Reparative effects of chronic intermittent hypobaric hypoxia pre-treatment on intervertebral disc degeneration in rats

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Received September 6, 2021; Accepted February 25, 2022

DOI: 10.3892/mmr.2022.12689

Abstract. Previous studies have indicated that chronic intermittent hypobaric hypoxia (CIHH) preconditioning can inhibit TNF-α and other related inflammatory cytokines and exerts protective effect on intervertebral disc degeneration disease (IDD) in rats; however, the mechanism is still unclear. The present study aimed to explore the repair mechanisms of CIHH on IDD in rats. In the experiment, 48 adult Sprague-Dawley rats were selected and randomly divided into an experimental group (CIHH-IDD), a degenerative group (IDD) and a control group (CON). The CIHH-IDD group of rats (n=16) were treated with CIHH (simulated 3000 m altitude, 5 h per day, 28 days; P_{O2}=108.8 mmHg) before disc degeneration surgery. The IDD group of rats (n=16) underwent tail-vertebral intervertebral disc surgery to establish a model of intervertebral disc degeneration. The CON group of rats (n=16) did not receive any treatments. After surgery, the disc height index was calculated using X-ray analysis of rat tail vertebrae, the degeneration process was observed and repair was evaluated by chemically staining degenerative intervertebral disc tissue slices. The expression levels of basic fibroblast growth factor (bFGF), TGFβ1, Collagen I and Collagen II were measured in the intervertebral disc tissue using western blotting; while the expression levels of bFGF, TGFβ1 and hypoxia-inducible factor 1-α (HIF-1α) were measured in rat serum using ELISA. The results demonstrated that: i) The degree of intervertebral disc height degeneration in CIHH-IDD rats was significantly lower compared with that in IDD rats (P<0.05); ii) the expression levels of bFGF, TGFβ1 and HIF-1α were higher in CIHH-IDD rat serum compared with those in IDD rat serum (P<0.05); iii) optical microscopy revealed that the degree of disc degeneration was relatively mild in CIHH-IDD rats; and iv) the protein expression levels of bFGF, TGFβ1 and collagen II were increased in CIHH-IDD rat intervertebral disc tissues compared with those of IDD rats, while the overexpression of collagen I protein was inhibited. Overall, after CIHH pre-treatment, the expression levels of bFGF and TGFβ1 were up-regulated, which play notable roles in repairing degenerative intervertebral discs in rats.

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Key words: chronic intermittent hypobaric hypoxia, intervertebral disc degeneration, basic fibroblast growth factor, TGFβ1, Collagen I, Collagen II, Sprague-Dawley rats

Introduction

Intervertebral disc degeneration disease (IDD) is one of the major causes of low back pain (LBP) (1). Since 2015, overall >540 million individuals worldwide suffer from varying degrees of LBP, which causes pain and loss of function. LBP imposes huge burdens on society, the economy and families; seriously reduces the quality of life of patients (1). The present authors have been engaged in orthopaedic trauma-related clinical work. In clinical practice, we noticed the present of varying degrees of disc degeneration (most frequent at the level of L4/L5) in patients with lumbar spine fractures after surgery. Intervertebral disc degeneration is a very complex process that is mainly associated with the abnormal apoptosis of intervertebral disc cells, biomechanical mechanisms and the autoimmune response (2,3). Regardless of the aetiology, the end result is that the proliferation capacity of the intervertebral disc is weakened and the function is reduced, which leads to the occurrence of disc degeneration (2,3). As a family of proteins that regulate cell proliferation and cell matrix biosynthesis, growth factors play important roles in stimulating cell proliferation and repairing intervertebral disc injury (3).

The IDD rat model is a good experimental model for studying the mechanism of intervertebral disc degeneration. Because the pathological changes in the blood and intervertebral discs in the model are similar to those of human intervertebral disc degeneration, the IDD rat model has been widely used to study human intervertebral disc degeneration (4). Fine needle puncture is currently the preferred method for establishing models of intervertebral disc degeneration due to its simplicity, ease of operation and high repeatability.

Masuda *et al* (4) attempted to puncture rabbit fibre rings using different sizes of needles (16G, 18G and 21G) and successfully established an animal model of disc degeneration that led to decreases in both disc height and magnetic resonance imaging grading.

Chronic intermittent hypobaric hypoxia (CIHH) pre-conditioning is the practice of simulating altitude sickness in humans or animals through intermittent exposure to low pressure and low oxygen. Moderate low-pressure hypoxia stimulation can effectively mobilize the body's endogenous protective mechanism to counter the subsequent stimulation by more serious external injury (5). CIHH has been widely used in sports training to enhance the resistance of organs and tissues to anoxia (6). Previous studies have demonstrated that CIHH pre-treatment has a protective effect against collagen-induced arthritis in rats through the down-regulation of hypoxia-inducible factor 1-α (HIF-1α) and NF-κB, and through the inhibition of the inflammatory cytokines TNF-α and IL-17 (5). Therefore, it was hypothesised that CIHH promotes the expression of basic fibroblast growth factor (bFGF) and TGFβ1, which play important regulatory roles in cell proliferation, differentiation and tissue repair, thereby promoting intervertebral disc tissue repair. The main objective of the present study was to investigate the reparative effect of CIHH pre-treatment on degressive intervertebral disc tissue in rats.

Materials and methods

Chemicals and reagents. The Haematoxylin-Eosin/HE Staining kit, Modified Safranine O-Fast Green FCF Cartilage Stain kit, and Masson's Trichrome Stain kit were purchased from Beijing Solarbio Science & Technology Co., Ltd. Rat bFGF ELISA kit (cat. no. E-EL-R0091c), Rat HIF-1α ELISA kit (E-EL-R0513c) and TGF-β1 ELISA kit (E-EL-0162c) were purchased from Elabscience Biotechnology, Inc. For the western blotting experiments, collagen I(WL0088), collagen II(WL03082) and TGF-β1 (WL02193) antibodies were purchased from Wanleibio Co., Ltd., and the bFGF (E-AB-15525) antibody was purchased from Elabscience Biotechnology, Inc.

Animals and treatments. All experiments were performed in accordance with the guidelines for the Care and Use of Experimental Animals (National Research Committee, 1996) and approved by the Ethics Committee for the Use of Experimental Animals of Hebei Medical University (approval no. Z2019-012-1; Hebei, China). At total, 48 adult male Sprague-Dawley rats (provided by Hebei Medical University Experimental Animal Centre; weight, 320±20 g; 8 weeks old) were randomly divided into three groups: The experimental group (CIHH-IDD), degenerative group (IDD) and control group (CON). The IDD model was established in rats in the IDD group (n=16) by puncturing the tail discs after 28 days of normal feeding. CIHH + IDD rats (n=16) were treated with CIHH (simulated altitude of 3,000 m, 5 h per day, for 28 consecutive days; P_{O2}=108.8 mmHg) before undergoing the same treatment as the IDD rats. CON rats (n=16) were normally bred without IDD induction. At 1, 2, 4 and 8 weeks after IDD surgery, four rats from each group were randomly selected for X-ray imaging, after which blood from the heart and tissue from the tail disc were collected after the animals were anaesthetised.

All animals were kept in a temperature-controlled room (22±1˚C; relative humidity, 40-80%) with a 12 h light/dark cycle and free access to water and food. The health status and physical activity of the rats were monitored every day. At the end of the experiments, rats were fasted overnight and anaesthetised with pentobarbital sodium (50 mg/kg; intraperitoneal injection). Left index finger to find the heart apex pulse, right hand holding blood needle puncture and 3 ml of blood were collected from the heart. The rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg; intraperitoneal injection), and then tail disc specimens were isolated. In the present study, the preparation of IDD rats, the collection of samples and the measurement of the results were performed by the same skilled researchers to reduce errors caused by subjective and human errors.

IDD establishment. The rats were anaesthetised using pentobarbital sodium (50 mg/kg, intraperitoneal injection) and fixed in the supine position. The rat tail discs at C0 6/7, C0 7/8 and C0 8/9 were selected as the research objects. The tail vertebrae were first disinfected, and then an X-ray instrument was used to locate the intervertebral disc nucleus centre. A 21G needle was passed through the disc nucleus, slowly rotated 180˚ and maintained for 5 sec, after which the needle was removed, pressure was applied to stop the blood and the area was disinfected again. Because the endplate is an important structure for maintaining nutrition in the intervertebral disc, damage to the endplate can exacerbate or accelerate the degeneration of the intervertebral disc (7-9); thus, the surgeon avoided damaging the endplate as much as possible.

CIHH treatment. For CIHH treatment, the animal was placed in a low-pressure oxygen chamber, and the air was pumped away using a vacuum, resulting in a pressure of 108.8 mmHg, which represents an altitude of 3,000 m. At the same time, fresh air flowed into the chamber through a small ventilation hole to keep enough fresh air for the animal to breathe. An intermittent oxygen environment controller was used to maintain a low oxygen environment for 5 min, and then the pressure was restored to normal. For safety, the reduced pressure and boost pressure speed were controlled at 2.5 m/s with the vent valve. The time from the low oxygen concentration to the high oxygen concentration was 30 sec, and the intermittent low-pressure hypoxia experiment lasted for 5 h.

X-ray calculation of the disc height index (DHI). After being anaesthetised, the rats were placed in the prone position for X-ray scans and radiographs before IDD surgery. The same operation was performed at 1, 2, 4 and 8 weeks after IDD surgery. According to the method described by Han *et al* (10), the width of the intervertebral disc was divided into four equal points, the image analysis software ImageJ (V1.8.0.112, National Institutes of Health) was used to measure the height of the intervertebral disc and its adjacent vertebral body, and the intervertebral DHI was calculated, which represented the change in height relative to the rate of change in the DHI

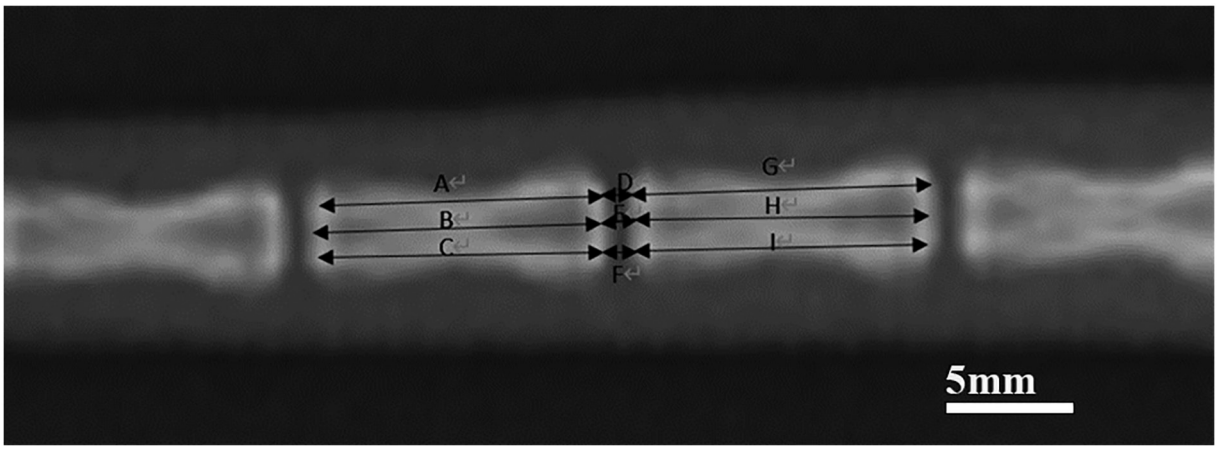

Figure 1. Disc measurement. Width of the intervertebral disc was divided into four equal points; the height value of the intervertebral disc (D + E + F); the height value of adjacent vertebral body (A + B + C + G + H + I).

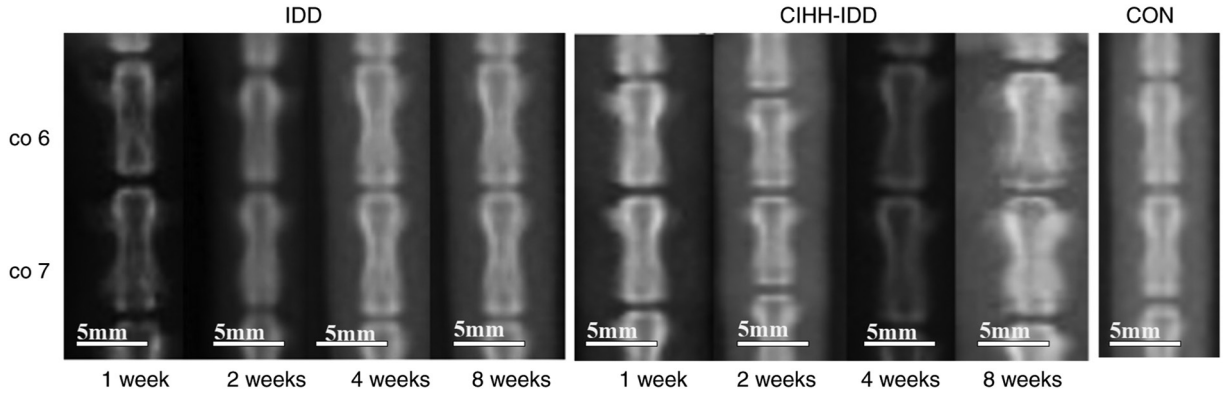

Figure 2. Images of intervertebral discs in rats. Images of CO6, CO7 and intervertebral space in rats of IDD group and CIHH-IDD group, at 1, 2, 4 and 8 W after surgery. Scale bar, 50 nm. W, weeks; IDD, CIHH-IDD; CON, control; Scale bar, 50 nm. W, weeks; IDD, CIHH-IDD; CON, control.

value (10). The specific calculation method was calculated as follows (Fig. 1): DHI=2(D+E+F)/(A+B+C+G+H+I), where DHI %=post-operative DHI/pre-operative DHI x100%.

Intervertebral disc tissue specimen collection. At 1, 2, 4 and 8 weeks after the IDD operation, four rats in each group were sacrificed by an intraperitoneal injection of excessive levels of anaesthetic drugs. The tail skin was carefully removed, the tail was detached and the C0 6/7, C0 7/8 and C0 8/9 discs were separated. One intervertebral disc tissue sample was soaked in 10% neutral-buffered formalin fixation solution for 48 h (23-26˚C). The specimens were routinely dehydrated, decalcified and paraffin-embedded to prepare wax blocks. The intervertebral disc tissue was cut into 5-μm thick slices that were used for haemotoxylin-eosin (HE), modified Safranine O-Fast Green and Masson's trichrome staining. HE staining can clearly show the layers of tissue, modified Safranine O-Fast Green staining can better show the cartilage layers and subchondral bone structure, and Masson's trichrome staining can clearly show fibrous ring tissue and nucleus pulposus tissue (11). The other two intervertebral disc tissues were quickly placed at -80˚C and used to measure the protein expression levels of bFGF, TGFβ1, Collagen I and Collagen II using western blotting.

ELISA determination of bFGF, TGFβ1 and HIF-1α in serum. The rats were anaesthetised with pentobarbital sodium (50 mg/kg; intraperitoneal injection), and 3 ml of blood were collected from the heart and centrifuged for 5 min (2716 x g, 23-26˚C) to get serum. The serum was collected and stored at -20˚C for later use. The expression levels of bFGF, TGFβ1 and HIF-1α in the serum were determined using ELISA kits as aforementioned. According to the manufacturer's procedures indicated in the kits, the optical density values were measured at 450 nm (ELX-800, BioTek Instruments, Inc.) 15 min after the cessation of the reaction. The concentrations of bFGF, TGFβ1 and HIF-1α were determined from a standard log-log graph.

Western blotting. The expression levels of bFGF, TGFβ1, Collagen I and Collagen II in degenerative disc tissue were measured using western blotting. Degenerated intervertebral disc tissue was frozen at -80˚C, homogenised and lysed and then placed on ice for 5 min. The samples were centrifuged at 23,188 x g. and 4˚C for 10 min, and the protein was extracted using RIPA lysis buffer (WLA019, Wanleibio Co.). and separated by electrophoresis. The protein concentration in the supernatant was determined using the BCA method, and each sample was boiled in water for 5 min at 100˚C. The samples (20 μl per lane, containing 40 μg of protein)

Table I. Intervertebral disc height index (DHI %).

Post-operation	CIHH-IDD	IDD	CON
1 week	98.20±4.61	97.20±5.37	99.20±2.31
2 weeks	96.45±5.24[b]	94.83±4.97[a]	98.90±2.78
4 weeks	93.67±6.17[a,b]	88.26±5.71[c]	99.56±2.51
8 weeks	87.54±4.76[b,c]	81.79±4.96[c]	99.12±2.47

[a]P<0.05 vs. control; [b]P<0.05 vs. IDD; [c]P<0.01 vs. control. All data are expressed as mean ± SEM (n=16 for each group; n=4 for each time point). CON, control group; IDD, intervertebral disc degeneration disease group; CIHH-IDD, chronic intermittent hypobaric hypoxia intervertebral disc degeneration disease group.

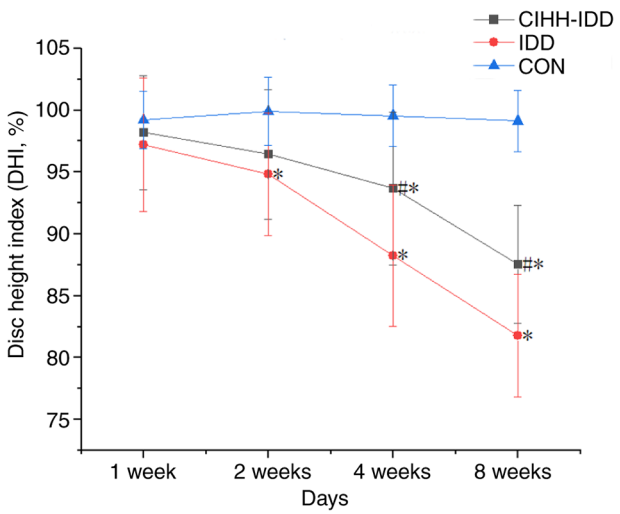

Figure 3. DHI % trend of rats in each group. The trend of DHI% represented the change of intervertebral disc height and reflected the degree of intervertebral disc degeneration in each group of rats. n=16 for each group, n=4 for each time point. *P<0.05 vs. CON group; #P<0.05 vs. IDD group. CON, control group; IDD, intervertebral disc degeneration disease group; CIHH-IDD, chronic intermittent hypobaric hypoxia-IDD group; DHI, disc height index.

were examined using 7.5-15% SDS-PAGE and transferred to PVDF membranes. The PVDF membrane was blocked with 5% (M/V) non-fat milk powder for 1 h at 4˚C. The blots were incubated first at 4˚C with primary antibodies against bFGF (1:2,000), TGFβ1 (1:500), Collagen I (1:500) and Collagen II (1:500) overnight. The samples were then incubated with secondary antibodies (Goat Anti-rabbit IgG-HRP, 1:5000, WLA023, Wanleibio Co.) for 45 min at 37˚C. The reaction was visualised by chemiluminescence, and the optical density of the target band was analysed by a gel image processing system (Gel-Pro-Analyzer 6.0; Media Cybernetics, Inc.). The protein levels were normalised to that of β-actin (1:1,000, WL01845, Wanleibio Co.).

Statistical analysis. Statistical analysis was performed using SPSS 26.0 (IBM Corp.). Experiments were repeated three times. The data are expressed as the mean ± SEM. n represents the number of animals in functional or western blotting experiments. Statistical analysis was conducted using one-way ANOVA followed by Student-Newman-Keuls's post hoc test for comparisons among multiple groups. The paired Student's t-tests were used for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Table II. Effect of chronic intermittent hypobaric hypoxia on the expression of bFGF, TGF 1 and HIF-1 in rat blood.

Group	bFGF	TGFβ1	HIF-1α
CIHH-IDD			
1 week	11.12±3.41[a,b]	48.53±7.97[a,b]	7.76±2.23[a,b]
2 week	9.78±3.12[a,c]	42.19±6.76[a,b]	4.88±1.56
4 week	9.85±3.31[a]	36.85±6.45[a,b]	4.01±1.22
8 week	8.52±2.76[a]	32.88±5.87[a,b]	4.39±1.32
IDD			
1 week	7.53±2.74	30.91±5.45[a]	4.13±1.01
2 week	8.31±3.25[d]	34.47±5.94[a]	4.12±1.13
4 week	9.83±3.49[a]	26.32±2.67[d]	4.22±1.22
8 week	8.73±2.78[a]	26.98±3.83[d]	4.32±1.32
CON			
1 week	7.75±2.46	23.59±2.97	4.01±1.21
2 week	7.57±2.66	24.37±3.42	4.02±1.01
4 week	7.49±2.58	23.26±3.17	4.22±0.99
8 week	7.53±2.62	23.63±3.29	4.21±1.02

[a]P<0.01 vs. CON; [b]P<0.01 vs. IDD; [c]P<0.05 vs. IDD; [d]P<0.05 vs. CON. All data are expressed as mean ± SEM (n=16 for each group; n=4 for each time point). CON, control group; IDD, intervertebral disc degeneration disease group; CIHH-IDD, chronic intermittent hypobaric hypoxia intervertebral disc degeneration disease group; bFGF, basic fibroblast growth factor; HIF-1α, hypoxia-inducible factor 1-α.

Results

Imaging analysis of the effect of CIHH on intervertebral disc degeneration in rats. Vertebral imaging of each group of four rats was performed using digital X-ray instruments at 1, 2, 4 and 8 weeks after surgery, and the results are shown below. The DR images indicated that the density of the intervertebral space in the CIHH-IDD and IDD groups increased gradually after surgery (Fig. 2). The intervertebral space height (DHI %) of each group of rats at different times was measured using image analysis software and is presented in Table I. In the second week after surgery, the differences between the IDD group and CON group were significant (P<0.05), the results of the CIHH-IDD group were not significant compared with those of CON group (P>0.05), and the CIHH-IDD group was significantly different compared with the IDD group (P<0.05). At 4 weeks after surgery, The DHI (%) of the IDD group was significantly different compared with that in the CIHH-IDD group (P<0.05), and the difference between the CIHH-IDD group and the CON group was significant (P<0.05).

The results indicated that the intervertebral disc height of rats in the IDD group decreased at 2 weeks after surgery compared with the control, and the decreasing trend was

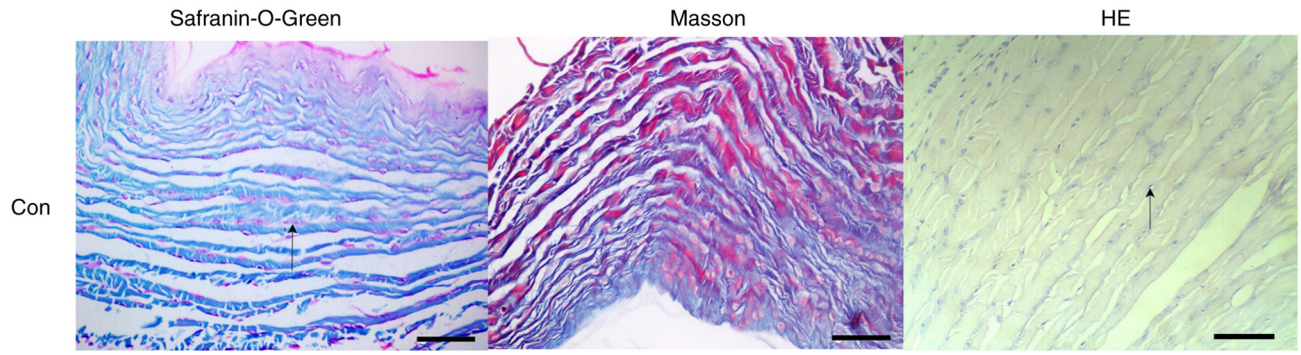

Figure 4. Histopathological sections of rat intervertebral disc in the CON group. Sections were stained using safranin-O-Green, Masson trichromatic staining and HE staining. Arrow indicates fusiform fibroblasts. Scale bar, 100 μm. HE, haematoxylin-eosin staining; Con, control.

gradually but significantly worsened over time (Fig. 3). By contrast, the intervertebral disc height of rats in the CIHH-IDD group demonstrated a significant decrease at 4 weeks after surgery compared with the control, with a slower downward trend compared with that in the IDD group. Overall, CIHH treatment significantly inhibited the degree of disc degeneration in the CIHH-IDD group compared with the IDD group.

Effect of CIHH on disc pathology in rats. Disc tissue staining in the CON group indicated that the intervertebral discs of rats presented a similar oval appearance and that the nucleus pulposus was increased, accounting for more than half of the total intervertebral disc volume. The proteoglycans in the nucleus pulposus were stained red and have small round cells spaced at intervals. The boundary between nucleus pulposus tissue and the surrounding fibre ring was clear. The fibre ring was arranged in an orderly and concentric circular lamella, and there was no obvious fracture or crack between each lamella. Fusiform fibroblasts were observed between each lamella (Fig. 4).

Pathologically stained disc slices from the IDD group showed that intervertebral disc histology was mainly changed in the following manners (Fig. 5): Reduced volume of the nucleus pulposus, fractures or disordered arrangement between the annulus fibrosus, decreased numbers of nucleus pulposus cells and extracellular matrix (ECM) and metaplasia of cells. After the operation, the nucleus pulposus became irregular and smaller, and some tissue of the nucleus pulposus was lost, resulting in space. Over time, the degree of shrinkage gradually increased, and the space in part of the nucleus pulposus increased. Furthermore, the number of cells in the nucleus pulposus was markedly reduced. Although some small round cells could be seen in the nucleus pulposus, these cells were significantly different compared with the uniformly distributed normal cells in the nucleus pulposus, which were separated by ECM and distributed in clusters. The original small circular spinal cord cells became large round cartilage-like cells, some cells appeared to have large empty bubbles and the cells and their surroundings were dyed red by safranin-O-green staining or blue by Masson staining. The boundary between the nucleus pulposus and the annulus fibrosus became unclear. The annulus was disorganised, with radiation-like or edge tearing, which sometimes extended from the inside out to the perimeter of the fibre ring. Metaplasia of cells was also observed in the annulus fibrosus; in particular, the number of chondroid cells between the lamellar layers of the inner annulus fibrosus increased, and the amount of red-stained proteoglycan increased. Masson staining showed blue collagen fibres, which were markedly increased. Chondroid cell proliferation in the annulus fibrosus was present. The structure of cartilage, subchondral bone and bone tissue can be indicated by safranin-O-green staining. The cartilage matrix appears red, and the subchondral bone and bone tissue appear green, which can robustly the cartilage tissue.

By observing the pathological sections of CIHH rats with intervertebral disc disease, the arrangement of the annulus fibrosus was observed to be more similar to that of the CON group compared with the IDD group, and the arrangement disorder and tearing degree were reduced compared with those in the IDD group (Fig. 6). Both nucleus pulposus volume reductions and extracellular matrix reductions occurred but did not further worsen. Cavitation of the nucleus pulposus was rare. CIHH treatment resulted in some repair of the degenerative discs in rats.

Effect of CIHH on the expression levels of bFGF, TGF 1 and HIF-1 in rat blood. The serum level of HIF-1α at 1 week after surgery was significantly higher in the CIHH-IDD group compared with the CON and IDD groups ($P<0.01$). There was no significant difference in the level of HIF-1α between the three groups beginning at 2 week ($P>0.05$). The expression level of HIF-1α in CIHH-pre-treated rats returned to normal after normal feeding for 2 weeks (Table II).

The serum level of TGFβ1 in the IDD group was higher compared with that in the CON group beginning in the first week after surgery ($P<0.05$), indicating that the expression of TGFβ1 was increased in rats after disc puncture (Table II). After CIHH pre-treatment, the serum level of TGFβ1 in the CIHH-IDD group was significantly higher compared with that in the IDD group beginning in the first week after surgery ($P<0.01$), indicating that CIHH pre-treatment significantly increased the serum level of TGFβ1 in rats.

The expression of bFGF was measured, and the serum levels of bFGF in rats that were pre-treated with CIHH were observed to significantly increase compared with that in the IDD groups and CON groups at week 1 ($P<0.01$). Furthermore, the serum level of bFGF in rats in the IDD group showed a tendency to increase over time (Table II).

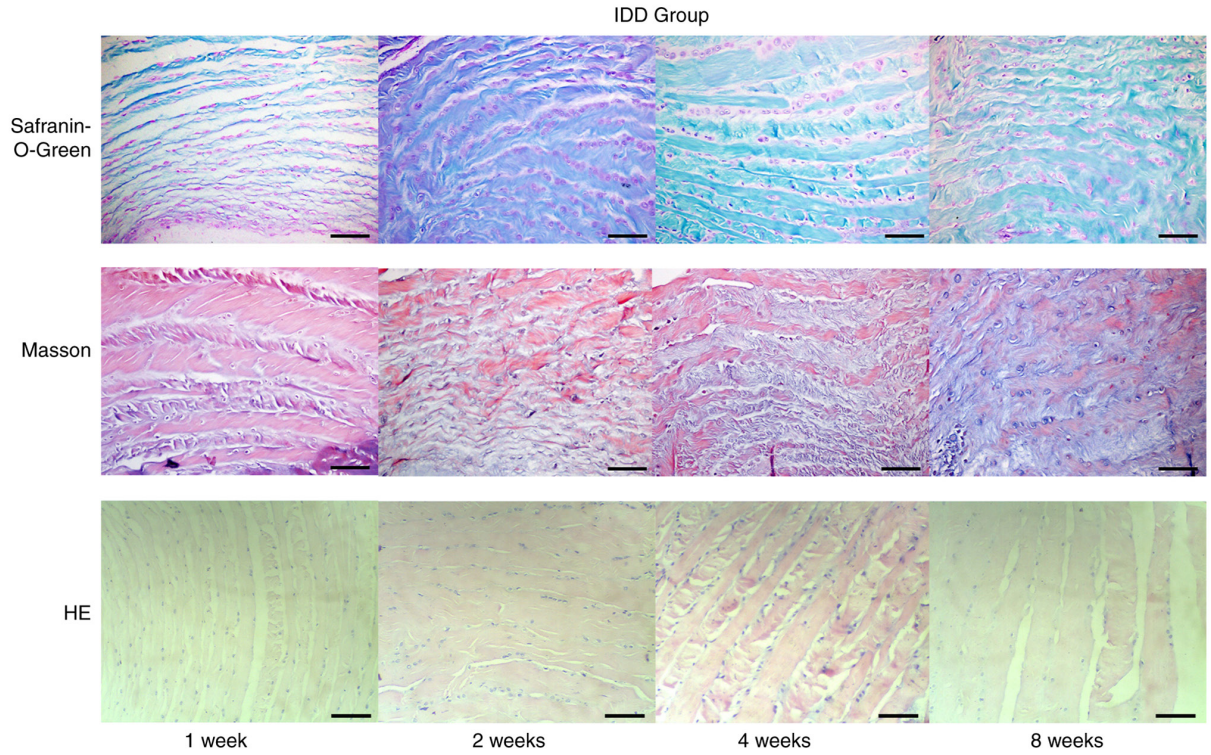

Figure 5. Histopathological sections of rat intervertebral disc in the IDD group at 1, 2, 4 and 8 weeks after surgery. Sections were stained using Safranin-O-Green staining, Masson trichromatic staining and HE staining. Scale bar, 100 μm. HE, haematoxylin-eosin staining; IDD, intervertebral disc degeneration disease group; W, week.

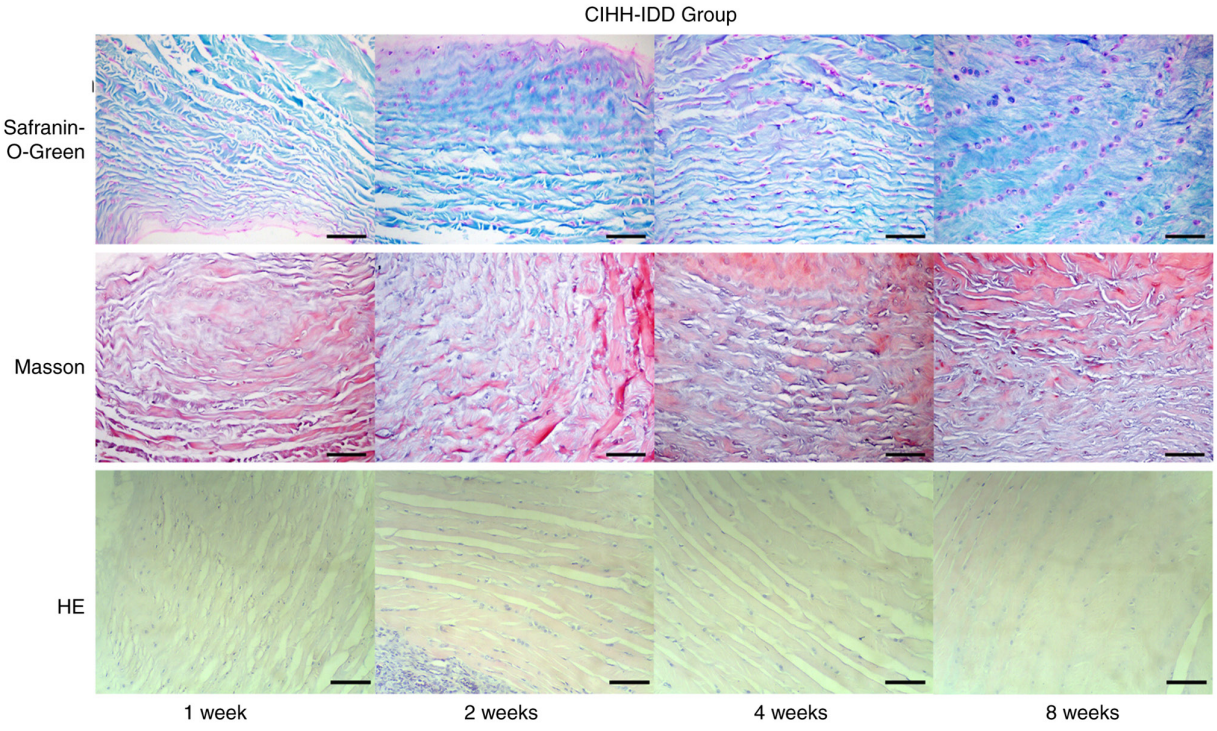

Figure 6. Histopathological sections of rat intervertebral disc in the CIHH-IDD group at 1, 2, 4 and 8 weeks after surgery. Sections were stained using Safranin-O-Green staining, Masson trichromatic staining and HE staining. Scale bar, 100 μm. HE, haematoxylin-eosin staining; HE, haematoxylin-eosin staining; CIHH-IDD, chronic intermittent hypobaric hypoxia-intervertebral disc degeneration disease group; W, week.

Effect of CIHH on bFGF, TGFβ1, Collagen I and Collagen II expression in degenerative disc tissue in rats. At 1 week after surgery, the expression of bFGF in the three groups was not statistically significant. At 2 weeks after the operation, the expression of bFGF in the CIHH-IDD group was significantly increased compared with that in the CON group ($P<0.01$).

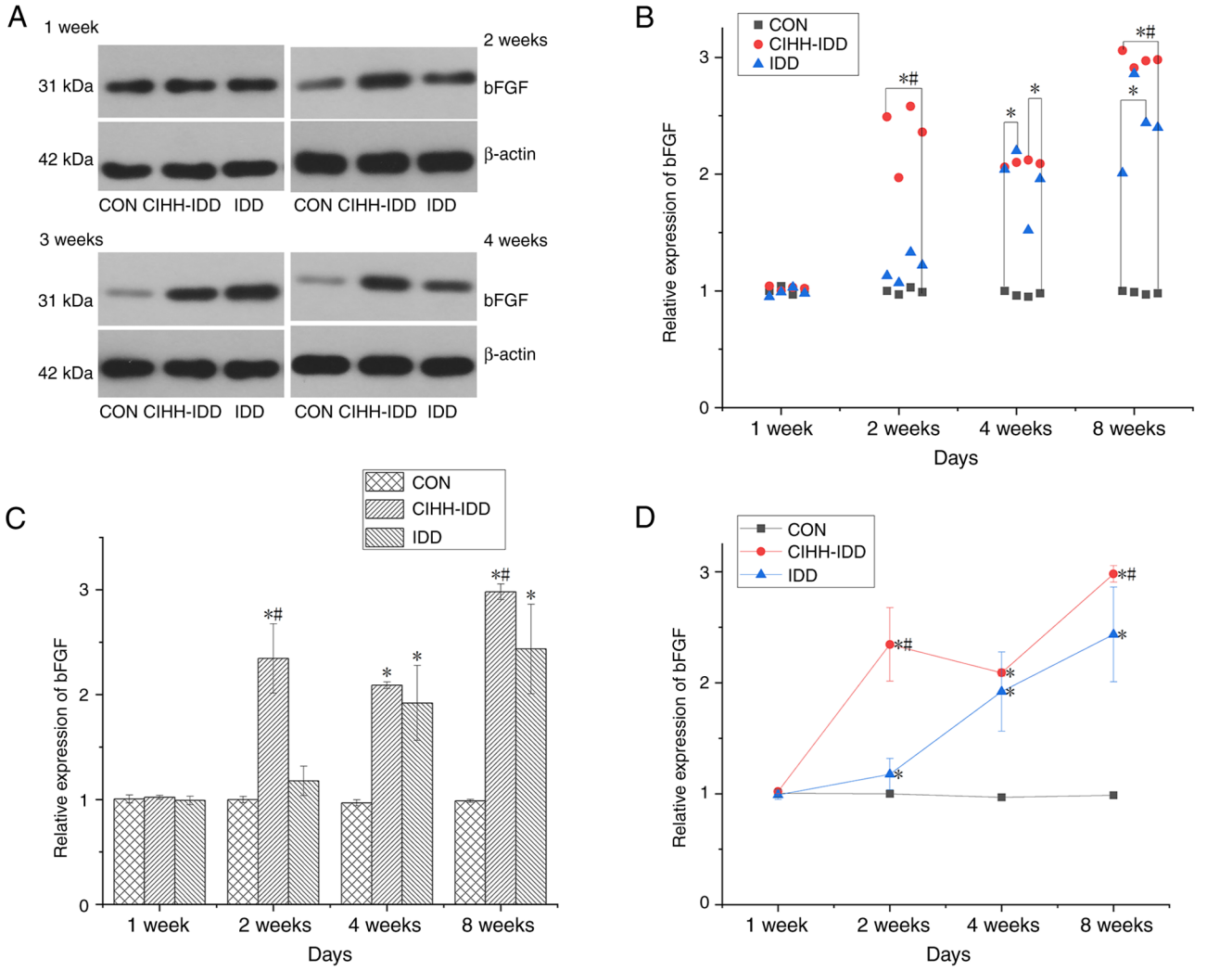

Figure 7. Effect of CIHH on the expression of bFGF protein in degeneration disc tissue. (A) Western blotting of bFGF protein at 1, 2, 4 and 8 weeks. (B) Western blotting data distribution of bFGF proteins at 1, 2, 4, and 8 weeks. (C) bFGF protein expression levels at 1, 2, 4, and 8 weeks in three groups. (D) Expression trend of bFGF protein. n=16 for each group; n=4 for each time point. *P<0.05 vs. CON group; #P<0.05 vs. IDD group. bFGF, basic fibroblast growth factor; CIHH, chronic intermittent hypobaric hypoxia; IDD, intervertebral disc degeneration disease group; CON, control.

Compared with that in the IDD group, the expression of bFGF in the CIHH-IDD group was significantly up-regulated at 2 and 8 weeks (P<0.05), but there was no significant difference between the two groups in the fourth weeks (Fig. 7).

Compared with that in the CON group, the expression of TGFβ1 in rat degenerative discs (IDD group) demonstrated a significant trend of increased expression beginning at 1 week (P<0.01). By contrast, the expression of TGFβ1 in the CIHH-IDD group was not significantly different at 1 week compared with that in the IDD group. Starting at 2 weeks, the protein expression of TGFβ1 in the CIHH-IDD group increased significantly compared with both the CON and IDD groups (P<0.05) (Fig. 8).

After establishing the animal models of IDD, the expression of Collagen I in the degenerative intervertebral discs of rats in the CIHH-IDD group and IDD group was significantly increased compared with that in the CON group at week 1 (P<0.05). The data demonstrated that at 2 weeks after surgery, the protein expression of Collagen I was not significantly different among the three groups, and it was considered that this effect might be associated with deviations during surgery and the experiments. At 1, 4 and 8 weeks after surgery, the protein expression of collagen I in the CIHH-IDD group was inhibited compared to that in the IDD group (P<0.05) (Fig. 9).

At 1 week after surgery, the protein expression of collagen II in the CIHH-IDD group and IDD group was significantly increased compared with that in the CON group (P<0.05). Compared with that in the IDD group, the protein expression of collagen II in the CIHH-IDD was significantly increased from 2 weeks after surgery (P<0.05) (Fig. 10).

Discussion

Intervertebral disc degeneration is a complex process that gradually occurs under the combined effects of various factors, including natural and environmental factors (12). In recent years, due to the increasing burden of lumbago on individuals, families and society, research on the aetiology and pathogenesis of disc degeneration has increased (13). A number of clinical studies have demonstrated that the mechanism of intervertebral disc degeneration may be a complex pathological phenomenon caused by reduced nutrient supply

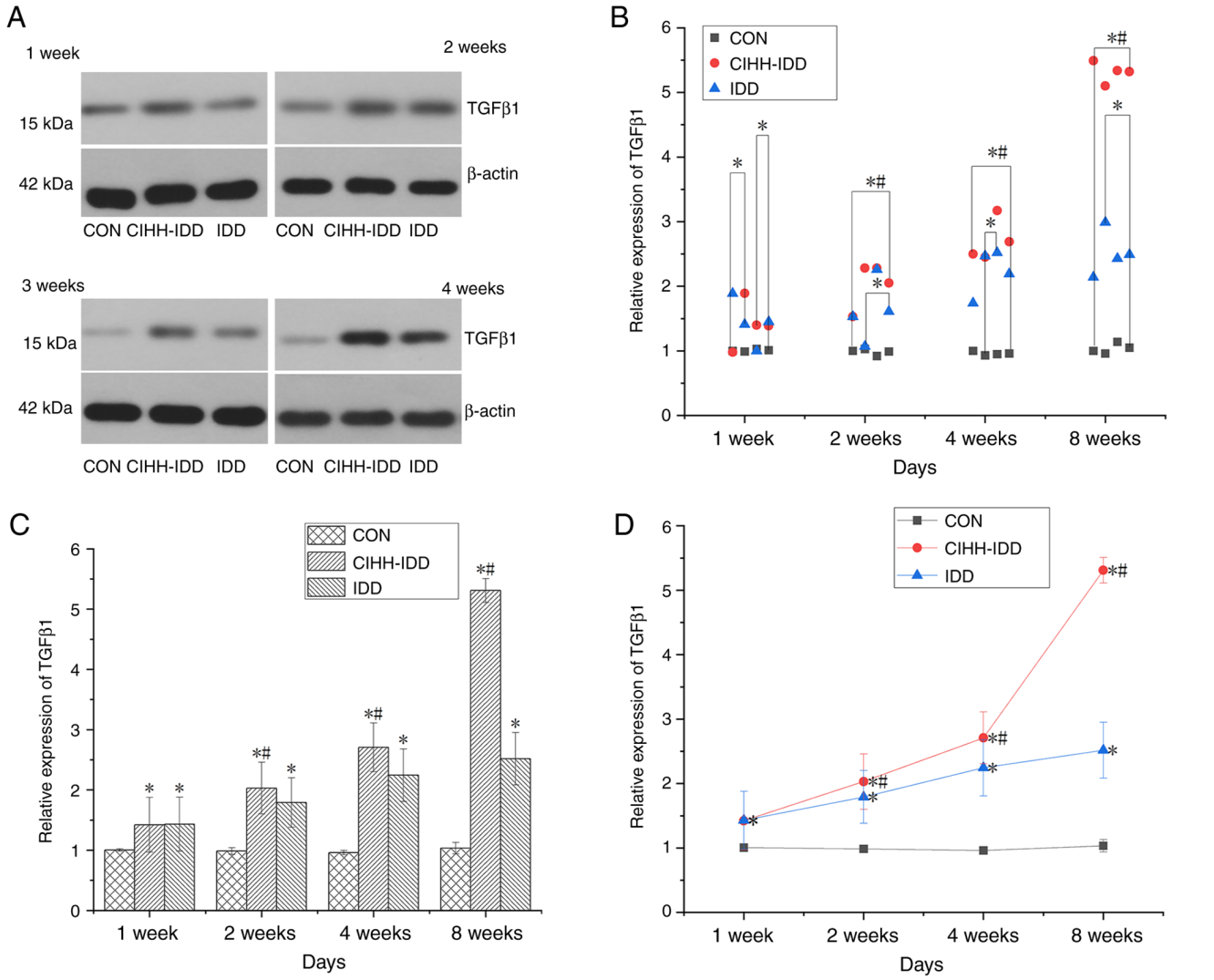

Figure 8. Effect of CIHH on the expression of TGFβ1 protein in degeneration disc tissue. (A) Western blotting of TGFβ1 protein at 1, 2, 4 and 8 weeks in the three groups. (B) Western blotting data distribution of TGFβ1 proteins at 1, 2, 4, and 8 weeks. (C) TGFβ1 protein expression levels at 1, 2, 4, and 8 weeks in three groups. (D) The expression trend of TGFβ1 protein. n=16 for each group, n=4 for each time point. *P<0.05 vs. CON group; #P<0.05 vs. IDD group. CIHH, chronic intermittent hypobaric hypoxia; IDD, intervertebral disc degeneration disease group; CON, control

in intervertebral disc tissue, changes in extracellular matrix components in intervertebral discs, excessive apoptosis, biomechanical changes and autoimmunity, but the exact mechanism has not been clarified (14-16).

The present study investigated the internal mechanism by which CIHH promoted disc repair in IDD rats. It was revealed that that CIHH pre-treatment could significantly promote the expression of bFGF and TGFβ1 in blood and intervertebral disc tissue, thus effectively reducing the degree of intervertebral disc degeneration and playing a role in the repair of intervertebral disc degeneration. Of course, the effect of CIHH on degenerative intervertebral discs in rats not only involved repair but was also associated with the suppression of inflammatory factors, Such as IL-4, TNF-α, and IL-17.

Through CIHH pre-treatment, an increase in HIF-1α was observed in the serum of rats. Increased HIF expression is a marker of the hypoxia response and a signal of hypoxia in tissues. The body's adaptation to a low-pressure, low-oxygen environment is achieved by enhancing HIF-1 levels (17). Hypoxia has both positive and negative effects on the body. Severe hypoxia is involved in the occurrence and prognosis of acute and chronic diseases such as diabetes, cardiovascular disease and pulmonary oedema (18). On the other hand, controlled hypoxia (intensity and time) plays a beneficial role through the adaptation mechanism of body's response (19). For example, hypoxia can resist heart ischaemia/reperfusion injury (20), inhibit arrhythmia (21) and protect the liver (22). Ambalavanan *et al* have demonstrated the presence of low levels of bFGF in serum under normal conditions, while in the case of hypoxia, the pulmonary vascular endothelium is damaged, which destroys cellular integrity and then bFGF is released (23).

The present study observed that the expression of bFGF in blood peaked at 4 weeks after CIHH pre-treatment and then declined steadily to the same level as that in IDD rats (Table II), which was consistent with the conclusion reported in the literature that bFGF is released after vascular endothelial injury in a hypoxic environment (24). TGF-β1 is a multifunctional growth factor with fibrotic and immunoregulatory properties. TGF-β1 is considered to be an important regulatory factor in COPD and other inflammatory lung

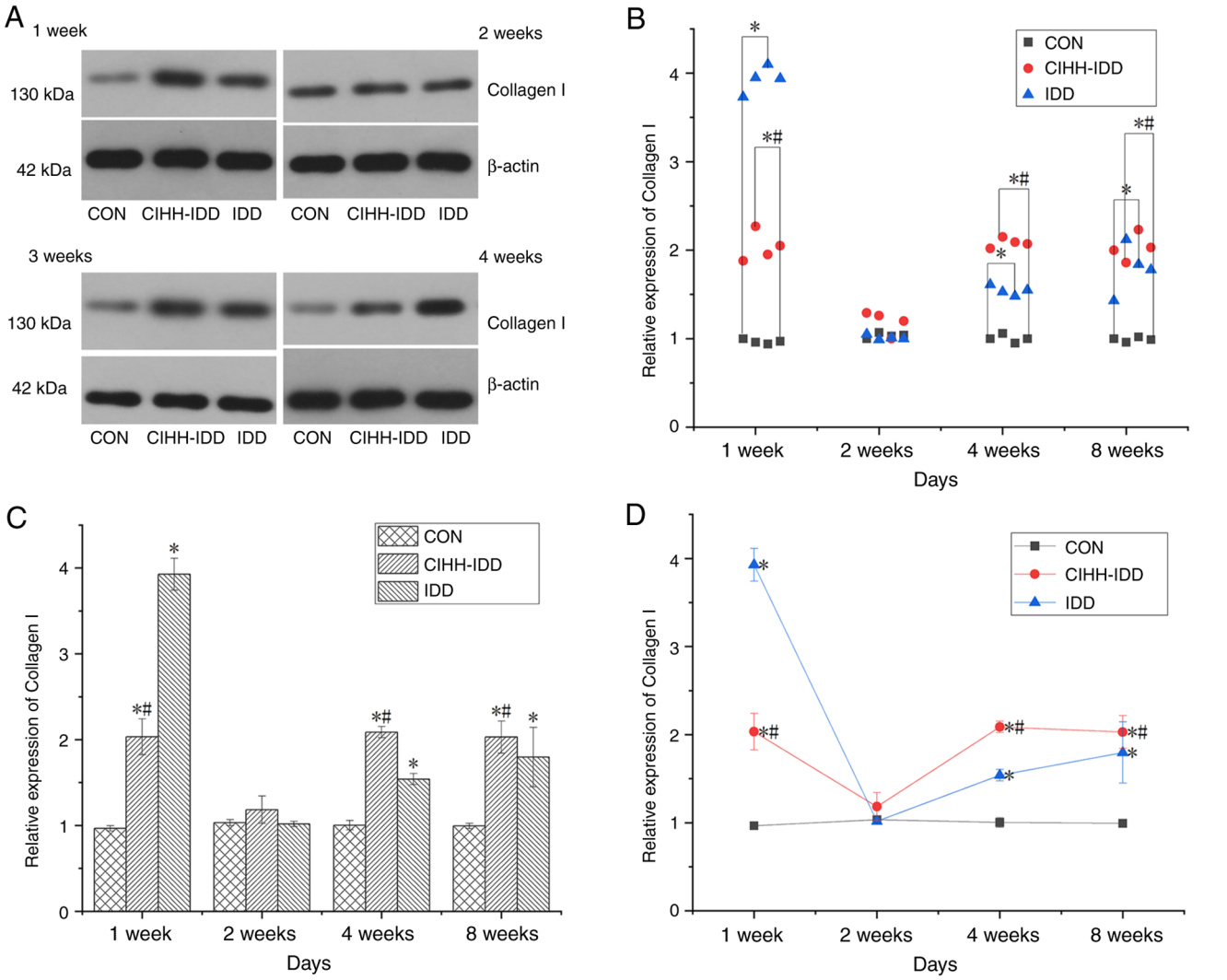

Figure 9. Effect of CIHH on the expression of Collagen I protein in degeneration disc tissue. (A) Western blotting of Collagen I protein at 1, 2, 4 and 8 weeks in the three groups. (B) Western blotting data distribution of Collagen I proteins at 1, 2, 4, and 8 weeks. (C) Collagen I protein expression levels at 1, 2, 4, and 8 weeks in three groups. (D) The expression trend of Collagen I protein. n=16 for each group, n=4 for each time point. *P<0.05 vs. CON group; #P<0.05 vs. IDD group. CIHH, chronic intermittent hypobaric hypoxia; IDD, intervertebral disc degeneration disease group; CON, control.

diseases (25-27). Notably, hypoxia can induce the up-regulation of TGF-β1, platelet-derived growth factor (PDGF) and HIF-1 protein expression in pulmonary artery smooth muscle cells, thereby promoting the occurrence of pulmonary arterial hypertension (28-30). In addition, TGF-β1 and PDGF play important roles in hypoxia-induced lysyl oxidase expression and the promotion of vascular smooth muscle cell growth (31). In the present study, the serum level of TGF-β1 was significantly up-regulated in rats that were pre-treated with CIHH compared with those in the CON and IDD groups. Hypoxia caused an increase in TGF-β1 expression, which played a role in inducing tissue differentiation.

Degeneration of the intervertebral disc first affects the nucleus pulposus through necrosis and apoptosis of nucleus pulposus cells. Then, the synthesis and secretion of extracellular matrix proteins such as proteoglycan is decreased, Collagen II is transformed to Collagen I and the decrease in extracellular matrix changes the microenvironment of the nucleus pulposus, further reducing the number of nucleus pulposus cells and forming a vicious cycle (32).

The role of bFGF in intervertebral disc degeneration and repair is relatively complex, and some studies have suggested that bFGF has dual effects on intervertebral disc degeneration, such as preventing and accelerating disc degeneration (33-35). bFGF, as a powerful mitogen, stimulates the proliferation of capillary endothelial cells and chondrocytes via the ERK and AKT signalling pathways (36). Since there are no blood vessels in mature intervertebral disc tissue, oxygen and nutrients are obtained by the intervertebral disc through penetration of the endplate (37). Therefore, in intervertebral disc development, bFGF is highly expressed in the capillary inner skin, suggesting that it promotes the growth and development of intervertebral discs through blood vessel formation. The present study demonstrated that there was no significant change in the expression of bFGF in the degenerated disc tissues of CIHH-IDD rats compared with those of IDD rats and CON rats at 1 week after surgery.

TGF-β1 induces the differentiation and proliferation of intervertebral disc mesenchymal cells and participates in the repair process in damaged tissue (38). TGF-β1 is present

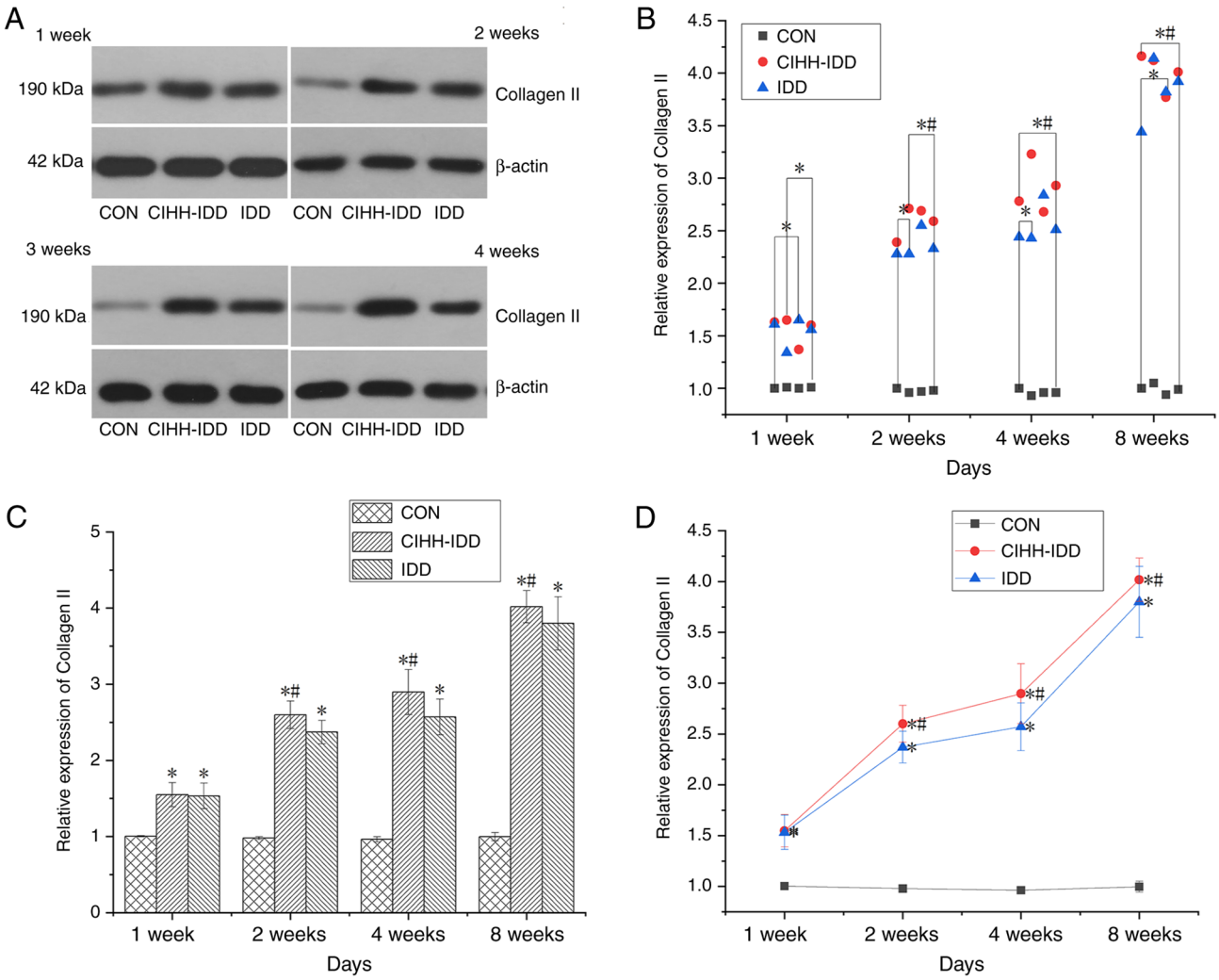

Figure 10. Effect of CIHH on the expression of Collagen II protein in degeneration disc tissue. (A) Western blotting of Collagen II protein at 1, 2, 4 and 8 weeks in the three groups. (B) Western blotting data distribution of Collagen II proteins at 1, 2, 4, and 8 weeks. (C) Collagen II protein expression levels at 1, 2, 4, and 8 weeks in three groups. (D) Expression trend of Collagen II protein. n=16 for each group; n=4 for each time point. *P<0.05 vs. CON group; #P<0.05 vs. IDD group. CIHH, chronic intermittent hypobaric hypoxia; IDD, intervertebral disc degeneration disease group; CON, control.

in normal human disc tissue, and the level of TGF-β1 can increase when disc degeneration occurs. TGF-β1 repairs degenerative discs in the early stages by promoting ECM synthesis (39). The present study observed increased levels of TGF-β1 in rats in the IDD and CIHH-IDD groups at the early stage of postoperative disc degeneration. The expression of TGF-β1 in the IDD group decreased with time, while that in the CIHH-IDD group remained increased. Light microscopy was used to observe that the number of nucleus pulposus cells and amount of ECM in the degenerated intervertebral discs of IDD rats were reduced compared with those of CIHH-IDD rats. In addition, it was observed that with increasing degeneration degrees, the nucleus pulposus decreased, the boundary between the nucleus pulposus and annulus fibrosus became increasingly blurred and the annulus arrangement was disordered and broken. The differentiation and proliferation of degenerated mesenchymal cells were observed in CIHH-IDD rats, and the degree of intervertebral disc degeneration was significantly inhibited.

Walsh *et al* (40) used static pressure to induce disc degeneration, and then injected TGF-β1 and bFGF into intervertebral discs. This study demonstrated that the number of fibre cells, the expression of proteoglycan and collagen II and the height of the intervertebral disc increases in the exogenous growth factor injection group compared with those of the control group (injected with normal saline) (40). Walsh *et al* (40) demonstrated that bFGF can repair the degenerative disc. The present study observed that CIHH-IDD rats still had degeneration after disc injury compared with those in the CON group. However, the present study showed that the degree of intervertebral disc degeneration was significantly reduced compared with that in the IDD group (Fig. 3). These results indicated that CIHH treatment could relieve and repair intervertebral disc degeneration in rats.

The present study observed the effect of CIHH pre-treatment on degenerative discs and obtained positive results, which suggested that CIHH pre-treatment had a preventive effect on IDD in the clinic. However, the therapeutic effect of CIHH pre-treatment on degenerated discs could not be determined because no recovery of disc height was observed. This is a shortcoming of the present study, and further studies need to be performed.

In summary, the present study experimentally demonstrated that CIHH preconditioning had a protective effect

on the degeneration of intervertebral discs in rats. CIHH played a role in repairing and preventing disc degeneration by increasing the expression of related inflammatory factors, such as TGFβ1 and bFGF, in blood and disc tissues.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Hebei Province (Shijiazhuang, China).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PCW conceptualised the study and investigated and analysed the data. SRL, DR, HTW, SQY, ZHS and LDG collected and analysed the data and developed the methodology. PCW and SRL investigation and developed methodology. SRL wrote the manuscript. All authors have read and approved the final manuscript. PCW and SRL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All experiments are in accordance with the guidelines for the Care and Use of Experimental Animals (National Research Committee, 1996), and approved by the Ethics Committee for the use of experimental animals of Hebei Medical University (approval no. Z2019-012-1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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