased the BV/TV while markedly suppressed the Tb.sp and SMI in trabecular bone. On the other hand, CT treatment markedly increased maximum load, yield stress, maximum stress and elastic modulus. Thus, CT not only promoted the bone formation in guinea pig vertebral body, but also improved the microstructure and biomechanical proper-

ties. In light of the pathogenesis of disc degeneration, the microstructure and biomechanical properties of the vertebrae logically influence the disc biological environment. However, it should be acknowledged, although the intervention of CT was 9 months long, the obvious limitation of the present study is that we only collected the data in early phase of the IVD degeneration, whether CT treatment could reverse the naturally occurred IVD degeneration at late period is still unclear.

Taken together, the data presented here provide new evidence support that spontaneous intervertebral disc degeneration occurred in guinea pigs, though occurred later than osteoarthritis did. Moreover, under the regime in the present study, CT pretreatment showed several beneficial effects and few opposite effect in guinea pig IVD, and finally, the modulation of ECM metabolism, coupled with improving the microstructure and biomechanical properties weight more than increasing bony endplate ratio and decreasing porosity.

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Disclosure of conflict of interest

None.

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