Biomedicine & Pharmacotherapy 101 (2018) 745–753

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Halofuginone attenuates intervertebral discs degeneration by suppressing collagen I production and inactivating TGFβ and NF-κB pathway

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ARTICLE INFO

Keywords:
Halofuginone
Intervertebral discs degeneration
Collagen I
NF-κB
TGFβ

ABSTRACT

Most low back pain is caused by intervertebral discs (IVD) degeneration, a disease that prevalence is increasing with age. Halofuginone, an analog of ferbrifugine isolated from plant *Dichroa febrifuga*, has drawn much attention in recent years for the wide range of bioactivities in malaria, cancer, fibrotic and autoimmune diseases. In this study, we evaluated the benefit effects of halofuginone in IVD degeneration treatment in a validated rabbit puncture model. Halofuginone treatment could attenuate disc degeneration by suppressing the decrease of discs height and nucleus pulposus signal strength. Besides, halofuginone treatment could suppress mRNA and protein expression of collagen I in nucleus pulposus. This might possibly due to the inactivation of transform growth factor-β (TGFβ) signal pathway by down-regulating p-Samd3 and up-regulating inhibitory Smad7. Then, we evaluated the effects of halofuginone treatment on nuclear factor of kappa B (NF-κB) signal pathway and its downstream pro-inflammatory cytokines. The level of p-p65 and p-IκBα was down-regulated in halofuginone treated group, indicating the inactivation of NF-κB signal pathway. The mRNA expression of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 8 (IL-8) was decreased in nucleus pulposus too, indicating the down-regulation of pro-inflammatory cytokines. In conclusion, halofuginone treatment could attenuate IVD degeneration and this was possibly due to suppressing of collagen I production and inactivation of TGFβ and NF-κB signal pathway in nucleus pulposus of degenerated discs. These results suggest that halofuginone has the potential for IVD degeneration treatment, but more research is needed to validate this.

1. Introduction

Low back pain is the main cause of disability in developed countries. It is estimated that nearly 84% people suffers from low back pain during their lifetime [1]. Intervertebral discs (IVD) degeneration is the main cause of low back pain [2]. Nowadays, novel strategies such as gene therapy, cell-based therapy, tissue engineering and growth factor injection are developed to treat IVD degeneration, but much more efforts are needed to achieve full IVD regeneration [3]. The IVD is composed by three interdependent and structurally distinct regions: nucleus pulpous (NP), annulus fibrosus (AF) and cartilaginous endplates (CEPs). Nucleus pulpous, the core region of IVD, is surrounded by lamellas of annulus fibrosus and outermost layer of cartilaginous endplates. The extracellular matrix (ECM) of IVD is normally composed by collagens, proteoglycans, elastin and glycoproteins. These molecules play a central role in maintaining normal functions of IVD. Collagens comprise about 70% dry weight of IVD and their concentration decreases from AF to NP [4]. Collagen fibre provide a strong and mechanically resilient network to support the discs cells. Collagen I and collagen II are dominating collagens in mature discs, which make up to 80% of the total amount [5]. Their distributions in the discs are reciprocal. Collagen I dominates in the outer AF and Collagen II dominates in the inner AF and NP [6]. This arrangement is important to maintain the normal function of mature discs. During the progression of IVD degeneration, there is a progressive decrease in the expression of collagen II with increased expression of collagen I [7–10]. This ultimately leads to a shift from predominately collagen II to collagen I in NP, resulting in a loss of water-binding potential [11].

Abbreviations: IVD, intervertebral discs; ECM, extracellular matrix; TGFβ, transform growth factor-β; NF-κB, nuclear factor of kappa b; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; NP, nucleus pulpous; AF, annulus fibrosus; CEPs, cartilaginous endplates; DHI, discs height index; IHC, immunohistochemistry; TNF-α, tumor necrosis factor α

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https://doi.org/10.1016/j.biopha.2018.01.100
Received 24 October 2017; Received in revised form 22 January 2018; Accepted 24 January 2018

Inflammation plays an important role in the progression of IVD degeneration and is possibly related to low back pain. IVD degeneration normally starts with decreasing of nutrition cells in central IVD and followed by accumulation of cell wastes and degraded matrix molecules [12]. It remains unclear that trigger the recruitment of immune cells to IVD and inflammatory response, but one hypothesis is that ECM breakdown products may induce inflammatory response as demonstrated in various models [13–15]. NF-κB is the core component of cellular response to damage, stress and inflammation. It consists of five subunit: RelA or p65, c-Rel, RelB, p50 and p52. NF-κB exists as homodimer or heterodimer. The heterodimer of p50-p65 is most abundant and controls the majority of NF-κB regulated genes. There are more than 150 genes regulated by NF-κB including proinflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8, cyclooxygenase-2 (COX-2), MMPs and adhesion molecules [16]. In degenerated IVD, there is increasing levels of TNF-α, IL-1β, IL-6 and IL-8 [17–19]. Immunohistochemical studies have demonstrated the activation of NF-κB signal pathway in human IVD degeneration *in vivo*, especially in NP tissue [20].

Halofuginone (HF) is an analogue of febrifugine isolated from plant *Dichroa febrifuga*. In recent years, halofuginone has attracted much attention for its wide range of biological activities in malaria, cancer, fibrosis-related and autoimmune diseases [21–23]. Halofuginone has been shown to attenuate osteoarthritis by suppressing TGF-beta activity [24]. Fibrosis is characterized by high levels of ECM proteins especially collagen I. Halofuginone is shown to elicit resolution of pre-existing fibrosis possibly by reducing collagen I synthesis and increasing collagenase activity [25,26]. Halofuginone also plays a role in inflammation and autoimmune diseases by selecting inhibition of CD4 + T helper cell subset, Th17 [27].

IVD degeneration is correlated with deregulation of ECM components and inflammatory response, so we speculated that halofuginone might have some beneficial effects on IVD degeneration. Therefore, a rabbit IVD degeneration model was established to evaluate the potential of halofuginone in treatment of IVD degeneration in this study.

2. Materials and methods

2.1. Animal grouping and treatment

The experimental protocol was approved by Animal Care and Experimental Committee of Huazhong University of Science and Technology. Thirty healthy female New Zealand white rabbits (3.0 ± 0.3 kg) were randomly divided into Sham group, IVDD group and HF group on average. Animals of Sham group underwent spinal surgery without annulus fibrosus puncture. The rest twenty animals that underwent annulus fibrosus puncture were randomly divided into two group: IVDD group and HF group. The animals of HF group received an oral halofuginone treatment of 1 mg/kg daily. The Sham group and IVDD group received an equal volume of saline at the same time. Half animals of each group were sacrificed at 4 weeks post-operation and the rest animals were sacrificed at 8 weeks post-operation. The lumbar spines were harvested and stored at −80 °C.

2.2. Spinal surgery

A validated rabbit annular puncture model was used to induce disk degeneration [45]. Before spinal surgery, animals were anaesthetized with xylazine (5 mg/kg) and ketamine hydrochloride (35 mg/kg) by subcutaneous injection. Under general anesthesia, the rabbits' spine were exposed from an anterolateral retroperitoneal approach. A 16-gauge hypodermic needle was used to puncture the L2-L3, L3-L4, L4-L5 discs at a depth of 5 mm. The L5-L6 discs were left undisturbed as an internal control discs. The surgical incisions were closed routinely. All animals were injected intramuscularly with 800,000 U penicillin after surgery. Animals were kept in separated cages with free activity, food and water.

2.3. Magnetic resonance imaging (MRI)

All animals underwent sagittal T2WI MRI examination pre-operation, 4 and 8 weeks post-operation. The L2-L3, L3-L4, L4-L5 and L5-L6 discs were evaluated. A3-T Siemens magnet and standard human knee coil were used to obtain T1-(repetition time = 650 ms, echo time = 14 ms, slice thickness = 0.6 mm) and T2-weighted images (repetition time = 3800 ms, echo time = 114 ms, slice thickness = 0.6 mm). The rabbits were sedated and placed in the knee coil in supine position. The T1-weighted images were used to evaluate the bone abnormalities in spine. The T2-weighted images were used to evaluate the amount of degeneration in the discs. The discs morphologic change were graded into five categories according to the methods of Pfirrmann et al [46].

2.4. Radiographic analysis

Discs height of the lumbar spine was evaluated by lateral plain digital radiographs (DR; Simens, Erlangen, Germany) pre-operation, 4 and 8 weeks post-operation. The lateral discs height (DH), upper vertebral height (UB), lower vertebral height (LB), discs height index (DHI) and %DHI (post-operation DHI/ pre-operation DHI × 100%) were analyzed from images according to a previous method [47].

2.5. Immunohistochemistry (IHC)

Immunohistochemistry was performed as described previously [48]. Briefly, IVD samples were fixed in formalin, embedded in paraffin and sectioned at 5 um thick. Sections were stained with hematoxylin and eosin (H&E) for histological changes. Antigen retrieval were done in citrate buffer and washed by PBS. Tissue sections were blocked with 10% goat serum (Sigma, St. Louis, MO, USA) for 1 h at room temperature and incubated with primary antibody overnight at 4 °C. The anti-rabbit second antibody was diluted (1:300) and incubated at room temperature for 1 h. All slides were incubated in avidin biotin peroxidase complex (Sigma, St. Louis, MO, USA) diluted 1:300 in PBS for 30min at 37 °C. Rabbit polyclonal collagen I (1:100 dilution; Boster, Wuhan, China) were used to evaluate the changes in collagen I in the discs.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from nucleus pulposus of IVD samples using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The quality and quantity of extracted RNA was measured by Nano Drop ND-2000 spectrophotometer. Complementary DNA was amplified using ReverTra Ace kit (Toyobo, Japan) and quantified using a standard SYBR-Green PCR kit protocol (Takara, Japan) in ABI 7900 Real Time PCR system. All samples were done in triplicate and normalized to GAPDH, the relative expression levels were calculated by the equation $2^{-\Delta\Delta CT}$. The primers for qRT-PCR were list in Table 1.

2.7. Western blot analysis (WB)

Nucleus pulposus tissues from IVD samples were lysed in RIPA buffer containing protease inhibitors (Sigma-Aldrich, Carlsbad, CA, USA). Protein concentration was quantified using BCA protein assay kit (Thermo Scientific, Grand Island, NY, USA). A same amounts of protein was electrophoresed by 10% SDS-PAGE and transferred onto nitrocellulose membranes, then incubated with specific first antibodies and corresponding second antibodies. The specific first antibodies were list as follows: Anti-Collagen I antibody [COL-1] (SantaCruz#ab6308); anti-phospho-Smad3 antibody (R&D Systems, Minneapolis, MN); Smad3 (Zymed Laboratories, San Francisco, CA); anti-Smad7 antibody