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Significant Effect of Crocin on the Gene Expression of MicroRNA-21 and MicroRNA-155 in Patients with Osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis associated with gradual joint destruction. The current treatment aims to alleviate pain and inflammation and improve the quality of life. Crocin is an active ingredient in saffron, with anti-inflammatory properties. MicroRNAs are small, non-coding RNAs that regulate gene expression. We aimed to evaluate the effect of crocin on the gene expression of microRNA-146a, microRNA-155, microRNA-223, and microRNA-21 in OA patients and compare it with a placebo.

This study was approved and registered in the Iranian Registry of Clinical Trials (2015021910507N2) and ClinicalTrials.gov identifier: NCT03375814. Forty OA patients were randomly divided into two equal groups, receiving either crocin or placebo. Peripheral blood samples were collected before and four months after the intervention. The pain was assessed using the visual analog scale, and laboratory tests included C-reactive protein and erythrocyte sedimentation rate. The expression levels of microRNA-146a, microRNA-155, microRNA-223, and microRNA-21 genes were evaluated by SYBR Green real-time PCR.

The results showed that the gene expression levels of microRNA-21 and microRNA-155 in patients receiving crocin were significantly decreased and increased, respectively. No significant changes were observed in microRNA-146a and microRNA-223 gene expression levels.

In conclusion, crocin's anti-inflammatory role might be partly attributed to its effects on the gene expression of microRNA-21 and microRNA-155.

Keywords: Clinical trial; Inflammation; MicroRNA; Osteoarthritis

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INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis, associated with progressive articular cartilage

destruction and synovium inflammation. It is a principal reason for pain and disability, usually in old age.[1] OA influences approximately 3.8% of the global population.[2] Many risk factors, such as age, gender, hormones, genetics,[3] obesity, ethnicity, race, occupation, and physical activity, contribute to the etiology of OA.[4-6] Pathological changes include gradual destruction of articular cartilage, thickening of the subchondral bone, subchondral cysts, pain, changes in the bone structure, hypertrophy of the joint capsule, osteophyte formation, synovial inflammation, and stiffening of tissues.[7,8]

There is no definite treatment for OA,[9] and conventional therapies mainly include nonsteroidal anti-inflammatory drugs (NSAIDs), such as sodium diclofenac and aspirin; side effects are a primary concern with NSAIDs.[10,11,12] Hence, herbal medicines exhibit beneficial effects for the treatment of OA.[13]

The Saffron Plant *Crocus sativus* belongs to the lily family (Iridaceae), which originates from Central Asia and is generally considered a valuable medicinal plant. Saffron has four biologically active compounds: crocin, crocetin, picrocrocin, and safranal.[14] These compounds act as antioxidants and can protect against oxidative stress and free. As an active ingredient in saffron, crocin exerts anti-inflammatory activity by inhibiting nitric oxide, tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β. Crocin also inhibits the expression of nuclear factor (NF)-κB-induced Matrix metalloproteinases (MMPs). Hence, cartilage breakdown in OA.[13] Saffron also displays a high safety profile: daily consumption of less than 1.5 g of saffron is considered safe. Toxic effects have been reported with doses of 5 g and more elevated.[15] The anti-inflammatory effects of saffron are attributed to crocin and crocetin, which are related to their antioxidant effects.[16] Studies have described the efficacy of one crocin tablet (15 mg) per day in osteoarthritis and other inflammatory diseases such as diabetic maculopathy.[17,18] Krocina is a nanomicelle form of crocin, an active ingredient of saffron.

MicroRNAs (miR) are a category of endogenous non-encoding single-stranded RNAs.[19] They have epigenetic properties and are involved in regulating post-transcriptional gene expression.[20,21] miR-223 plays an essential role in the development and homeostasis of the immune system. It stimulates osteoclast differentiation and function by suppressing nuclear factor 1 A (NFIA) expression.[22] miR-155 is vital in inflammatory and anti-inflammatory responses depending on the type of tissue involved.[23] Moreover, overexpression of miR-155 reduces chronic inflammation.[24] The miR-146 family includes the miR-146a and miR-146b genes, which act as regulators of inflammation[23] miR-146a is a conserved class of regulatory microRNAs that play a crucial role in regulating the immune system and stimulating inflammatory responses.[25] Abnormal expression of miR-146a is also involved in forming chronic inflammation.[26] MiR-146a is highly expressed in the cartilage of OA patients in the early stages of the disease and may regulate pain in osteoarthritis.[27] Recent studies have demonstrated the critical role of miR-21 in reducing inflammation and negatively regulating the pro-inflammatory responses.[28]

The conventional treatments used in OA are associated with various side effects. Given the regulatory role of microRNAs and the anti-inflammatory effect of crocin, we aimed to study the effect of crocin (Krocina) on the gene expression levels of microRNA-146a, microRNA-155, microRNA-223, and microRNA-21 in OA patients.

MATERIALS AND METHODS

Study Design

This study was conducted as a double-blind, randomized placebo-controlled clinical trial at the Rheumatology Clinic of Emam Reza Hospital, Mashhad University of Medical Science, Mashhad, Iran. The microRNA samples were prepared from archived samples stored in the biobank, previously approved by and registered in the Iranian Registry of Clinical Trials (2015021910507N2) and ClinicalTrials.gov identifier: NCT03375814. The Ethics Committee approved the trial of Mashhad University of Medical Sciences, Mashhad, Iran (code: IR.MUMS.FM.REC.13940279).

Patient Selection

Forty patients with idiopathic knee OA aged 40-75 years old, who matched the American College of Rheumatology (ACR) criteria for knee OA, were enrolled in the study. The exclusion criteria were as follows: (1) age ranges under 40 or over 75 years; (2) Kellgren-Lawrence (KL)[29] grades I or IV based on radiography within the last 6 months; (3) history of joint surgery or injury; (4) history of diabetes mellitus,

rheumatoid arthritis, osteonecrosis, or gout; (5) history of corticosteroid consumption within the past 3 months; 6) pregnant women; (7) OA patients who had a body mass index (BMI) of ≥ 30; (8) occupations with heavy jobs, and (9) consent to participate in the study. The inclusion criteria were as follows: (1) non-traumatic knee pain for the past 6 months; (2) KL classification grades II or III, within the last 6 months; (3) ages between 40 and 75 years; (4) no history of joint surgery; (5) no history of osteoarthritis of the hip, septic arthritis, or active gastrointestinal bleeding; and (6) BMI of <30. According to our recent publication by the same team,[17] Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaires, the visual analog scale (VAS) for pain, clinical history, and radiographic images of the knee joint were evaluated. All patients enrolled in the study had grade 2 or 3 knee OA bilaterally, which was radiologically confirmed according to the KL grading system. Demographic data, including weight, height, BMI, and laboratory indices, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, were investigated before and four months after the intervention. ESR>30 or CRP>5 indicate an ongoing inflammation.[17]

Written consent was obtained from 40 OA patients divided into two groups: 20 received Krocina tablets, 20 received placebo tablets, and both groups continued the conventional treatment during the intervention. Five patients decided to drop out of the study. A total of 18 patients in the Krocina group and 17 in the placebo group finished the clinical trial after four months.

Krocina was purchased from Samisaz Pharmaceutical Company (www.samisaz.com) with the patent certificate 83115 approved by (https://irangov.ir/ministry-of-health-and-medical-education), Iran. The prescribed dose of Krocina was 15 mg per day for four months. All patients also took diclofenac sodium (50 mg daily) as a conventional painkiller. Krocina tablets contain 15 mg of crocin with a purity of>90%.

Crocin Extraction and Purification

Crocin extraction and purification were done according to our previously published work from saffron stigmas by crystallization method at Bu-Ali (Avicenna) Research Institute, Mashhad University of Medical Sciences.[30] Each tablet contained 15 mg total crocin (>90% purity) extracted from saffron stigmas. Other excipients were microcrystalline cellulose (Avicel), polyvinylpyrrolidone (PVP), and magnesium stearate.[31] Crocin and placebo tablets were prepared in the Pharmaceutical Department, School of Pharmacy, Mashhad University of Medical Sciences laboratory.

Sample Preparation

Peripheral blood samples were collected in EDTA tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Cedarlane, Canada). MicroRNA was extracted using a microRNA extraction kit (GeneAll Company, South Korea) according to the manufacturer's instructions. The extracted microRNA was quantified by NanoDrop Spectrophotometer (Thermo Scientific. CO), and the absorbance ratios at 260/280 nm and 260/230 nm were measured to ensure microRNA purity. The extracted microRNA was immediately stored at -80 °C. Complementary DNA (cDNA) was prepared according to the manufacturer's instructions (EXIQON Company, USA).

SYBR Green Real-time Polymerase Chain Reaction

Primers were purchased from EXIQON (EXIQON Company, USA). Forward primer sequences were patented. Supplementary Table 1 presents the sequence of the forward and reverses primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene and the sequence of the reverse primers for all four microRNAs.

SYBR Green RT-PCR Master Mix kit (Yekta Tajhiz Azma, Iran) was used for real-time quantitative polymerase chain reaction (RT-qPCR) according to the kit's instructions. Rotor-Gene 6000 (QIAGEN, USA) was used to perform the RT-qPCR technique. PCR temperature of 95°C for 10 minutes was set for primary activation, followed by 40 cycles, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds. All RT-qPCR reactions were performed in duplicate. The $2^{-\Delta\Delta CT}$ method was used to assess the expression level of microRNA-146a, microRNA-155, microRNA-223, and microRNA-21 genes.[32,33]

Statistics and Data Analysis

All statistical analyses regarding microRNA data were compared before and four months after the intervention using SPSS 20 (IBM SPSS, Armonk, NY, USA) and Graph Pad Prism version 8 (Graph Pad

Prism Systems, Inc. CA, USA) software programs. The Kolmogorov-Smirnov test was used for analyzing normality. The paired t-test and Wilcoxon were used for parametric and nonparametric data, respectively. Cohen's d test was used to examine the effect size. $p<5\%$ was considered statistically significant.

RESULTS

Clinical and Laboratory Factors

Forty patients with idiopathic knee OA between 40 and 75 years old were included in the study, and 35 completed the four-month follow-up. All patients were assessed for VAS, and CRP and ESR were measured. Figure 1 depicts the CONSORT flow diagram.

Previously published works indicated that administering one or two crocin tablets daily is safe without serious side effects.[31,34] Also, some studies showed the efficacy of one crocin tablet (15 mg) per day in OA and other inflammatory diseases such as diabetic maculopathy.[17,18] This research selected 15 mg crocin/day as a safe and effective dose.

ESR, CRP and VAS Scores

CRP is an acute-phase plasma protein used as a marker to represent immune system activity.[35] It is also a serum biomarker in patients with OA.[36] The results showed that the CRP and VAS levels in OA patients receiving Krocina decreased significantly over 4 months. No change was observed in the ESR levels. Clinical symptoms were improved in OA patients after 4 months of receiving Krocina. For further details, please see our recent publication.[17]

Gene Expression of microRNA-146a, microRNA-155, microRNA-223, and microRNA-21 Before and After the Intervention

To determine the size of the SYBR Green Real-Time RT-PCR reactions for target microRNAs and the housekeeping genes, the product was electrophoresed on 2.5% agarose gel. Based on the Basic Local Alignment Search Tool (BLAST) query for primer specificity, the amplification fragment length of target microRNA genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected at 75 and 101 base pairs, respectively (Figure 2).

In the intervention group, the gene expression of miR-146a was 85.56±41.93 and 167.9±90.96 before and four months after the intervention, respectively. Moreover, in the placebo group, the expression of the miR-146a gene was 27.52±19.47 and 46.28±40.20 before and after the intervention, respectively. The results showed no considerable increase in miR-146a gene expression in the placebo (p=0.38) or Krocina (p=0.70) groups four months after the intervention. The effect sizes were 0.30 in the Krocina group and 0.15 in the placebo group.

In patients receiving Krocina, the gene expression of miR-155 was 0.22±0.072 and 1.86±0.7 before and after the intervention, respectively. In the Krocina group, a significant increase was found at the end of the study compared to the beginning (p=0.018). Moreover, the gene expression of miR-155 in the placebo group was 2.90±1.82 before and 0.56±0.16 after the four months (Figure 3).

Our results showed a non-significant reduction (p= 0.43) in the miR-155 gene expression in the placebo group. The effect size was 1.02 in the Krocina and 0.67 in the placebo group. This data indicates the significant effect of Krocina on miR-155 gene expression.

In the Krocina group, the mean±SEM gene expression of the miR-223 was 26.37±12.59 and 56.30±48.05 before and four months after the follow-up. The gene expression of miR-223 indicated a non-significant increase (p= 0.89) in the Krocina group. In the placebo receivers, the mean±SEM gene expression of miR-223 was 16.76±7.19 and 22.56±17.21 before and after the intervention, respectively, indicating a non-significant increase (p=0.89). The effect size was 0.24 and 0.11 in the Krocina and placebo groups, respectively.

The mean±SEM gene expression of miR-21 in Krocina receivers before and four months after the follow-up was 5.23±2.97 and 0.34±1.19, respectively. A significant reduction was found in the gene expression of miR-21 in the Krocina group (p= 0.023). The mean ± SEM gene expression of miR-21 in the placebo group was 7.88±4.19 before and 8.63±5.39 four months after the intervention. Our results showed no notable change between the two-time points (p=0.357). The effect size was 0.80 and 0.03 in the Krocina and placebo groups, respectively. This data shows a significant effect of Krocina on the *miR-21* gene expression.

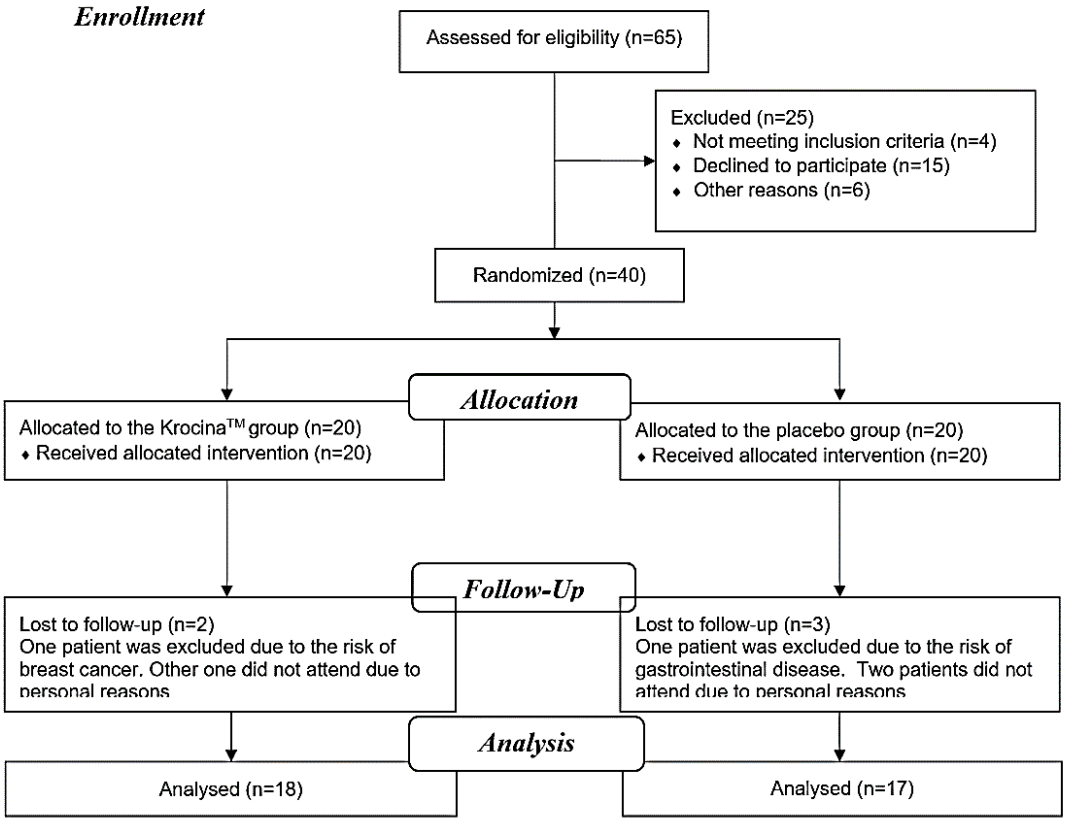

Figure 1. CONSORT flow diagram.

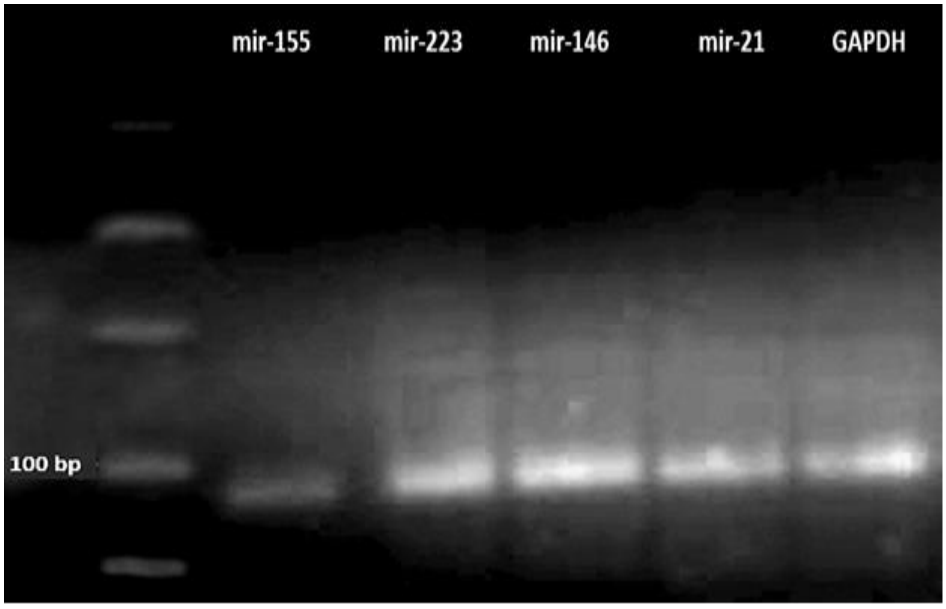

Figure 2: Gel electrophoresis of the SYBR Green real-time PCR products shows the specificity of the primers.

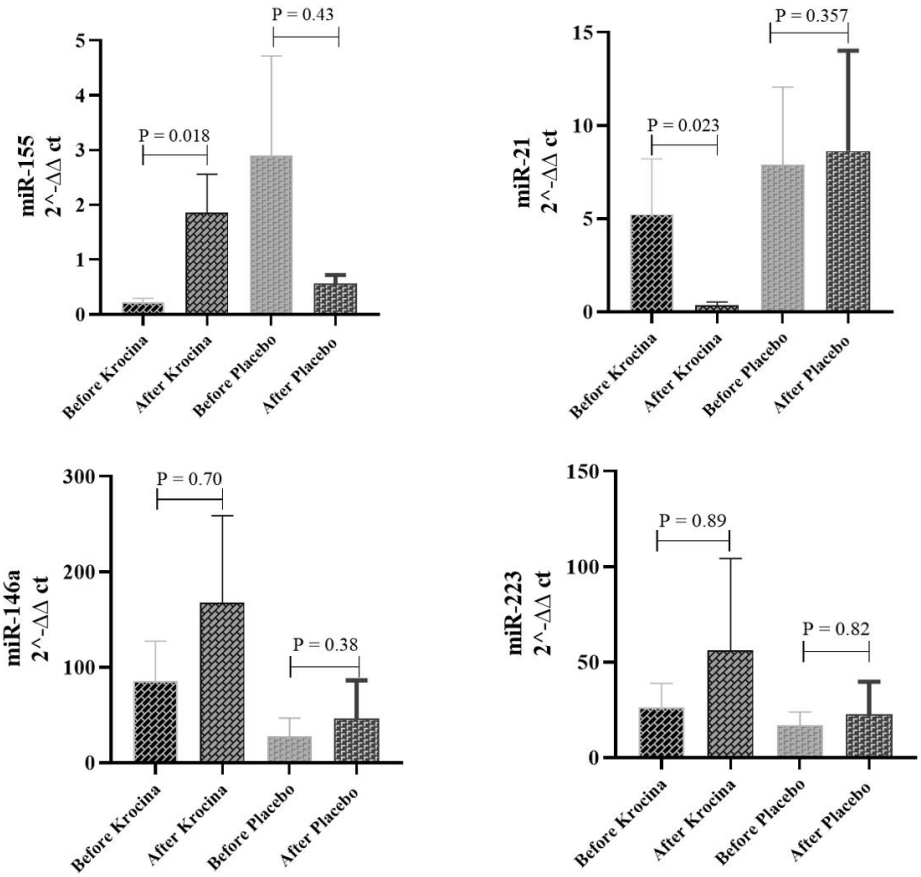

Figure 3. Gene expressions of miR-146a, miR-155, miR-223, and miR-21 in the placebo and Krocina groups before and four months after the intervention in osteoarthritis patients.

DISCUSSION

In this research, we aimed to assess the effect of Krocina on changes in the gene expression of miR-146a, miR-155, miR-223, and miR-21 in patients with OA compared with a placebo group. Our results show that (1) crocin demonstrated a significant effect in reducing inflammation, improving the clinical symptoms, and reducing pain in patients with OA; (2) CRP levels in patients receiving Krocina decreased significantly, but no change was observed in the patients' ESR levels; (3) the expression of the miR-155 gene was significantly increased in patients who received Krocina; (4) the expression of the miR-21 gene was significantly decreased in patients who received Krocina; and (5) no side effects were observed in patients who consumed Krocina. A previous study showed that saffron may reduce CRP levels due to its antioxidant and anti-inflammatory effects.[37]

Hemshekhar et al, examined the effects of crocin on reducing arthritic inflammation in rats. Their results demonstrated that crocin effectively neutralized the increased levels of enzymatic and non-enzymatic inflammatory mediators. Moreover, they showed that crocin, with its antioxidant properties, enhanced bone resorption, thereby restoring the damaged bone during inflammation and preventing recurrent arthritis.[38]

Ding et al, studied the effect of different doses of crocin on the expression of matrix metalloproteinases (MMPs) 1, 3, and 13 in an experimental model of OA in rabbits chondrocytes in the presence and absence of IL-1β. Their results revealed that cartilage destruction was improved after an intra-articular injection of crocin. Amelioration of the symptoms was due to the repressor effects of crocin on the expression of MMPs

1, 3, and 13 by suppressing the NF-κB transcription factor in the cartilage.[13]

A study on the functional effects and toxicity of crocin was conducted in 2014 and regarded 20 mg of crocin per kilogram of body weight for one month to be safe in healthy individuals.[39] In a study by Lee et al, daily administration of 30 mg of crocin for 10 days in OA rat models decreased muscle IL-6 levels, increased the citrate synthesis activity, and increased the synthesis of the myosin chain. [40] The results of another study showed no side effects after administering doses of up to 1.5 grams of saffron.[15] In our study, patients received a 15 mg tablet of Krocina daily for 4 months, and no side effects were observed.

Our results demonstrated improvements in the clinical and para-clinical indices of OA patients who received Krocina, which is in line with previous findings.[17] Our study showed that CRP and VAS levels of patients receiving Krocina decreased significantly during this period, but no change was observed in the patients' ESR levels. Crocin may be able to lower CRP levels due to its antioxidant and anti-inflammatory effects, resulting in reduced pain. Accordingly, CRP may be a relevant factor in predicting the OA progression and response to the treatment.

In the present study, a non-considerable rise was observed in the gene expression of miR-146a in Krocina receivers. The expression of this gene in the placebo group also showed a non-significant increase. Some microRNAs, including miR-146a, regulate knee joint homeostasis, cartilage inflammation, and the associated pain. Studies showed that the expression of miR-146a, an important therapeutic target for OA, was closely related to the stimulation of inflammatory mediators.[22]

Jones et al, described an association between microRNA expression in OA patients and cartilage function. Their results indicate that the gene expression of miR-146a is significantly higher in the early stages of OA compared with later stages. TNF-α production was also shown to be substantially reduced by overexpression of miR-146a, suggesting an anti-inflammatory effect of miR-146a; this causes it to be a valuable target for investigating the prevention of the OA progression.[41]

In the present study, the expression of the microRNA-155 gene was notably increased in patients receiving Krocina. However, the expression of this gene in the placebo group exhibited a significant decrease. Previous studies showed that miR-155 had effects on inflammatory and anti-inflammatory responses depending on the type of tissue involved.[23]

Okuhara et al. evaluated the gene expression patterns of miR-146a, miR-155, miR-188a, and miR-223 in PBMCs of OA patients. Their results revealed increased gene expressions of miR-146a, miR-155, and miR-223. They also found a significant increase in the expression of miR-146a and miR-223 genes in the early stages of OA compared to the late stages. However, they showed that miR-155 had a higher expression in the late stages of the disease (grades or III) compared with early stages (grade 0). Higher expression of these microRNAs increased the production of pro-inflammatory cytokines from PBMCs, which exacerbated the disease.[42]

Soyocak et al, investigated the role of miR-146a, miR-155, and c-Jun N-terminal kinase (JNK) in patients with OA. They observed a considerable increase in the miR-155 gene. They showed that the correlation between the regulatory effects of microRNAs and signal transduction pathways could enroll a fundamental role in OA pathogenesis and disease progression.[43]

Our results revealed a non-remarkable increase in the gene expression of miR-223 in both Krocina and placebo groups. MicroRNA-223 plays a crucial role in the homeostasis of the immune system. Studies have shown that miR-223 plays a role in various types of cancers and inflammatory and autoimmune diseases. MiR-223 stimulates osteoclast differentiation and function by suppressing NFIA expression. MiR-223 also plays a critical role in osteoclast formation and bone regeneration regulation. MiR-223 affects IL-1β production through the NF-κB signaling pathway. Moreover, it modulates macrophage differentiation by targeting the transcription factor IκB kinase-α (IKKα), and modulating the NF-κβ pathway. Decreased expression of miR-223 prevents the over-activation of macrophages.[22]

In our study, the gene expression of miR-21 was significantly decreased in patients receiving Krocina, but a non-significant increase was detected in the placebo group. Previous research has shown that miR-21 plays a vital role in the pathogenesis of OA and may have a potential therapeutic target. Furthermore, studies reported increased miR-21 expression in OA.[44] MicroRNA-21 was induced by many pro-inflammatory stimuli, such as pathogen-associated molecular patterns

(PAMP) and damage-associated molecular patterns (DAMP). It could trigger an inflammatory cycle activating immune cells.[28] Additionally, miR-21 can inhibit the expression of growth differentiation factor-5 (GDF-5), and its overexpression can slow OA progression. Moreover, miR-21 can significantly increase the levels of MMP1, MMP2, MMP3, and MMP9.[44] Delayed induction of miR-21 in inflammatory reactions indicate that miR-21 can negatively regulate the inflammatory process and play a crucial role in inhibiting inflammation and maintaining homeostasis.[28,45]

Wang et al. aimed to assess the expression of microRNA-21 in the cartilage of OA mouse models compared with the control group. Their results revealed that the expression of the miR-21 gene in the OA cartilage tissue was significantly increased. Moreover, they showed that selective removal of miR-21 considerably reduced the destruction of articular cartilage in mice.[46] In our study, the gene expression of miR-21 was decreased with Krocina consumption, which might be related to a subsequent decrease in inflammation and improvements in OA patient signs and symptoms.

In conclusion, this investigation aimed to assess the effect of Krocina on the gene expression of miR-146a, miR-155, miR-223, and miR-21 in patients with OA. Crocin has anti-inflammatory activities and can effectively reduce inflammation and clinical symptoms. Our results demonstrated that crocin treatment had no side effects; therefore, in patients with OA, it can be described as a possible herbal medicine with analgesic and anti-inflammatory effects. Moreover, given the results of crocin on the regulatory role of miR-21 and miR-155, targeting these small molecules may be the subject of future studies on the progression, diagnosis, and therapy of OA. Including a group of OA patients who just received the conventional treatments to compare the results with intervention and placebo groups is suggested to get a better conclusion in future works. The main limitations of our study include small sample size and a lack of long-term follow-up. As our study was designed as a pilot clinical trial, we suggest enrolling more patients in future studies and increasing the follow-up period. Considering the limitations of the previous studies, we tried to select a dose and an appropriate follow-up time for crocin according to the standardization protocol. We also included only the patients in grades 2 and 3 knee OA. Inclusion and exclusion criteria were precisely considered to select patients with OA who were eligible to participate in our study. It was one of the most vital points to overcome the limitations of the previous studies.

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest to disclose.

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