International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2022.1278.1286

Research Article
Response of Heme Oxygenase-1 in Intervertebral Disc Degeneration by Promoting Autophagy and Apoptosis

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Abstract

Background and Objective: Recent years have witnessed the increasing incidence of Intervertebral disc degeneration (IDD), the basic pathological changes of many spinal degenerative diseases. The purpose of this research was to explore the influence of HO-1 on autophagy of nucleus pulposus cells (NPCs) in IDD rats, to provide a new target for IDD treatment. **Materials and Methods:** About 40 SD rats were randomly divided into four groups: Model, blank, HO-1 and control group. Model group rats were established IDD model only. The blank group was infected with an Adeno-associated virus packaged with an empty vector. HO-1 group rats were infected with HO-1 overexpression Adeno-associated virus vector. Control group rats were fed normally. After modelling, HO-1 mRNA levels, pain threshold and inclined plane test (IPT) score were measured. IL-1β, IL-6, TNF-α, SOD and MDA were detected by ELISA. NPCs were isolated to detect cell multiplication and apoptosis. Beclin-1 and LC-3 in NPCs were determined by western blot. **Results:** The model and the blank group had similar HO-1 mRNA ($p>0.05$), pain threshold and IPT scores were lower than the control group and higher than the HO-1 group ($p<0.05$). IL-1β, IL-6, TNF-α and MDA increased, while SOD decreased ($p<0.05$). *In vitro*, the multiplication capacity of NPCs was highest in the control group ($p<0.05$), Beclin-1 and LC-3 protein expression in blank and model groups were the same ($p<0.05$) but was higher than control and lower than HO-1 group ($p<0.05$). **Conclusion:** Elevation in IDD, HO-1 can exacerbate inflammation and oxidative stress via activating NPC autophagy, which may be the key to future treatment of IDD.

Key words: HO-1, IDD, NPC, autophagy, inflammatory response, ELISA, SOD

Citation: Wang, Y., H. Wang, Z. Sun, H. Sun, U. Ur-Rehman and J. Tian, 2022. Response of heme oxygenase-1 in intervertebral disc degeneration by promoting autophagy and apoptosis. Int. J. Pharmacol., 18: 1278-1286.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Intervertebral disc degeneration (IDD) is the basic pathological change of many spinal degenerative diseases[1].In a modern society with an increasing global ageing and changing lifestyles, cervical spondylosis and lumbar disc herniation incidence are ascending at a high pace[2,3]. At present, more than 20% of the world's middle-aged and elderly people are affected by IDD[4]. Clinically, surgery is still the mainstay of treatment for IDD, which, however, may affect human body structure with serious problems including adjacent segment syndrome, wearing a prosthesis, failure of internal fixation and new surgery because of failed implant[5]. Biological treatment for IDD has attracted the extensive attention of scholars in recent years. The goal of such therapies is to directly act on the pathological changes of IDD at the molecular level and to block, delay or even reverse the process of degeneration[6]. Unfortunately, lack of understanding and data available about the molecular mechanism of IDD makes IDD biological treatment impossible to be implemented effectively[7]. Therefore, in-depth exploration of IDD changes has become a hotspot as well as a conundrum to decipher in the current clinical research.

IDD involves all components of the intervertebral disc, including nucleus pulposus cells (NPCs), annulus fibrosus and endplate[8]. Among these, NPCs are of utmost importance for the development of IDD. It is pointed out that the interference of inflammatory mediators can cause the disorder of extracellular matrix metabolism of NPCs, which greatly reduces the ability of bone metabolism and bone synthesis and promotes the occurrence and development of IDD[9]. Autophagy, as a biological behaviour of cell self-regulating growth, differentiation and metabolic cycle, has also been proved to have obvious changes when IDD occurs[10,11]. Moreover, autophagy has been proposed to antagonize the body's excessive inflammatory response[12]. Therefore, regulating the autophagy of NPCs may be the key to treating IDD in the future.

In previous studies, we found that Heme Oxygenase-1 (HO-1) mediated metabolic disorders of the extracellular matrix and participated in the development of several immune diseases and inflammatory diseases[13,14]. Vasconcellos's research further showed that HO-1 can act on the development of diseases by changing the autophagy capacity of cells[15]. At present, HO-1 is also known to have an abnormal expression in IDD[16], but the exact connection between the two remains uncharacterized. The current study speculated that HO-1 can also affect NPC autophagy and mediate the process of inflammatory response. If this hypothesis holds, it is expected to find a new imbalance mechanism of inflammatory response in IDD and provide a new target for the treatment of IDD. Accordingly, this study verified the above inference through experiments.

MATERIALS AND METHODS

Study area: This study was carried out at the Department of Orthopedic Center, Nanjing Jiangbei Hospital Affiliated with Nantong University, Nanjing, Jiangsu, China from October, 2020-June, 2021.

Animal data: Offered by Beijing Kangchuanglian Biopharmaceutical Technology Research Co., Ltd. with the animal license number SYXK (Jing) 2020-0046, 40 clean SD rats (half male and half female, 6 months old, weighing 280-320 g) were reared at 24±2 and 50±5% humidity.

Modelling method: Thirty rats were randomly selected for IDD modelling and the other 10 rats in the control group were fed normally. Before modelling, all rats were reared adaptively for 3 days, eating and drinking freely. Rats were intraperitoneally injected with anaesthesia and placed in a prone position on the operation table, disinfected with iodophor, Co7/8, Co8/9 and Co9/10 intervertebral spaces were identified and 20 G injection needles were used to carry out annulus fibrous puncture to establish the IDD model.

Encapsulation of HO-1 recombinant Adeno-associated virus: The pAOV-CMV-EGFP Adeno-associated virus vector was subjected to double enzyme digestion and the coding region of HO-1 was cloned and verified by sequencing. Then, the pAOV-CMV-EGFP plasmid or empty vector cloned with HO-1 was packaged with helper plasmid and packaging plasmid, respectively to obtain HO-1 and recombinant Adeno-associated virus without exogenous gene expression. The titers of the virus solution were determined after dilution and those of empty vector and HO-1 recombinant Adeno-associated virus were adjusted to 7×10^{12} gene copies/mL and 5×10^{12} gene copies/mL, respectively.

Intervention treatment: The 30 modelled rats were randomized into three groups: Model group, blank group and HO-1 group. The model group rats only established the IDD model. Empty vector rats were infected with Adeno-associated virus packaged with empty vector 7 days before modelling. HO-1 group rats were infected with HO-1 overexpression Adeno-associated virus vector 7 days before modelling.

HO-1 level detection: Four weeks after modelling, the rat tail vein blood was collected to measure HO-1 expression by PCR. HO-1 sense: 5'-TCTGGAATGGAAGGAGATGC-3', anti-sense: 5'-AGTTCTGGGGCTCTGTTGC-3', GAPDH sense: 5'-AAGGTCATCCATGACAACTTTGGC-3', anti-sense: 5'-ACAGTCTTCTGGGTGGCAGTGAT-3'. Calculation of the relative expression employed $2^{-\Delta\Delta CT}$.

Rat behaviour testing: Rat behaviour tests were performed before (T_0), 4 weeks (T_1) and 8 weeks after (T_2) modelling. Mechanical pain threshold test: The pain threshold of the hindfoot in rats was measured by an electromechanical pain meter. Before measurement, the rat's hind feet were washed and the measuring needle was inserted into the centre of the rat's right hind feet until the rat had evasive behaviour. After the rat dodged, the force on the measuring needle was reduced to 0, which was regarded as a measurement completed. Inclined plane test (IPT): The rats were put into the closed end of the self-made 4-sided inclined wooden channel and the closed end was raised slowly at a constant speed after adaptation for 3 min. The maximum angle of the rat staying for 5 sec without falling was recorded as the score of IPT.

Inflammation and oxidative stress detection: Before and 8 weeks after modelling, 3 mL of rat tail vein blood was extracted into the procoagulant tube, which was then left at room temperature for 30 min and centrifuged to obtain serum, for the identification of inflammatory factors IL-1β, IL-6 and TNF-α and oxidative stress indicators SOD and MDA by ELISA with kits all provided by Jiangxi IBIO Co., Ltd.

Isolation and cultivation of NPCs: Eight weeks later, all rats were killed under anaesthesia. After body surface disinfection and PBS rinsing, the thoracolumbar spine of rats was isolated in an aseptic environment. The gelatinous nucleus pulposus tissue was obtained by cutting the fibrous ring of the aseptic scalpel. After PBS washing, the nucleus pulposus tissue was cut into small pieces, which were moved into an aseptic centrifuge tube, digested with trypsin and centrifuged to get the supernatant. DMEM medium containing 10% FBS and 1% penicillin-streptomycin were added for cultivation at 37 in a 5% CO_2 incubator.

Cytotoxicity test: NPCs in the logarithmic growth phase were inoculated into 48-well plates, which were taken out at 24, 48, 72 and 96 hrs, respectively for the addition of 90 μL fresh culture solution and 10 μL MTT solution. After 4 hrs, the supernatant was removed and the absorbance value (OD 490 nm) was measured.

NPC apoptosis detection: NPCs were digested with pancreatic enzyme and prepared as 1×10^9 cells/L with 1× binding buffer. Then, 100 μL cell suspension was added with 5 μL Annexin V-FITC and PI and the apoptosis of NPCs was detected by flow cytometry.

Protein detection: The NPCs cracked by RIPA were subjected to protein detection by BCA. Then electrophoresis and membrane transfer, as well as 1 hr of blocking with 5% skim milk were performed on the proteins, followed by overnight cultivation with I antibodies. The next day, the II antibody was added to the membrane and rinsed with TBST 3 times for 2 hrs of incubation. After ECL development, the relative expression of the target protein was detected by a gel imager.

Statistical methods: In this experiment, all tests were repeated three times and the statistics and drawing software was GraphPad Prism 9. Statistical calculations include independent samples t-test, one-way ANOVA and LSD *post hoc* test and the differences were deemed significant when $p<0.05$.

RESULTS

Comparison of HO-1 expression: First, the level of HO-1 mRNA in rats was detected by PCR and the results were shown in Fig. 1. The HO-1 mRNA of the control group was the lowest among the 4 groups of rats ($p<0.05$). The HO-1 mRNA of the model group and the blank group showed no difference ($p<0.05$), while the HO-1 mRNA in the HO-1 group was the highest among the 4 groups ($p<0.05$).

Comparison of behavioural results: The behavioural test results of rats were shown in Table 1. The pain thresholds at T_1 and T_2 and IPT were the highest among the 4 groups ($p<0.05$). In the model group, there was no difference in pain thresholds compared with the blank group ($p>0.05$). In the HO-1 group, the pain thresholds and IPT at T_1 and T_2 were the lowest among the 4 groups ($p<0.05$). The pain threshold and IPT of the control group did not change significantly at T_0, T_1 and T_2 ($p>0.05$), while the pain threshold and IPT of the other three groups showed a gradual decrease ($p<0.05$).

Comparison of inflammatory factors: ELISA test results showed that the IL-1β of the control group before modelling had no difference compared with after modelling ($p>0.05$), while the levels of IL-1 β in the HO-1 group, model group and blank group before modelling were lower than those after modelling ($p<0.05$, Fig. 2a). The IL-6 of the control group

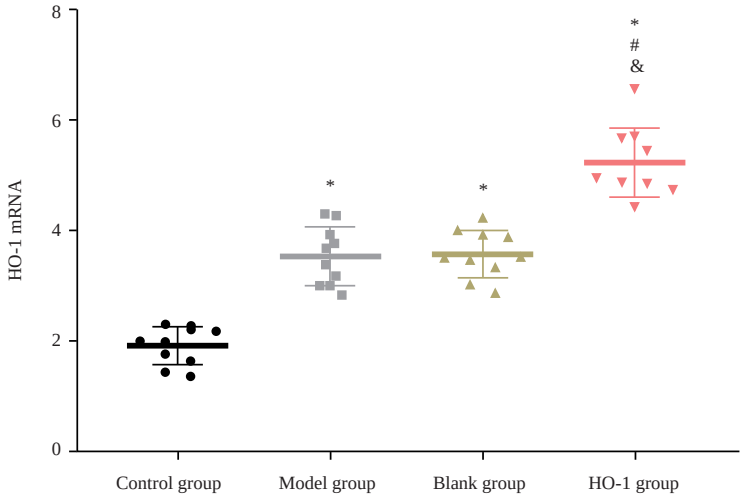

Fig. 1: Comparison of HO-1 expression

*vs control group, #vs model group &vs blank group and p<0.05

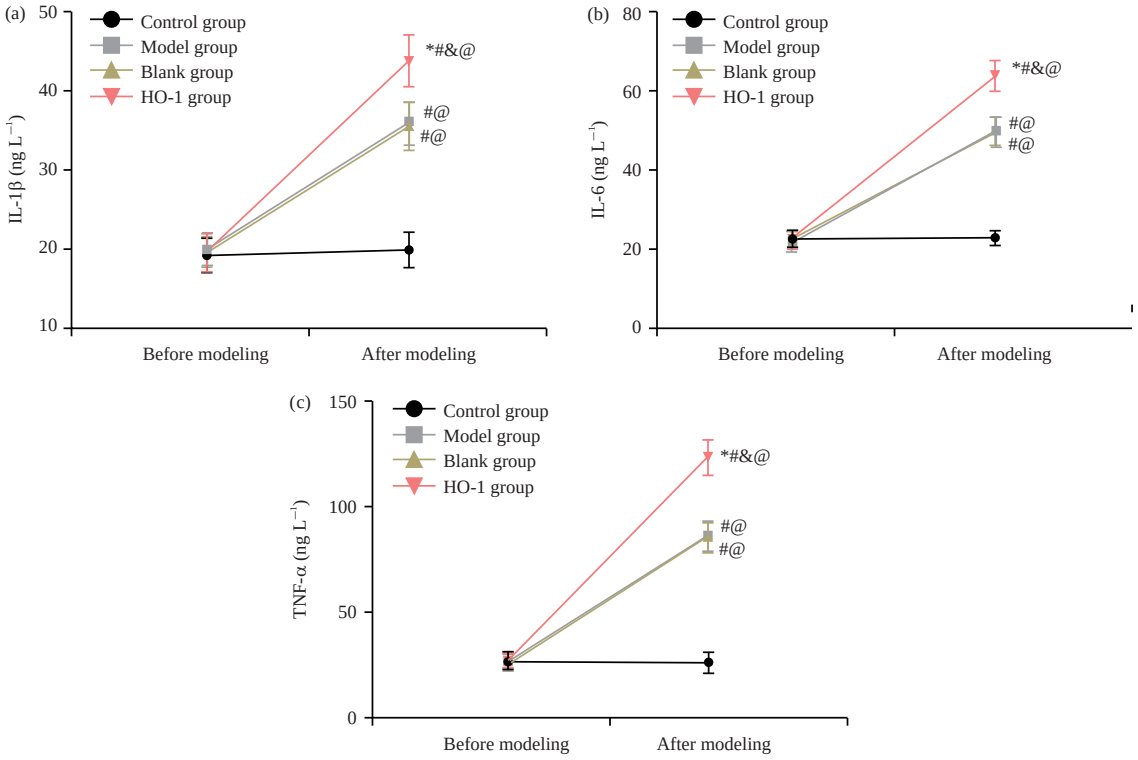

Fig. 2(a-c): ELISA was used to detect the levels of inflammatory factors in each group of rats, (a) Comparing IL-1β levels, the control group had no difference before and after modelling, while the HO-1 group, model group and blank group increased after modelling, the model group had no difference from the blank group, which was higher than the control group but lower than the HO-1 group, (b) Comparing IL-6 levels, the control group had no difference before and after modelling, while the HO-1 group, model group and blank group increased after modelling, the model group had no difference from the blank group, which was higher than the control group but lower than the HO-1 group and (c) Compared with the level of TNF-α, the control group had no difference before and after modelling, while the HO-1 group, model group and blank group increased after modelling, there was no difference between the model group and the blank group, which was higher than the control group but lower than the HO-1 group

*vs control group, #vs model group, &vs blank group, @vs before modelling in the same group and p<0.05

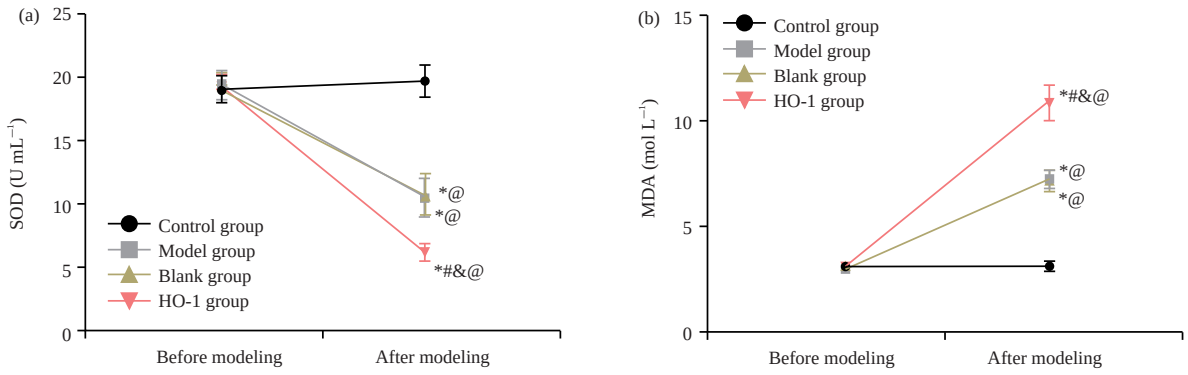

Fig. 3(a-b): Comparison of oxidative stress responses, (a) Comparison of SOD levels and (b) Comparison of MDA levels
*vs control group, #vs model group, &vs vector group, @vs before modelling in the same group and p<0.05

Table 1: Comparison of behavioral results

	Pain threshold			IPT score		
	T_0	T_1	T_2	T_0	T_1	T_2
Control group	39.59±0.88	40.08±0.94	39.84±0.87	47.81±0.82	49.04±0.93	49.01±0.86
Model group	39.42±0.92	36.28±1.07$^{*@}$	33.16±1.08$^{*@\wedge}$	48.06±0.94	45.14±0.82$^{*@}$	40.81±1.08$^{*@\wedge}$
Blank group	39.43±0.95	36.14±1.14$^{*@}$	33.81±0.94$^{*@\wedge}$	47.94±0.86	45.81±0.69$^{*@}$	40.24±1.24$^{*@\wedge}$
HO-1 group	39.42±0.87	32.81±1.14$^{*\#\&@}$	27.93±1.07$^{*\#\&@\wedge}$	47.93±0.94	42.14±1.06$^{*\#\&@}$	36.14±1.14$^{*\#\&@\wedge}$
F	0.085	76.330	240.50	0.131	102.20	245.13
P	0.968	<0.001	<0.001	0.941	<0.001	<0.001

*vs control group, #vs model group &vs blank group, @vs at T_0 in the same group and ^vs at T_1 in the same group

before modelling showed no difference after modelling (p>0.05), while the levels of IL-6 in the HO-1 group, model group and blank group before modelling were lower than those after modelling (p<0.05, Fig. 2b). The TNF-α of the control group before modelling showed no difference after modelling (p>0.05), while the levels of TNF-α in the HO-1 group, model group and blank group before modelling were lower than those after modelling (p<0.05, Fig. 2c). Before modelling, there was no difference in inflammatory factors in the four groups of rats (p>0.05), but after modelling, there was no difference between the model group and the blank group (p>0.05), which was higher than the control group but lower than the HO-1 group (p<0.05)). It can be seen that HO-1 can promote the inflammatory response in IDD rats.

Comparison of oxidative stress responses: ELISA test results showed that the SOD of the control group before modelling had no difference compared with after modelling (p>0.05), while the levels of SOD in the HO-1 group, model group and blank group before modelling were higher than those after modelling (p<0.05, Fig. 3a). The MDA of the control group before modelling had no difference after modelling (p>0.05), while the levels of MDA in the HO-1 group, model group and blank group before modelling were lower than those after modelling (p<0.05, Fig. 3b). Before modelling, there was no difference in SOD and MDA of the 4 groups of rats (p>0.05), but after modelling, there was no difference between the model group and the blank group (p>0.05). SOD was lower than the control group but higher than the HO-1 group and MDA was higher than the control group but lower than the HO-1 group (p<0.05). It can be seen that HO-1 can aggravate the oxidative stress response in IDD model rats.

Biological behaviour changes of NPCs: The MTT experiment showed that the cell absorbance value at 96 hrs in the control group was the highest among the 4 groups (p<0.05). The cell absorbance value at 96 hrs in the model group was the same as in the blank group. There was no difference (p>0.05), while the 96 hrs cell absorbance value of the HO-1 group was the lowest among the 4 groups (p<0.05, Fig. 4a). The results of flow cytometry showed that the apoptosis rate in the control group was the lowest among the 4 groups (p<0.05) and the apoptosis rate in the model group was the same as the blank group. There was no difference (p>0.05), while the apoptotic rate of the HO-1 group was the highest among the 4 groups (p<0.05, Fig. 4b). This shows that HO-1 can inhibit the proliferation of NPC and accelerate apoptosis.

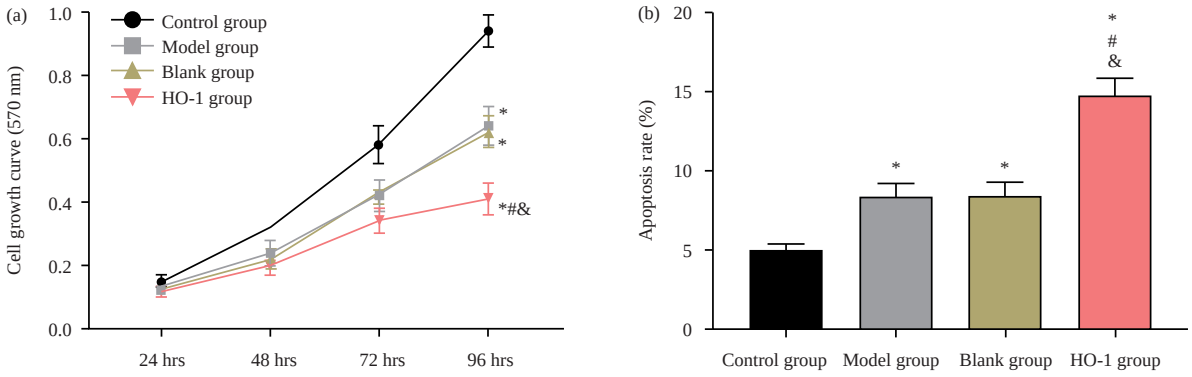

Fig. 4(a-b): Comparison of biological behaviour changes of NPCs, (a) NPC growth curve and (b) Apoptosis rate of NPCs
*vs control group, #vs model group, &vs vector group and p<0.05

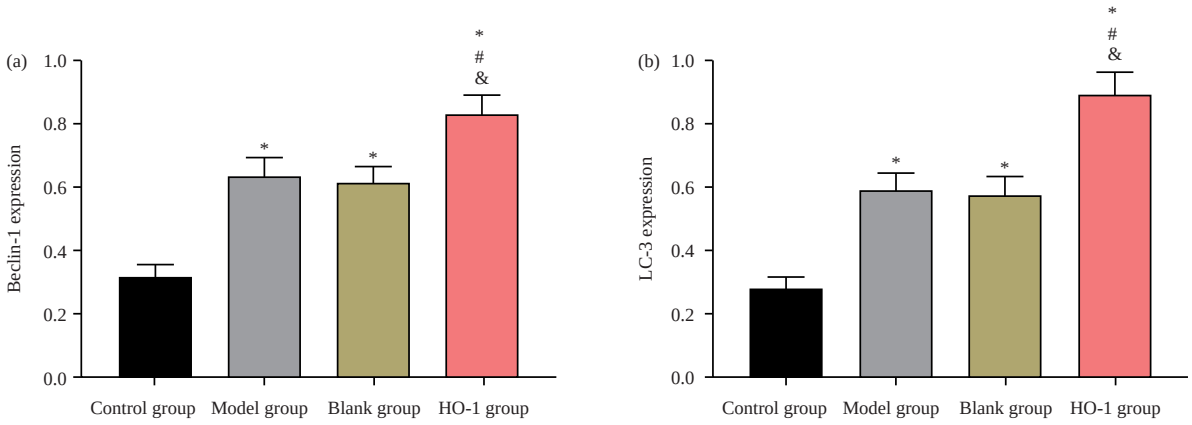

Fig. 5(a-b): Comparison of NPC protein expression, (a) Comparison of Beclin-1 protein expression and (b) Comparison of LC-3 protein expression
*vs control group, #vs model group, &vs vector group and p<0.05

Comparison of NPC protein expression: Western blot results showed that the Beclin-1 protein expression in the control group was the lowest among the 4 groups ($p<0.05$). The expression of Beclin-1 protein in the model group showed no difference compared with the blank group ($p>0.05$), while the expression of Beclin-1 protein in the HO-1 group was the highest in the group ($p<0.05$, Fig. 5a). The expression of LC-3 protein in the control group was the lowest among the 4 groups ($p<0.05$). The expression of LC-3 protein in the model group showed no difference compared with the blank group (0.58 ± 0.06) ($p>0.05$), while the expression of LC-3 protein in the HO-1 group was the highest among the 4 groups ($p<0.05$, Fig. 5b). It can be seen that HO-1 can promote the autophagy ability of NPC.

DISCUSSION

Often accompanied by lower back pain, acute lower limb radiculopathy and other adverse symptoms, IDD has extremely complex pathogenesis, which seriously affects the normal life of patients[17]. At present, it is clinically believed that the occurrence of IDD has a close connection with inflammatory response, oxidative stress and NPC degeneration[18-20]. Therefore, a full understanding of its pathogenesis and changing the process of inflammatory response and NPC degeneration through a molecular perspective may be the breakthrough for the future treatment of IDD[21]. The concept of this study to explore the influence of HO-1 on NPC autophagy is of great clinical implications.

In this experiment, HO-1 levels in the four groups of rats were first detected and the results identified noticeably higher HO-1 levels in the model group versus the control group, suggesting that HO-1 may be involved in the occurrence and development of IDD. This was also consistent with previous literature, which can support our experimental results[22,23]. The increase of HO-1 level in rats infected with HO-1 expression lentiviral vector fully indicated the successful construction of the lentiviral vector and the initial success of targeted

regulation. Subsequently, the behaviour of four groups of rats was tested and found that under HO-1 infection, the pain threshold and the IPT score of the HO-1 group were higher than those of the blank group and model group, suggesting that the increase of HO-1 can effectively improve the condition of IDD. However, the exact mechanism remains to be explored. As we all know, inflammatory response and oxidative stress reaction are the keys to IDD[24,25]. Therefore, the inflammatory factors IL-1β, IL-6, TNF-α and oxidative stress indicators SOD and MDA in four groups of rats were detected. The results showed that IL-1β, IL-6, TNF-α and MDA in the HO-1 group were all increased, while SOD was decreased, indicating that HO-1 can activate the inflammatory response and oxidative stress response of IDD. This may also be the preliminary mechanism for HO-1 to participate in IDD. As classic inflammatory factors, IL-1β, IL-6 and TNF-α are extremely sensitive to inflammation[26]. In previous studies, it was found that HO-1 can regulate NLRP-3 corpuscles in acute liver injury[27]. NLRP-3 is recognized as the key factor mediating inflammatory response[28], so this may also be the way that HO-1 affects inflammatory cytokines. SOD and MDA, as the most effective indicators of oxidative stress reaction, are also the most common clinical observation indicators[29]. Due to the reduction of the metabolic capacity of spinal cells in the process of IDD, the original intact and compact intervertebral disc tissue will be damaged, which is very easy to produce a variety of ischemic and hypoxic injuries caused by insufficient cell function, resulting in a significant aggravation of intervertebral disc oxidation reaction[30]. Referring to previous studies, we also found that HO-1 had a very significant regulatory effect on the autophagy of kidney cells[31]. Autophagy, as a necessary way for cells to maintain internal environment stability after stimulation, enhances the reuse of nutrients by removing damaged or ageing proteins, organelles or pathogens, thereby promoting cell survival and reducing excessive apoptosis[32]. In IDD, autophagy is also an extremely important pathological development process[33]. First of all, NPCs were isolated from four groups of rats and conducted biological behaviour detection. It was found that the multiplication capacity of NPCs in IDD model rats decreased notably, while the apoptosis rate increased, indicating that IDD can cause accelerated apoptosis of NPCs. Moreover, the decrease in multiplication ability and the increase of apoptosis of NPCs in the HO-1 group were more significant, which indicated that the increase of HO-1 could further promote and induce the apoptosis of NPCs. The detection of autophagy proteins Beclin-1 and LC-3 in each group further showed that Beclin-1 and LC-3 in NPCs of the HO-1 group increased obviously, which suggested that this group had the strongest autophagy ability. Combined with the above experimental results, we can preliminarily know that HO-1 mediates inflammatory response and oxidative stress response of IDD by regulating the autophagy of NPCs.

However, there are still many aspects to be improved in this study. For example, the way of HO-1 regulates NPC autophagy is still unclear. In addition, due to certain differences between human body structure and animals, there may be some discrepancies in the influence of HO-1 on NPC autophagy in human experiments. Moreover, more experiments are needed to confirm the application of HO-1 targeted therapy for IDD, which is also the direction and focus of our follow-up research.

CONCLUSION

HO-1 is elevated in IDD and can aggravate inflammation and oxidative stress by activating autophagy in NPCs, which may be the key to the future treatment of IDD.

SIGNIFICANT STATEMENT

In this study, it was found that HO-1 can activate NPC autophagy, promote cell apoptosis and at the same time aggravate inflammation and oxidative stress to promote the development of IDD. This reminds researchers that in the future, by inhibiting the expression of HO-1, the effect of treating IDD may be achieved. This may be a breakthrough for IDD with a high incidence and disability rate and lack of clinical treatment methods.

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