Original Article

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The Comparison of the Effect of the Aqueous Extract of the Elaeagnus Angustifolia with Ibuprofen on the Expression of the iNOS and COX2 Genes in the Cartilage Tissue of Rat Rheumatoid Arthritis Model

A. Moradi (MSc)[1], M. Korani (PhD)*[2], G.H. Alishiri (MD)[3]

1.Department of Biochemistry, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran
2.Chemical Injuries Research Center, System Biology and Poisoning Institute, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran
3.Department of Internal Medicine, Rheumatology Ward, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Rheumatoid Arthritis (RA) is a chronic autoimmune disease is treated by non-steroidal anti-inflammatory drugs. Considering that use of medicinal plants has lower side effects than chemical drugs and Elaeagnus angustifolia is one of the plants used in the traditional medicine is, the present study was conducted to investigate the anti-inflammatory property of the aqueous extract of the Elaeagnus angustifolia by assessing the expression of the iNOS and COX2 as inflammatory genes.

METHODS: This experimental study was performed on 35 male Wistar rats in 5 groups of 7. Seven rats were considered as control group, and in the rest of rats, rheumatoid arthritis was induced according to the CIA protocol with collagen type 2 injection. One group was not treated as the control group and three groups were treated orally for a period of 30 days by ibuprofen with the concentration 15 mg/kg and the aqueous extract of the Elaeagnus angustifolia with the concentration 350 mg/kg and their combination, then expression of the COX2 and iNOS genes was measured by real-time PCR technique.

FINDINGS: Treatment with ibuprofen and the aqueous extract reduced the genes expression. In other words, the expression of the iNOS gene decreased more in the ibuprofen group (6.34±0.49) than the Elaeagnus angustifolia (7.71±0.61; $p<0.001$) and the expression of the COX2 gene decreased more in Elaeagnus angustifolia (7.70±0.77) than ibuprofen group (9.93±0.68; $p< 0.001$).

CONCLUSION: It seems that the aqueous extract of the Elaeagnus angustifolia can be used independently or together with ibuprofen to treatment the rheumatoid arthritis.

KEY WORDS: *Rheumatoid Arthritis, Elaeagnaceae, Ibuprofen, Cyclooxygenase-2 ،Nitric oxide synthase type II.*

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*Corresonding Author: M. Korani (PhD)
Address: System Biology and Poisoning Institute, Baqiyatallah University of Medical Sciences, Tehran, I.R.Iran
Tel: +98 21 82483417
E-mail: mohsenkorani@gmail.com

Introduction

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease that affects one percent of the world's population (1). Synovium inflammation, advanced bony erosion, abnormal joints displacement, and degeneration and weakness of the surrounding muscles occur. Treatment of this disease is based on the use of non-steroidal anti-inflammatory (NSAIDs) drugs and cardiovascular and gastrointestinal problems are considered as long-term side effects.

Therefor investigating new drugs with lesser side effects than the NSAIDs is considered as a new challenge. Medicinal plants due to their natural nature and because of their biological balance, have fewer side effects than chemical drugs. One of the plants used in traditional medicine in Iran is Elaeagnus angustifolia (2). Studies have shown that the aqueous extract of Elaeagnus angustifolia contains various compounds including flavonoids that have antioxidant, antiviral, anti-allergic and anti-inflammatory properties and can inhibit inflammatory mediators, such as cyclooxygenase and nitric oxide synthase enzymes (3). It has been shown that the anti-inflammatory effect of Elaeagnus angustifolia extract is significant on the acute and chronic edema induced by formalin in a dose-dependent manner and is comparable to sodium salicylate (4).

The aqueous extract also has a similar therapeutic effect on knee aesthetic arthritis with ibuprofen (5). NO and PGE2 are two important mediators in the inflammation process that are produced by Inducible Nitric Oxide Synthase (iNOS) and COX2 (Cyclooxygenase 2) enzymes and exacerbate inflammation in the joint (6). There is an interaction between these two pathways so that NO can increase the production of PGE2 by increasing the activity or COX2 protein (7). On the other hand, PGE2 can also increase the expression of the iNOS gene (8). There are numerous evidences about the presence and role of NO and its producing enzyme (iNOS) in the pathogenesis of rheumatoid arthritis (10, 9). A sharp increase in the concentration of nitrites in the articular fluid in patients with rheumatoid arthritis has been reported (11). According to studies, prostaglandins also play an important role in the pathophysiology of cancer and inflammation (12). There is sufficient evidence that COX2 enzyme is present in chronic human inflammation such as RA and even similar inflammations in the animal model (13, 12). Inhibition of prostaglandin production through selective inhibition of COX2 production is a promising and practical way of treating these diseases. Different studies have shown that ibuprofen, in addition to inhibiting COX2 activity, can also affect the amount of its mRNA and protein (15, 14). It also reduces the iNOS mRNA (16,15). Flavonoids which are components of various plants, such as Elaeagnus angustifolia can reduce the levels of COX2 and iNOS mRNA (17).

Given that there is not any study to date on the effect of the aqueous extract of Elaeagnus angustifolia on the expression of molecules involved in inflammation, such as COX2 and iNOS genes in rheumatoid arthritis, the aim of this study was to compare the effect of ibuprofen and the aqueous extract of Elaeagnus angustifolia on the expression of COX2 and iNOS genes in rheumatoid arthritis model of rats as a potential supplement or replacement for ibuprofen in the treatment of rheumatoid arthritis.

Methods

This experimental study was approved by the Committee of the Ethics of the Baqiyatallah University of Medical Sciences with registration code IR.BMSU.REC.1396.563 On 35 wistar male rats (200-250 gr). All experiments were carried out in accordance with the guidelines for the care and use of laboratory animals. After one week, when the rats were used to the environment, 7 mice were selected as control group and rheumatoid arthritis was induced in the rest of the rats by injection of collagen type 2 emulsion and complete Freund's adjuvant (CFA). To create a model of rheumatoid arthritis, the equivalent ratio of bovine collagen type 2 (2 mg/ml) was mixed with CFA and then 200 µl at day 0 and 100 µl at day 14 was injected subcutaneously to the tail of the rats (20-18). To confirm the induction of model, the joint diameter, CBC and ESR were examined before and after the induction of the model. Histological sections were also taken from cartilage tissues of their toes and compared with the control group.

They were randomly divided into four groups: the first group received no treatment as the control group; the second group received aqueous extract of Elaeagnus

angustifolia, the third group received ibuprofen and the fourth group received both aqueous extract of Elaeagnus angustifolia and ibuprofen. To prepare the aqueous extract, the fresh and dried fruits were harvested. 80 g of powder were added to 400 ml of distilled water, and the boiling was continued for 15 minutes, after cooling the contents were filtered with filter paper. Then, it was placed in a Bain-marie at a temperature of 40 °C and then drained, then the extract powder was stored in the refrigerator at -10 °C (21). One week after immunization, the treated groups received in addition to water and food for 30 days, ibuprofen (15 mg/kg), aqueous extract (350mg/kg) or the combination (ibuprofen 15mg/kg and aqueous extract 350mg/kg) in the form of single dose and gavage. For analyzing the genes desired, after the anesthesia of the rats, the cartilage tissue was isolated from their feet and washed with liquid nitrogen and kept at 70 °C for the extraction of the RNA. The steps for extracting RNA were performed by (Invitrogen) TRIZOL. Then the total RNA was used to make the cDNA by the QuantiTect Rev trnscriptase kit (Qiagen product). Subsequently, quantitative fast SYBR Green PCR (Qiagen) kit and specific primers for COX2, iNOS and β-actin genes were amplified using Real Time PCR technique. The primers were designed by the genscript real time taqman program and then blast was done in NCBI. They were also analyzed using the oligo software for loop and dimmer formation. The sequence of primers was shown in Table 1. In this study, the β-actin gene was used as an internal control gene and the CtΔΔ-2 method was used to examine the relative expression of COX2 and iNOS genes in different groups. Statistical analysis was performed using SPSS 18 software, one-way ANOVA and Tukey multiple comparison tests. P<0.05 was considered significant.

Table 1. Primer sequence of the studied genes

Gene	Primer sequence	Base pair
iNOS	Forward: TCCCAGCACAAAGGGCTCAA Reverse: TGCGGACCATCTCCTGCATT	106
COX2	Forward: AGCTTCACTTGCCACCAACG Reverse: TCGGAAGAGCATCGCAGAGG	70
β-actine	Forward: AGCCATGTACGTAGCCATCC Reverse: CTCTCAGCTGTGGTGGTGAA	141

Results

The erythrocyte sedimentation rate (ESR) in the RA group (12.76±0.77 mg/hr) was significantly higher than the control group (3.85±0.52) (p<0.01). Also, the number of white blood cells in the RA group 16710±2117.28 per µl) compared to the control group (7415±327.51) (p<0.01). Additionally, the joint in the RA group was inflated (Fig. 1). Moreover, intense infiltration of the inflammatory cells and abnormal cells of the synovium were observed in the histopathological examination (Fig. 2) which all represent the success of the model. The relative expression of COX2 gene in the control group was 14.92±1.16 and in the Ibuprofen group was 9.93±0.68 and in the aqueous extract group was 7.7±0.77 and in the combination group of ibuprofen and aqueous extract was 5.94±0.34 higher than control group (Fig. 1). As it can be seen, various treatments by drug and aqueous extracts have been able to reduce the expression of COX2 gene in comparison with the control group (p<0.001). This decrease was higher for the aqueous extract group than the ibuprofen group (p<0.001). The combination of these two drugs alone reduced the COX2 gene (p<0.001) relative to each of the treatments alone. The relative expression of iNOS gene in the control group was 13.59±1.05, in the Ibuprofen group was 6.34±0.49, in the aqueous extract group was 7.71±0.67 and in the combined group was 3.5±0.27 (Fig 2). As it has been shown, the development of inflammation in rheumatoid arthritis has increased the expression of iNOS gene in all groups. On the other hand, treatment with aqueous extract has been able to reduce the expression of iNOS gene compared to the control group (p<0.001). Decreased expression in the ibuprofen group was higher than that of the aqueous extract (p<0.001). The combination of ibuprofen and aqueous extract in comparison to each of the treatments alone led to a further decrease in iNOS gene expression (p<0.001).

Figure 1. Increased joint diameter of RA group (a) compared to control group (b) on day 21

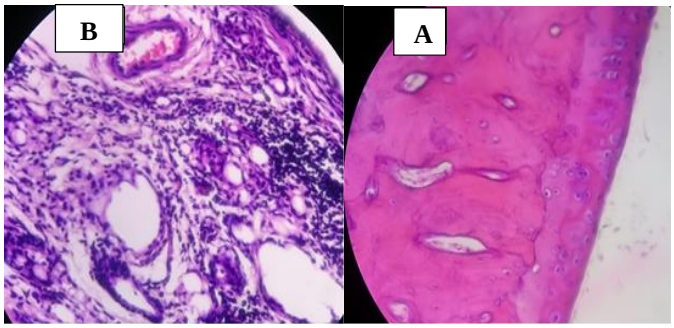

Figure 2. Cartilage tissue of the control group with a normal synovial layer and normal chondrocytes (a). The cartilage tissue of the RA group shows a high concentration of inflammatory cells (b).

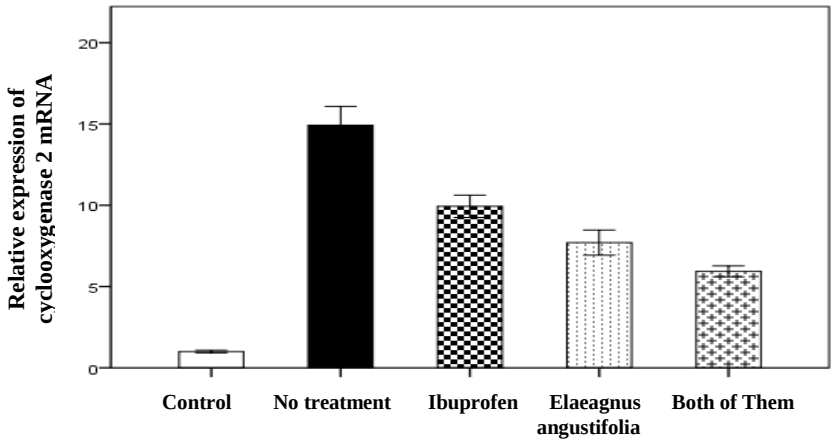

Figure 3. Comparison of the relative expression of cyclooxygenase 2 gene in the studied groups. a, b, c and d were meaningful in all groups (P<0.001)

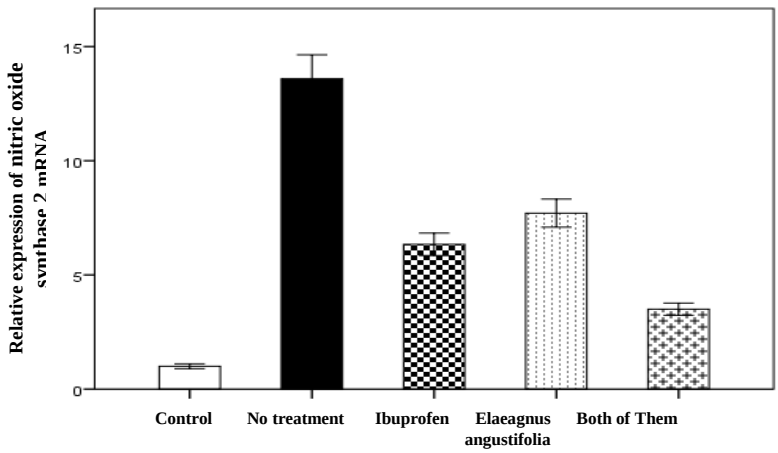

Figure 4. Comparison of relative expression of nitric oxide synthase 2 gene in the studied groups. a, b, c and d were meaningful in all groups (p <0.001)

Discussion

In the present study, ibuprofen and aqueous extract of Elaeagnus angustifolia reduced the expression of iNOS and COX2 genes in articular cartilage of the rats' carpal joints in the rheumatoid arthritis model. The results of research by Motevalian et al. showed that the extract of Elaeagnus angustifolia has anti-inflammatory effects comparable to sodium salic acid (4). The results of Panahi et al. indicated that the effectiveness of aqueous extract of Elaeagnus angustifolia in the treatment of chronic inflammation (5).

Liu et al. Investigated the effect of Camellias herbal flavonoids on pro-inflammatory agents such as TNFα, iNOS, COX2 and MIP-1α. The results of this study showed that the total flavonoid of this plant can decrease the mRNA of the TNFα, iNOS, COX2 and MIP-1α genes which was induced by LPS in 7/264 RAW cells (22). The results of Liu et al. showed the effectiveness of the flavonoid family on the reduction of inflammatory precursors. In the study of Chai et al., Ibuprofen increased the expression of COX2 gene both in the level of mRNA and protein levels in Urotsa cells line of bladder cancer (23), which is not consistent with our results, probably due to dose and course of drug use and cell lineage used in that study.

Moreover, Liu et al., examined the effect of ibuprofen on diabetic encephalopathy and indicated that chronic treatment with ibuprofen significantly reduced the activity of the protein and mRNA of the COX2 and iNOS gene in the brain, but increased the protein and mRNA associated with PPARγ in the brain of diabetic rats. PPARγ inhibits the expression of pro-inflammatory genes (24). Crofford's study showed that the expression of the COX2 gene in the synovial tissue of rheumatoid arthritis patients increases through activation of NFκB transcription factors by cytokines IL-1 and TNFα (25). On the other hand, D'Acquisto's study showed that ibuprofen inhibits the expression of COX2 and PGE2 in T cells and rodent macrophages by inhibiting NFκB (26). In the present study, the expression of iNOS and COX2 genes in all treatment groups and the control group was increased in comparison with the control group, which indicates that these genes play an important role in the inflammatory process and their expression in rheumatoid arthritis increases.

On the other hand, the expression of these genes was reduced by the aqueous extract and ibuprofen extract compared to the control group, indicating the effectiveness of the treatment process that reduced the progression of inflammation, but the reduction of iNOS gene expression by ibuprofen was more that the aqueous extract of Elaeagnus angustifolia and decreased expression of the COX2 gene was more pronounced in the aqueous extract of Elaeagnus angustifolia than ibuprofen. In addition, the combination of ibuprofen and aqueous extract reduces the expression of these genes, which suggests that if the aqueous extract and ibuprofen be co-administered simultaneously, the effect of them on the reduction of the expression of these inflammatory genes would be strengthened.

It is suggested that Elaeagnus angustifolia be used as an appropriate candidate for formulation in a pharmaceutical industry as an alternative to ibuprofen or co-administered with it in treating patients with rheumatoid arthritis which requires further studies on the extraction of the active ingredient and the full recognition of the mechanism of action, complications and toxicity.

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