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Original article

Potentiality and safety assessment of combination therapy with silymarin and celecoxib in osteoarthritis of rat model

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ABSTRACT

Osteoarthritis (OA) is a common form of arthritis leading to cartilage degeneration and inflammation. We used rat model to evaluate the protective effect of silymarin (SMN) as a known antioxidant agent individually and in combination with celecoxib (CLX) as a COX-2 inhibitor on MIA-induced arthritis. The rats were assigned into five groups ($n = 6$) as control, sham, and tests. The objective was to improve adverse events occurring with celecoxib in the treatment of osteoarthritis. The biochemical alterations and its recovery by silymarin and combination of silymarin and celecoxibe were assessed by measuring enzymatic and non-enzymatic mediators. In the present study, our data demonstrated the significant ($P < 0.001$) elevated serum levels of hyaluronidase, C-reactive protein, 5-LOX and liver function enzymes, while both CLX and SMN reduced the level of these enzymatic and non-enzymatic mediators. Taken together, the study depicted the efficiency and protective role of combination of CLX with SMN in the treatment of OA. Thus it may be a safe compound to fight arthritis.

1. Introduction

Osteoarthritis (OA) is a common chronic disease, which leads to disability, pain in the affected area, which is mainly found in weight bearing joints [1,2]. Chronic inflammation has a role in development of diseases like arthritis, cardiovascular disease and cancer. Generation of cyclooxygenase and 5-lipooxygenase contribute to inflammation and finally joint damage in arthritis [3]. Elevated level of eicosanoids have been observed in patients serum of chronic inflammation, eicosanoids formed via COX and 5-LOX have been used as a target for drug developments [4]. Hyaluronic acid or hyaluronan (HA), a glycosaminoglycan, consists of repeating units of D-glucuronic acid and N-acetylglucosamin [5–7]. Wells et al. [8] reported that HA found in synovial fluid is essential for water retention and joint lubrication. Reduction in HA may increase susceptibility of the knee joint to physical damage [9]. C-reactive protein (CRP) is a 130 kd glycoprotein produced by the liver, which is a non-specific but sensitive marker for inflammation, infection, and tissue damage [10]. CRP has been recognized as a marker in association with OA, serum CRP concentrations are higher in individuals with OA [11] and are related with OA severity [12] and joint pain [13], and disease progression. Selective COX-2 inhibitors, like celecoxib, are widely used due to their efficiency, COX-2 inhibitors are a type of non-steroidal anti-inflammatory drugs (NSAIDs) useful for relieving pain and inflammation, and their use may be limited by toxic effects on the gastrointestinal tract, cardiovascular, kidneys and liver [14,15]. Medicinal plants cover a variety of bioactive agents that are applicable for disorders. Silymarin, an extract from the seeds of the milk thistle plant, has been used for decades as a hepatoprotectant and antioxidant agent [16]. *Silybum marianum* is one of the oldest plants for the treatment of liver and gallbladder disorders [17]. On these foregrounds, the current study has been designed to make an attempt to evaluate the possible safety and anti-inflammatory potentiality of Silymarin alone or in combination with celecoxib which can be as a new combination of this drug may prove to be useful using MIA-induced arthritis rats.

2. Material and methods

2.1. Chemicals

Silymarin (SMN), monoiodoacetate (MIA), celecoxib (CLX), Linoleic acid, ATP and DTT were purchased from Sigma–Aldrich (USA). Hyaluronic acid (251770010) is purchased from Acros

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Organics, New jersey, USA. Triton X100 is purchased from SRL Mumbai, India.

All other chemicals were of analytical grade.

2.2. Experimental design

Thirty male Wistar rats weighing appropriately 200–250 g were obtained from Mysore University, Mysore, India. Animals were housed in polypropylene cages in controlled room. The animals were supplied with standard pellet diet and water ad libitum. The animals used for the study were on approval by the Institutional Animal Ethical Committee (UOM/IAEC/36/2011). The animals were divided into five groups of six animals each.

Group I: control (normal diet); group II: OA induced; group III: MIA-induced OA + SMN treated (50 mg/kg b.wt.); group IV: CLX + SMN treated (25 mg/kg b.wt.); group V: CLX treated (100 mg/kg b.wt). Osteoarthritis was induced according to the Kalbhen method [18]. In brief, the rats were anaesthetized with diethyl ether and received single injection (2 mg) of MIA (Sigma–Aldrich) into the right knee joint in a total volume of 25 μl. Control animals were injected 25 μl of saline normal into the right knee joint under the anesthesia. The injection of MIA was performed once through the patellar ligament using a 27 gauge, 0.5 inch needle [19]. Celecoxib was used as a standard treatment control and saline as negative control. Animals were then killed and blood was collected through cardiac puncture. Serum and liver tissues were separated and stored at −20 °C for further estimation of different biochemical parameters.

2.3. Determination of liver aspartate aminotransferase/alanine transaminase (SGPT/SGOT)

The liver alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured spectrophotometrically by standard enzymatic method using commercial kits (Agappe Diagnostics Ltd., Kerala, India). The reaction mixture containing the serum/liver homogenate (10 μl) and AST/ALT reagent (1 mL) was mixed and incubated at 37 °C for 1 min and read at 340 nm for 3 min and expressed as activity/min.

2.4. Assessment of cartilage degradation

2.4.1. Hyaluronidase assay

HAase enzyme activity in the serum samples of control and experimental animals were evaluated according to the protocol of Guntenhöner et al. [20] but with slight modifications. HA was added at final concentration of 0.17% into the SDS polyacrylamide resolving gel matrix (10%). Serum samples (0.5 μl) were diluted in sample buffer, and gels were run at 100 V.

After electrophoresis, the SDS was removed by washing the gel with 2.5% Triton X100 and gel was incubated with 0.1 M sodium formate buffer pH 3.6 containing 0.1 mM NaCl at 37 °C for 20 h and the gel was stained with Alcian blue, then decolorized in 20% ethanol and 10% acetic acid for 2 h. HAase activity was detected as unstained translucent bands against blue background.

2.5. Assessment of inflammation

2.5.1. 5-lipoxygenase assay (5-LOX)

5-lipoxygenase was purified from Homogenates (10,000 × g supernatants) of leukocyte suspensions [21].

The enzyme assay was carried out essentially by modification of Aharony and Stein's [22] method. Briefly, the reaction was carried out in a final volume of 2 ml containing 50 μM DTT, 200 μM ATP, 300 μM $CaCl_2$, and 150 μM Linoleic acid. The reaction was started by adding 50 μl of the enzyme preparation. The formation of 5-HETE was followed for 3 min at 234 nm at room temperature. The results are expressed as percent inhibition of the 5-LOX activity.

2.6. Estimation of serum C-reactive protein (CRP)

With the ultra sensitive C-reactive protein Latex test Kit (Acros organics, Bangalore, India), latex particles coated with antibody specific to CRP aggregate in the presence of CRP in the sample, forming immune complexes. The immune complexes cause an increase in light scattering, which is proportional to the concentration of CRP in the sample. The light scattering is measured by reading turbidity at 550 nm.

3. Statistical analysis

The statistical analyses were performed using the statistical package SPSS (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 ($P < 0.05$). Values obtained are means of three replicate determinations ± standard deviation.

4. Results

4.1. Inhibitory study of 5-lipoxygenase in serum

The results showed that 5-LOX level significantly ($P < 0.0001$) elevated in arthritis. By contrast both CLX and SMN were able to reduce the 5-LOX level and the lowest amount of 5-lox was found in the test group of animals, which received the combination of test compounds (Fig. 1A).

4.2. Estimation of serum C-reactive protein level

Arthritic rats were observed with elevated levels of CRP (Fig. 1) significantly ($P < 0.001$; $n = 6$) in comparison with the saline control. While 14 days administration of CLX and/or SMN individually or in combination was able to decline the OA-increased level of CRP. But silymarin (50 mg/kg) and SMN along with CLX (25 mg/kg) administration re-established all the above modulated inflammatory mediators effectively ($P < 0.001$; $n = 6$), as compared to arthritic animals (Fig. 1B).

4.3. Effect of silymarin and silymarin along with celecoxib on cartilage degradation

The present study demonstrated a significant increase in serum HAase activity in the arthritic animals (lane B) compared to the saline (lane A). The intense clear zonal activity band with respect to lane A indicates augmented levels of HAase. However, silymarin (50 mg/kg) administration effectively abolished the increased HAase activity, suggesting the ECM-protective nature of silymarin (lane D). CLX + SMN (25 mg/kg) in lane C was more effective in neutralizing the augmented serum HAase levels compared to celecoxib (100 mg/kg) in lane E (Fig. 2).

4.4. Modulatory effect of silymarin and complex of silymarin with celecoxib on liver toxicity

Liver toxicity is further supported by the levels of liver ALT and AST. The ALT and AST levels were increased by 65% and 70%, respectively, in the serum of arthritic group. In contrast, both ALT and AST were found to be reduced by SMN (50 mg/kg) and SMN + CLX (25 mg/kg) by 53.5 and 56.1% in the arthritic liver, respectively