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Original article

Combined action of Silymarin and Celecoxib in modulating inflammatory mediators in osteoarthritis

Z. Ashkavand [a], H. Malekinejad [b], B.S. Vishwanath [a,*]

[a] Department of Studies in Biochemistry, University of Mysore, Mysore, India
[b] Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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ABSTRACT

Inflammation is the hallmark of osteoarthritis (OA) leading to pain and disability. Objective of this investigation was to evaluate the effect of Silymarin (SMN, an antioxidant), Celecoxib (CLX, a selective COX inhibitor) and their combination on chemically induced arthritis in rats. The biochemical parameters and radiology impact of the treatment was also determined. Wistar male rats were assigned into five groups including control, OA^+, OA^+ CLX (100 mg/kg), OA^+ SMN (50 mg/kg) and OA^+ CLX + SMN (25 mg/kg). Combined treatment returned the elevated levels of inflammatory mediators (ROS, TNF-α, ALP, COX-2) to normal levels in 14 days. In the SMN + CLX treated animals significant reduction in KL grade and normal joint space narrowing was observed. X-ray radiological studies supported the biochemical findings. These findings suggest that co-administration of SMN with CLX could be an effective antiarthritic treatment which can equally abolish the arthritis associated secondary complication.

1. Introduction

Osteoarthritis (OA) is a common form of joint disease affecting nearly 20 to 40 million people around the world [1]. OA is strongly associated with chronic pain, swelling, stiffness of joints and poses higher risk of liver and cardiovascular diseases. It is believed that inflammation is the main cause of joint damage [2]. In OA related inflammatory responses, cytokines such as interlukine-1 (IL-1) and tumour necrosis factor (TNF-α) are produced excessively by synoviocytes. Hence, the inhibition of IL-1 and/or TNF-α production appears to be a logical suppressive medical therapy for OA related complications [3,4]. Although non-steroidal anti-inflammatory drugs (NSAIDs) are useful for relieving pain and inflammation, their use is owing to their toxic effects towards the gastrointestinal tract, kidneys and liver. Therefore, there is urgent need for improving the conditions of people suffering from arthritis, especially with those at higher risk of adverse effects of due to administered antiarthritic drugs. In view of these limitations, enormous attention has been given for management of inflammatory diseases using herbal remedies. Many flavonoids with antioxidant and anti-inflammatory effects have been well documented [5]. Silymarin (SMN) is one of the flavonoids isolated from *Silybum Marianum L.* Seeds. It exhibits potent antioxidant activity along with hepatoprotective, chemopreventive, anti-inflammatory, and anti-aging properties [6,7]. The aim of this study is to determine the antiarthritic and anti-oxidative activities of SMN, CLX and SMN + CLX on the improvement of knee joint arthritis using rat model. In addition the biochemical and radiological examinations also were performed using these animals.

2. Materials and methods

2.1. Chemicals

Dihydrodichloro-fluorescein diacetate (DCFDA), Silymarin, Celecoxib, monosodium iodoacetate (MIA) were purchased from Sigma Aldrich, St. Louis, USA. All other chemicals were of analytical grade.

2.2. Experimental design

This study was performed on thirty male healthy Wistar rats, 10–15 weeks old, weighing between 200 and 250 g, kept at DOS in Zoology, University of Mysore. The rats were kept in ventilated room (22 ± 2 °C) with 12 h light/dark cycle. The rats were provided with food and water *ad libitum*. Following a week of acclimation, animals were assigned into five groups (n = 6/group) as control, animals with OA^+ (treated with normal saline), OA^+ treated with CLX

* Corresponding author. Tel.: +91 9845893634.
E-mail addresses: vishmy@yahoo.co.uk, natabiochem@gmail.com (B.S. Vishwanath).

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(100 mg/kg), OA+ SMN (50 mg/kg) and OA+ CLX + SMN (100 and 25 mg/kg, respectively). The dose levels were selected based on our previous study [8]. The animals received normal saline and/or test compounds via gastric gavages for 14 days. All animal experiments were performed in accordance with the guidelines of the ethical Committee University of Mysore (UOM/IAEC/36/2011).

2.3. OA induction

OA was induced according to Kalbhen method [9]. Rats were anesthetized using anaesthetic ether and injected once with 25 μL of monosodium iodoacetate (MIA) – (MIA-2 mg) into the right knee joint. Control animals received 25 μL of normal saline into the right knee joint under the anaesthesia. The injection of MIA was performed through the patellar ligament using a 27 gauge, 0.5 inch needle [10].

2.4. Radiological analysis

Before euthanizing the animals, X-rays were taken at the knee joint for evaluating the bone and cartilage damage. Radiographs were taken using X-ray apparatus (Siemens-60MA, Germany) and industrial X-ray film (Kodak Diagnostic film). The X-ray apparatus was operated at 220 V with a 40 V peak, 0.2 second exposure time, and a 60 cm tube-to-film distance for femur-tibia projection. The radiographic images were examined by the experienced radiologist. We have evaluated the X-ray images from the tibia femoral (TB) angle of the right knee joints of control, animals with MIA induced arthritis treated with SMN, SMN + CLX and CLX. The joint space narrowing (JSW) and other morphological changes in bone were classified based on the Kellgren-Lawrence (KL) classification grades.

According to the KL classification grades. The different stages of OA severity were classified as: 0 = normal; 1 = possible narrowing of the joint space, possible osteophytes; 2 = small osteophytes, narrowing of the joint; 3 = multiple, moderately sized osteophytes, definite joint space narrowing, some sclerotic areas, possible deformation of bone ends; 4 = severe case [11].

2.5. Serum preparation and tissue collection

On day 15, immediately after a light anesthesia with diethyl ether, to obtain serum, samples were allowed to clot at room temperature for 1 h, centrifuged at 3000 × g for 10 min. Following blood sample collection, animals were euthanized using CO_2 gas in special device. MIA- and saline-treated femorotibial joints were dissected immediately and stored at −20 °C for biochemical analysis.

2.6. Estimation of TNF-α as a pro-inflammatory cytokine

The levels of TNF-α in serum and tissue homogenate were measured by ELISA technique – using a high-sensitivity kit (R&D Systems Inc., Biosource, Minneapolis, MN, USA), according to manufacturer instructions. In brief, the measured levels of TNF-α are expressed as nanogram per dL (ng/dL). Absorbance was measured using a microtiter plate reader at 490 nm (Statfax 2100, USA).

2.7. Determination of serum ALP

The serum alkaline phosphatase (ALP) level was measured spectrophotometrically by standard enzymatic method [12]. Using commercial kits (Agappe Diagnostics Ltd., Kerala, India) in brief, the working reagents were prepared from PNPP substrate with 5.0 mL of buffer. To 20 μL of serum, 1 mL of working reagent, after 1 min the increase in absorbance was measured at 405 nm and ALP activity was expressed as IU/L activity.

2.8. Determination of endogenous reactive oxygen species (ROS) levels

The endogenous generation of ROS in the serum and synovial fluid of both treated and control groups of rats were quantified using dihydrodichloro-fluorescein diacetate (DCFDA) method as previously reported [13]. Briefly, an aliquot (0.2 mg protein) of the incubation mixture was dispensed into 96-well microtiter plate containing Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5 mM HEPES, 2 mM CaCl2 and 10 mM glucose, pH 7.4). To which 10 μL of DCFDA was added to obtain a final concentration of 5 mM. The reaction mixture was incubated for 30 min at room temperature to allow the cleavage of DCFDA by esterases and further conversion into the fluorescent product dichlorofluorescin. The fluorescence was measured using Multimode plate reader (Thermo scientific, USA) with excitation at 480 nm and emission at 530 nm. Data are expressed as percentage of DCF formed/min/mg protein for ROS.

2.9. Protein estimation

The protein estimation was done according to the method of [14] using bovine serum albumin (BSA) as standard.

2.10. Determination of COX assay

The bone-joint samples were macerated with a mortar and pestle and homogenized at 4 °C in 1.5 mL of 0.1 M phosphate buffer (pH 7.2). The homogenates were centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant was collected, in the supernatant cycloxygenase activity was measured using Amplex red reagent as previously described method of [15]. The assay reaction was carried out in final volume of 1 mL containing 100 μM Linoleic acid and 10 μM Amplex red reagent (prepared in pure DMSO and stored at −20 °C) in 50 mM Tris HCL buffer pH 9.0. The reaction mixture was incubated for 5 min at 37 °C. In the presence of cyclooxygenase, the nonfluorescent reagent is converted to resorcifin, a fluorescent molecule. The relative fluorescence intensity was measured in a fluorimeter using appropriate blanks. The excitation and emission wavelength were at 563 nm and was at 587 nm. Results are expressed as percent inhibition of the COX activity.

2.11. Statistical analyses

The statistical analyses were performed using Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA). Analyses of variance were performed by ANOVA procedures and significance of each group was verified with one-way analysis of variance followed by turkey post-hoc test. Values obtained are means of three replicate determinations ± standard deviation (SD). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. X-ray radiology analysis

The radiological examinations showed joint space narrowing with KL3 grade and sclerosis with cortical/articular surface irregularity, multiple osteophytes in the non-treated OA group (Fig. 1A and B). In the control group, all examined images were found to be normal and no signs of joint space narrowing, osteophyte formation were observed (Fig. 1C). Those OA positive animals, which were treated with SMN showed remarkable reduction in joint space