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Prevention of articular cartilage degeneration in a rat model of monosodium iodoacetate induced osteoarthritis by oral treatment with Withaferin A

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ABSTRACT

Withaferin A (WFA), a highly oxygenated withanolide is used for anti-osteoporotic, fracture healing, obesity control as medicine and dietary supplement in Ayurveda and Unani medicine but its potential remains to be investigate for the osteoarthritis studies. In the present study, chondro-protective effects of WFA, under *in vitro* and *in vivo* conditions were evaluated. *In-vitro* pharmacological activity of WFA was tested on rat articular chondrocytes through MTT, DPPH, different staining, FACS and translation studies. *In-vivo* studies of WFA were evaluated through monosodium iodoacetate (MIA) induced osteoarthritis studies. DPPH assay, alcian blue and toluidine blue staining indicated the chondrogenic potential of WFA. Similarly, WFA enhance chondrogenesis through up-regulation of SOX9 protein. In addition, WFA reduced the ROS generation, mitochondrial depolarization and apoptosis induced by inflammatory cytokines IL-1β and TNF-α. Furthermore, WFA treatment in MIA treated rats alleviated cartilage erosion and improvement in sub-chondral bone micro-architecture by decrease in Tissue volume (~32%), and trabecular bone pattern factor (~28%). Taken together, our study provides convincing evidence for the candidature of WFA (10 mg kg^{-1} day^{-1}) as a potential agent for the treatment of cartilage degenerative diseases like osteoarthritis.

1. Introduction

Articular cartilage is an important structural part of the body, which is mostly present between joints helps to reduce friction by acting as a cushion during running, bending, and stretching of body. Cartilage tissue is predominantly composed of chondrocytes and extracellular matrix of proteoglycan and collagen fibres [1–4]. This tissue is primary target of inflammatory cytokines (such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α)) and free radicals, (such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)), induced development and progression of osteoarthritis (OA) like disease [5,6]. Moreover, it is well known that cartilage tissues are devoid of or possess lesser number of blood vessels, lymphatic and nerves in comparison to other tissues of the body and thus exhibit inadequate capacity for intrinsic healing and repair [2]. Therefore, proper preservation and protection of the cartilage tissue are paramount to leading a healthy life.

In this direction, phyto-pharmacological products are nowadays attracting increased interest due to their important role in maintaining and improving skeletal health when consumed as part of nutritional supplementation and lack of adverse effects [7–12]. *Withania somnifera* (Ashwagandha, Winter Cherry) is a plant used in medicine as well as dietary supplement for various disorders in India from the time of Ayurvedic and Unani systems of medicine. The beauty of this drug is that it works in various disease conditions by numerous pathways as it kills cells in cancer by increasing inflammation and other side it reduce inflammation in rheumatism, neurological and cardiovascular disorders. Furthermore it is also used in anti-helminthic, diuretic, anti-ulcer, oligospermia, insomnia, nervous breakdown, obesity, osteoporosis, fracture healing and many more [13–20]. *Withania somnifera* different parts extract are widely used for anti-arthritic activity [21–24] but effect of Withaferin A (WFA), a highly oxygenated withanolide, the most abundant constituent and the major biologically active constituent

Abbreviations: WFA, Withania somnifera; RAC, rat articular chondrocytes cells; AB, alcian blue; GAGs, glycosaminoglycans; MIA, monosodium iodoacetate; OA, osteoarthritis; IL-1β, interleukin1β; TNF-α, tumour necrosis factor-α; ROS, reactive oxygen species; RNS, reactive nitrogen species; BV/TV, trabecular bone volume fraction; Tb.N, trabecular number; TB, toluidine blue; RAC, rat articular chondrocyte

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of this herb remains to study in monosodium iodoacetate (MIA) induced osteoarthritis. A previous study form our laboratory showed WFA to exhibit fractures healing potential and osteogenic efficacy in rodents [15]. Therefore, the present study was hypothesized to investigate the effect of WFA in cartilage degenerative disease at articular cartilage tissue region with an objective to search and develop novel therapeutic alternatives for the prevention and management of chondrocytes related disorders.

2. Materials and methods

2.1. Reagents and chemicals

Cell culture media and supplements such as Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media, Fetal bovine serum (FBS), antibiotic cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Alcian Blue, Dimethylmethylene blue (DMMB), 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), Alizarin Red were purchased from Sigma-Aldrich (St. Louis, MO, USA). tetraethylbenzimidazolylcarbocyanine iodide (JC-1), RNase/PI solution, 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA) and Griess reagent were purchased from Invitrogen (Carlsbad, CA, USA). Complementary DNA (cDNA) synthesis kit was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and SYBR Green kit was procured from Genetix (New Delhi, India). IL-1β and TNF-α were purchased from Pepro-tech (Rocky hills, NJ, USA). Withaferin A (purity ≥ 90%) from *Withania somnifera*, was received as a gift from Pharmanza Pvt. Ltd, Gujarat, India (Supp. Data 1, 2).

2.2. Animal ethical clearance

All experiments were performed in accordance with IAEC (Institutional Animal Ethical Committee, New Delhi, ref. no. IAEC/2013) guidelines and experimental protocols were approved by CSIR-Central Drug Research Institute, Lucknow.

2.3. Isolation and culture of rat articular chondrocyte (RAC) cells

Chondrocyte cells were isolated by enzymatic digestion from articular cartilage of one day old rat pups. In brief, chopped pieces of isolated articular cartilage were digested with trypsin (0.25 mg/mL) for 30 min and then with 2 mg/mL of collagenase in DMEM/F12 medium for 12 h at 37 °C followed by passing through a 70 μm filter. After achieving 70%–80% confluence, articular chondrocyte cells were trypsinized and seeded in respective plate. Afterwards, cells were cultured in DMEM/F12 medium supplemented with 15% BSA and antibiotics under static conditions in a humidified atmosphere with 5% CO_2 at 37 °C (Binder CO_2 incubator). After 48 h, medium was supplemented with GFs (1ng/mL bFGF + 1ng/mL TGF-β2) + 1.0% ITS (Insulin-transferrin-selenium) + 2.0% FBS for chondrocytes differentiation [25]. Medium was changed every alternative day

2.4. 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) assay

Antioxidant activity of WFA was monitored in the presence of DPPH. The oxidized DPPH molecule is reduced after encountering antioxidant molecule and this oxidation of DPPH can be monitored spectrophotometrically by reading the optical density (OD) of DPPH at 517 nm under a plate reader (Molecular Devices, USA) [26].

2.5. Cell cytotoxicity assay

The toxicity of the tested agents was done on rat articular chondrocyte (RAC) cells. Cells were seeded in 96 well plates at 2000 cells/well in growth medium. Cells were treated with different concentration of the test compound in differentiation medium for 48 h. The cell viability were determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. OD was taken 570 nm by spectrophotometer (Molecular devices, USA) [27].

2.6. Staining of RAC cells

After 14 days of WFA treatment in differentiation medium, RAC (1×10^4 cells/well) cells were fixed with methanol for 30 min at room temperature and stained separately with alcian blue (AB), toluidine blue (TB) and alizarin red-O (AR) dye, respectively. For AB staining, fixed cells were incubated in a readymade solution of alcian blue (Sigma-Aldrich, B8438) for 30 min before removing extra dye by washing with distilled water. TB dye was prepared by dissolving 1.0% toluidine in 70% ethanol solution with 1.0% solution of sodium chloride and pH was set at 2–2.5 using glacial acetic acid. In brief, cells were harvested and washed with PBS and fixed before incubating in1.0% toluidine blue for 30 min. After staining, the plates were with running distilled water until the water dripping from wells became clear. For AR staining, fixed cells were incubated with 40 mM Alizarin-red stain (pH 4.5) for 30 min followed by washing with tap water. Finally, all the cells samples stained with different dyes were analysed separately under microscope (EVOS XL digital inverted microscope, USA) and spectrophotometer (Molecular Devices, USA) [28,29].

2.7. Determination of glycosaminoglycans (GAGs)

The levels of GAGs in different samples were measured by spectrophotometry using a solution of dimethyl methylene blue (DMMB) based on the method described previously [30]. DMMB assay reagent was prepared by adding 16 mg DMMB in 1 L distilled water containing 2.37 g NaCl, 3.04 g glycine and 95 mL HCl. GAGs were estimated by mixing 100 μL sample of 14 days WFA treated differentiation medium with 900 μL DMMB and immediately absorbance values were measured at 535 nm. The amount of GAGs present in the sample was calculated from a standard curve of GAGs reference prepared using chondroitin sulfate sodium salt [31].

2.8. Analysis of apoptosis by flow-cytometry

RAC (1×10^6cells/T25 flasks) cells were grown in differentiation media for 24 h before treatment with four different groups (IL-1β and TNF-α, with and without WFA) along with control for next 24 h. At the end of treatment, cells were harvested by trypsinization and stained with Annexin-V/PI solution (100 μg/mL) for 10 min as per the protocol mentioned in Annexin-V/PI apoptosis detection kit (Invitrogen, USA) before analysis under flow cytometer using appropriate excitation and emission filters [32].

2.9. Mitochondrial membrane potential (MMP) analysis

MMPs of RAC cells (1×10^6cells/T25 flasks) treated with four different groups (IL-1β and TNF-α, with and without WFA) along with control for 24 h in differentiation media were determined using the cationic fluorescent probe JC-1 (Molecular Probes, USA). Briefly, cells were harvested, rinsed with PBS and stained with 1 mL culture medium containing 5 μM JC-1 for 30 min at 37 °C. Then, cells were rinsed twice with ice-cold PBS, re-suspended in 300 μL ice-cold PBS and instantly analyzed for fluorescence in red and green regions under a flow cytometer using appropriate filters for capturing emission around red (590 nm) and green (530 nm) regions respectively. A high value (>) of red to green ratio is indication of polarized mitochondria. In contrast, a low value is indicative of mitochondrial depolarization [33].

2.10. Intracellular ROS measurement

RAC cells (1×10^4/well) were seeded and grow for 24 h in