Biomedicine & Pharmacotherapy 96 (2017) 198–207

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

A standardized extract of *Butea monosperma (Lam.)* flowers suppresses the IL-1β-induced expression of IL-6 and matrix-metalloproteases by activating autophagy in human osteoarthritis chondrocytes

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ARTICLE INFO

Keywords:
Osteoarthritis
Butea monosperma (Lam.)
Autophagy
mTOR
Nutraceuticals

ABSTRACT

Background/Objective: Osteoarthritis (OA) is a leading cause of joint dysfunction, disability and poor quality of life in the affected population. The underlying mechanism of joint dysfunction involves increased oxidative stress, inflammation, high levels of cartilage extracellular matrix degrading proteases and decline in autophagy-a mechanism of cellular defense. There is no disease modifying therapies currently available for OA. Different parts of the *Butea monosperma (Lam.)* plant have widely been used in the traditional Indian Ayurvedic medicine system for the treatment of various human diseases including inflammatory conditions. Here we studied the chondroprotective effect of hydromethanolic extract of *Butea monosperma (Lam.)* flowers (BME) standardized to the concentration of Butein on human OA chondrocytes stimulated with IL-1β.

Methods: The hydromethanolic extract of *Butea monosperma (Lam.)* (BME) was prepared with 70% methanol-water mixer using Soxhlet. Chondrocytes viability after BME treatment was measured by MTT assay. Gene expression levels were determined by quantitative polymerase chain reaction (qPCR) using TaqMan assays and immunoblotting with specific antibodies. Autophagy activation was determined by measuring the levels of microtubule associated protein 1 light chain 3-II (LC3-II) by immunoblotting and visualization of autophagosomes by transmission electron and confocal microscopy.

Results: BME was non-toxic to the OA chondrocytes at the doses employed and suppressed the IL-1β induced expression of inerleukin-6 (IL-6) and matrix metalloprotease-3 (MMP-3), MMP-9 and MMP-13. BME enhanced autophagy in chondrocytes as determined by measuring the levels of LC3-II by immunoblotting and increased number of autophagosomes in BME treated chondrocytes by transmission electron microscopy and confocal microscopy. BME upregulated the expression of several autophagy related genes and increased the autophagy flux in human OA chondrocytes under pathological conditions. Further analysis revealed that BME activated autophagy in chondrocytes via inhibition of mammalian target of rapamycin (mTOR) pathway. Of importance is our finding that BME-mediated suppression of IL-1β induced expression of IL-6, MMP-3, -9, and -13 was autophagy dependent and was abrogated by inhibition of autophagy.

Conclusion: The above results show that the *Butea monosperma (Lam.)* extract has strong potential to activate autophagy and suppress IL-1β induced expression of IL-6 and MMP-3, -9 and -13 in human OA chondrocytes. This study shows that BME or compounds derived from BME can be developed as safe and effective chondroprotective agent(s) that function by activating autophagy to suppress the expression of inflammatory and catabolic factors associated with OA pathogenesis.

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis affecting knees, hips and spine with aging, gender and trauma as the most common risk factors [1]. The disease is characterized by inflammation, cartilage extracellular matrix (ECM) degradation, synovitis, chondrocytes apoptosis, and narrowing of the joint space leading to pain and limited joint movement. OA joints have high levels of oxidative stress,

Abbreviations: OA, osteoarthritis; BME, extract preparation of *Butea monosperma (Lam.)* flowers; IL-6, interleukin-6; MMP-3, matrix metalloprotease-3; MMP-9, matrix metalloprotease-9; MMP-13, matrix metalloprotease-13; LC3-I, microtubule associated protein 1 light chain 3-I; LC3-II, microtubule associated protein 1 light chain 3-II; mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; ECM, cartilage extracellular matrix

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http://dx.doi.org/10.1016/j.biopha.2017.09.140
Received 7 August 2017; Received in revised form 12 September 2017; Accepted 26 September 2017

inflammatory and catabolic mediators and decreased synthesis of type II collagen and aggrecan [2]. Other recently identified events that contribute to joint aging and dysfunction include decline in autophagy [3] resulting in compromised cellular function and decreased in the ability of chondrocytes (the only cell type present in the cartilage) to maintain homeostasis and survive under pathological conditions. Currently there are no disease modifying drugs available and the current treatment modalities include symptomatic treatments for pain and ultimately joint arthroplasty.

Autophagy is a highly conserved cellular process required for the clearance of protein aggregates and dysfunctional organelles to maintain cellular homeostasis, promoting cellular function and organism health [4,5]. Defective autophagy or lack of autophagy activation or autophagy inhibition by pharmacological inhibitors has been found to decrease cellular function and induce apoptosis [6]. Expression of autophagy related proteins were found downregulated in aged cartilage and in mouse joints with surgically induced OA indicating compromised autophagy as a contributing factor to the disease pathogenesis [7–9]. These observations necessitate the discovery and development of new agents, which can enhance and/or sustain autophagy in chondrocytes and improve their function and survival under pathological conditions to inhibit OA induction and/or progression.

Plant derived nutraceuticals have been widely used from centuries throughout the globe for the improvement of human health and treatment of diseases. Several nutraceuticals have been reported to improve joint health and prevent the progression of OA [10,11]. *Butea monosperma (Lam.)*, popularly known as 'Flame of the forest' or 'Palash' or 'Gul-e-tesu', a member of Fabaceae family, is a commonly grown tree in Indian subcontinent along Indo-Gangetic plains. For centuries products derived from different parts of the *Butea monosperma (Lam.)* including bark, leaves and flowers have been widely used for the treatment of several human ailments in the traditional ayurvedic/unani Indian medicine system. A methanolic extract from the bark of *Butea monosperma (Lam.)* has been reported to possess hepatoprotective activity [12]. Another study showed that an aqueous extract prepared from the leaves and bark of *Butea monosperma (Lam.)* has antidiabetic effect on rat model of diabetes [13]. A hydroethanolic extract of its brightly colored flowers was shown to decrease the secretion of inflammatory cytokines and matrix metalloproteases in human keratinocytes [14]. Previously we have shown that the reported anti-inflammatory effects of BME were mimicked by its bioactive constituents Butrin, Isobutrin, and Butein in human mast cells stimulated with IL-1β [15]. However, in our knowledge, there is no report investigating the role of *Butea monosperma (Lam.)* on cartilage health and the pathogenesis of osteoarthritis. The aim of this study was to investigated the effect of flower extract of *Butea monosperma (Lam.)* (BME) on chondrocytes under pathological conditions. We prepared a hydromethanolic extract of *Butea monosperma (Lam.)* (BME) flowers standardized to the concentration of its bioactive constituent Butein and treated primary human OA chondrocytes with BME for two hours prior to stimulation with IL-1β *in vitro* (pathological conditions). Pre-exposure of OA chondrocytes to BME induced the expression of several of autophagy related proteins and the pre-exposed OA chondrocytes showed enhanced and sustained autophagy flux under pathological conditions. Pre-exposure of OA chondrocytes to BME also inhibited the subsequent IL-1β induced expression of IL-6, MMP-3, -9 and -13 mRNA and protein. Inhibition of autophagy in human OA chondrocytes, either by treatment with bafilomycin A1 or 3MA abrogated the BME-mediated suppression of IL-6, MMP-3, MMP-9, and MMP-13 expression upon subsequent stimulation with IL-1β. Taken together these results demonstrate the potential of BME or compounds derived from it as effective and non-toxic disease suppressive agents for the prevention and/or management of OA.

2. Materials and methods

2.1. OA chondrocytes isolation and culture

Study protocol to use discarded, de-identified human cartilage samples was reviewed and approved by the Institutional Review Board of North East Ohio Medical University, Rootstown, Ohio as a "non-human subject study under 45 CFR". The chondrocytes from knee joint of patients aged 45–70, who underwent total knee arthroplasty were isolated as described previously [16]. The primary chondrocytes were maintained in DMEM/F-12 (#12-719Q, Lonza, Walkersville, MD, USA) and 10% fetal calf serum (#10437028, Life technologies).

2.2. Preparation of Butea monosperma (Lam.) flowers extract and treatment of OA chondrocytes in vitro

The *Butea monosperma (Lam.)* flowers were collected and the extract was prepared as described previously [15]. In brief, the dried flower powder (10 g) was extracted with 70% methanol in a Soxhlet extractor for overnight (20 h). Methanol was evaporated under reduced pressure in a rotatory evaporator system (Rotavapor R-210, BUCHI Rotavapor) and freeze dried. We obtained 3.31 g of the extract powder (33.1% yield), and was stored at 4 °C in a desiccator. The presence of different constituents in the flower extract was determined by HPLC and tandem mass spectrometry. Concentration of Butein in BME was determined by a standard curve prepared using a certified reference standard (Extrasynthese cat # 1103) and was found to be 0.5 ug/100 ug of extract. The required quantity of the extract was dissolved in DMSO to treat OA chondrocytes.

2.3. Chondrocyte viability assay

Chondrocytes viability was measured as described previously [17]. In brief, human primary OA chondrocytes were seeded in a 96 well plate at a density of 20,000 cells/well in complete media. OA chondrocytes were treated with BME for indicated time or with different concentrations of BME for 24 h followed by addition of 0.5% MTT (#M2128, Sigma-Aldrich) for 2 h. Supernatant was removed and the formazan crystals were solubilized in 200 µl DMSO (#D8418, Sigma-Aldrich) and absorbance was recorded at 570 nm using Synergy H1 hybrid microplate reader (Bio-Tek Instruments Inc, Winooski, VT, USA).

2.4. Total RNA isolation and gene expression analysis by RT-qPCR

Total RNA was isolated by Trizol (#15596018, Life Technologies)-Chloroform extraction and ethanol precipitation as described previously [16]. 1 µg of total RNA was used to prepare cDNA using high capacity cDNA synthesis kit (Life Technologies, #4368813) and mRNA expression was analyzed by TaqMan gene expression assays (IDT, Coralville, Iowa).

2.5. Western immunoblotting

The lysate preparation and immunoblotting of chondrocytes treated with IL-1β in the presence or absence of BME was done as described previously [18]. OA chondrocytes at the end of experiment were lysed in RIPA buffer supplemented with protease inhibitors (#11697498001, Roche) and phosphatase inhibitor. Lysate was clarified by centrifugation at 20,000*g* for 10 min at 4 °C and protein concentration was determined by Bradford assay (#500-0006, Bio-Rad) and was either used immediately or stored in −80 °C for later use. The lysate was resolved on 10 or 12% SDS-PAGE gels and transferred to PVDF membrane (#1704272, Bio-Rad), blocked with 5% BSA in TBS-T (0.1% Tween-20) and incubated with primary antibody (2% BSA in TBS-T) overnight at 4 °C. The membrane was washed with TBS-T, incubated with HRP