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A Significant Reduction in the Plasma Levels and Gene Expression of CCL2 in Patients with Osteoarthritis following Intervention with Krocina™

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Abstract

Background: inflammatory chemokines such as CCL2 and CCL5 are involved in the progress of osteoarthritis. Crocin with antioxidant and anti-inflammatory properties can reduce the symptoms of osteoarthritis (OA). This study was performed investigate the effect of Krocina™, on the gene expressions and plasma levels of CCL2 and CCL5 in OA patients.

Methods: The study included 35 patients that were randomized in the Krocina™ and placebo groups. The intervention was Krocina™ 15 mg daily for four months. Clinical and paraclinical parameters were measured. CCL2 and CCL5 genes expression and plasma levels were determined using the SYBR Green Real-Time RT-PCR and Enzyme-linked Immunosorbent Assay (ELISA) techniques.

Results: The C-reactive protein (CRP) value in the Krocina™ group and the visual analogue scale (VAS) value in the Krocina™ and placebo groups decreased significantly after the intervention. The gene expression of CCL2 in the Krocina™ and placebo groups decreased significantly. On the contrary, the gene expression of CCL5 in the Krocina™ and placebo groups increased significantly. Moreover, the plasma levels of CCL2 in the Krocina™ and placebo groups decreased meaningfully. There was no difference regarding the plasma levels of CCL5 within the Krocina™ and placebo groups before and after the intervention in either of the groups.

Conclusions: Administration of Krocina™ reduced the clinical signs of inflammation and CRP and VAS value. Also, Krocina™ significantly decreased the plasma levels and gene expression of CCL2 in osteoarthritis patients.

Keywords: CCL2, CCL5, Krocina™, Osteoarthritis.

Introduction

Osteoarthritis or arthrosis is one of the most common forms of joint disorders among the elderly affecting more than 25% of people over the age of 18 years (1). Osteoarthritis is associated with progressive destruction of the articular cartilage, subchondral bone remodeling, osteophytic formation, and synovium inflammation. The pathogenesis of osteoarthritis seems to result from a complex interaction between mechanical, biochemical,

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and cellular forces. The major risk factor of OA is obesity, and mechanical factors can contribute to its progression (2).

Inflammation of the synovial is a medical condition confirmed in OA. This inflammation is chronic and low-grade and mainly caused by the innate immune system and plays an important role in the pathogenesis of OA. Inflammation of the synovial lining is detected by the infiltration of the inflammatory cells into the synovium and may increase cartilage degradation (3). Previous studies showed the role of chemokines and their receptors in OA progress. (4). Chemokines, by altering the cellular reaction patterns of chondrocytes, caused changes in bone tissue and the destruction of articular cartilage via degradation of the extracellular matrix (ECM) with matrix metalloproteinases (MMPs) and N-acetyl-b-D-glucosaminidase (NAG) (5).

Various cell types such as epithelial cells, fibroblasts, monocytes, and chondrocytes are able to secrete CCL5 and CCL2 (6). These chemokines are capable of attracting monocytes, dendritic cells, basophils, and T cells to inflammatory regions (7).

In osteoarthritis, the gene expression and serum levels of inflammatory chemokines such as CCL2 and CCL5 are higher than healthy subjects (8–10). These chemokines not only stimulate the expression of MMP-3 but also inhibit the synthesis of proteoglycans and increase the release of proteoglycan from chondrocytes (5,11,12). Studies show that CCL5 and its receptor facilitate the onset and progression of OA (3). CCL2 and its high-affinity receptor (CCR2) also have a central role in the pain associated with osteoarthritis of the knee (8).

Treatments of osteoarthritis include drug therapy, surgery, and complementary therapies. Reduction of pain and inflammation, overcoming knee problems, growth in physical function and ultimate improvement in the individuals' quality of life are of most important purposes of its treatment. However, the treatments used for these patients are mainly nonsteroidal and anti-inflammatory drugs that are temporarily effective with cardiovascular, hepatic and digestive complications (2,13). Hence, the use of medicinal plant compounds, which has the least possible side effects can be very significant in the treatment of inflammatory osteoarthritis (14).

In recent years, multiple studies have been conducted on the use of saffron to reduce inflammatory symptoms in patients with OA. Picrocrocin, safranal, and crocin are the main metabolites of Saffron (a spice obtained from the flower stigmas of *Crocus sativus L*). Picrocrocins are the main components responsible for the bitter taste of saffron. The aroma of saffron is due to volatile oil called safranal and the color of saffron is due to carotenoids known as crocins (15,16). The crocin family contains various glycosyl esters that are obtained from the binding of glucose molecules to the two acidic groups found in Agclycon Crocetin, and six types of them are identified in saffron (17).

Crocin (a saffron-derived ingredient) has an anti-inflammatory function (18), which is done by reduction in the oxidative stress and absorption of free radicals, inhibiting nitric oxide induced by lipopolysaccharide (LPS) and inhibiting the synthesis of pro-inflammatory cytokines. Moreover, it can reduce the symptoms of osteoarthritis (14,19). With this consideration, we intended to evaluate the impact of a herbal medicine made of crocin called Krocina™ on plasma levels and gene expression of CCL2 and CCL5, in patients with knee OA.

Materials and Methods

Patients

According to the previous study by the same team (20), 40 patients with primary grade two or three knee osteoarthritis who did not undergo joint replacement surgery and were in accordance with the criteria of American College of Rheumatology (ACR) were identified and enrolled from July 2016 to June 2018. Inclusion and exclusion criteria were reported in our previously published article (20). All patients gave informed consent to participate in the study. Of these, 35 patients

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participated in the final follow-up, of which 18 patients were in the Krocina™ group and 17 patients in the placebo group. The placebo group received placebo in addition to conventional therapy and the Krocina™ group also received a 15 mg Krocina™ tablet daily for four months in addition to usual treatment.

All patients had the same drug regimen including diclofenac sodium tablets (two tablets of 25 mg daily). They were all examined in terms of clinical manifestations, paraclinical tests, gene expression, and plasma levels of CCL2 and CCL5 chemokines before and four months after the intervention**.**

The study was registered in IRCT (IRCT2015021910507N2) and ClinicalTrials.gov (identifier: NCT03375814) and was approved by the ethics committee of Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.sm.REC.1395.246).

SYBR Green Real-Time RT-PCR

Ten milliliter of venous blood was taken from patients prior to the administration of the Krocina™ or placebo and 4 months later. After the isolation of peripheral blood mononuclear cells, the total RNA extracted, and cDNA were synthesized by the Yekta Tajhiz kit (yektatajhiz.com) and in accordance with its instructions. The quality and quantity of RNA and cDNA were evaluated by agarose gel electrophoresis and NanoDrop apparatus (Thermo Fisher Scientific, USA), respectively**.**

Two microliter of extracted RNA was used for cDNA synthesis (www.yektatajhiz.com) (Biometra, Germany). Four milliliter cDNA was used for PCR reaction (Rotor-Gene 3000, Australia). Incubation steps were as follows**:**

5 minutes at a temperature of 60 ℃, 60 minutes at a temperature of 42 ℃, 5 minutes at a temperature of 70 °C.

Then four mL of cDNA diluted at a dilution of 1: 5 was added to five milliliter SYBR Green master mix made from TAKARA (www.takarabio.com), o.4 µl forward and reverse primer (10 pmol / µL) (www. afragen.com) and 0.2 µl of molecular water and then PCR performed with a 40 cycles program as follows;

95 ℃ for ten seconds for denaturation, 60 ℃ for 30 seconds for annealing and 72 ℃ for 20 seconds for extension. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was recruited as an internal and reference gene (21). Employing the double-delta CT method, the gene expression level of chemokines is compared with the expression of the GAPDH gene**.** Forward and reverse primers were designed in house and their sequences presented in Table 1.

Table 1. Primer sequence of GAPDH, CCL2 and CCL5 genes.

	GAPDH	**CCL2**	**CCL5**
Forward Primer	5'CACTAGGCGCTCACTGTTC TC3'	5'AAACTGAAGCTCGCACTCT CG3'	5'CCTGCTGCTTTGCCTACATT GC3'
Reverse Primer	5'CCAATACGACCAAATCCGT TGA3'	5'TTGATTGCATCTGGCTGAG CG3'	5'ACACACTTGGCGGTTCTTT CGG3'
Accession number	**P04406**	**J3KRT7**	**D0EI67**

ELISA

CCL2 and CCL5 plasma levels in patient samples were measured before and four months after intervention employing an ELISA kit (www.biolegend.com) according to the protocol (Bio Tek Instruments, Inc,USA)**.**

Statistical Analysis

All data were reported as mean ± SEM. Kolmogorov-Smirnov test was used to determine the normality of the data. The variables were non-parametric and the Mann-Whitney and Wilcoxon statistical tests were used to analyze the data by prism 7. In all

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calculations, p< 0.05 was defined for the statistical significance level**.**

Results

Patient information

Patients participating in the study (15 women and two men in placebo group/ 11 women and seven men in Krocina™ group) with an average age of 57.97 years and a range of 42 to 68 years old included 26 female patients with an average age of 58.23 years and a range of 46-68 years old and nine male with an average age of 57.22 years and range from 42 to 67 years old. The duration of the disease in patients from the placebo and Krocina™ groups was 4.39±2.28 and 4.26±2.38 years from the time of diagnosis (p-value= 0.88). Details of the demographic characteristics of patients with knee osteoarthritis participated in the study were reported in our previously published article (20).

Clinical and laboratory indicators

The clinical findings of this study included VAS. Reduction of VAS for pain index in each of the placebo and Krocina™ groups was significant after the intervention (p-value= 0.002 and 0.000, respectively)**.** Comparison of mean values of erythrocyte sedimentation rate (ESR) before and after the intervention was not significant in any of the placebo and Krocina™ groups (p-value= 0.11 and 0.67, respectively). The amount of CRP in Krocina™ group after the intervention compared to before the intervention decreased significantly (p-value equal to 0.046). Comparison of mean values of CRP in placebo group before and after the intervention was not significant (p-value= 0.95). Details of the clinical and paraclinical findings of the placebo and Krocina™ groups before and after the intervention were reported in our previously published article (20).

Gene expression of CCL2 and CCL5 chemokines in peripheral blood mononuclear cells of patients with knee osteoarthritis

Gene expression of CCL2 and CCL5 were calculated as $2^{-\Delta\Delta Ct}$ and are shown in Figure 1. The gene expression of CCL2 in both groups of Krocina™ and placebo decreased significantly after the intervention compared to the pre-intervention (Table 2). CCL5 gene expression in the groups studied at the end of 4 months drastically increased compared to the pre-intervention (Table 2). Comparison of CCL2 and CCL5 genes expression between Krocina™ and placebo groups before the intervention and also after the intervention was not significant (Table 2). Figure 1 compares the gene expression of CCL2 and CCL5 in peripheral blood cells of patients with osteoarthritis in the Krocina™ and placebo groups separately, before and after the intervention. Figure 2 compares the gene expression of CCL2 and CCL5 between the Krocina™ and placebo groups before and after the intervention.

Table 2. Comparison of Gene expression of CCL2 and CCL5 chemokines in the placebo and Krocina™ groups before and after the intervention.

Variables	Groups	Before the intervention (mean±SEM)	After the intervention (mean±SEM)	p- value
CCL2 gene expression	Placebo	2.35±0.73	0.62±0.17	**0.048**
	Krocina™	2.70±0.77	1.08±0.34	**0.014**
	p-value	0.790	0.206	**-**
CCL5 gene expression	Placebo	2.06±0.52	5.79±2.46	**0.004**
	Krocina™	1.83±0.48	5.26±2.47	**0.037**
	p-value	0.682	0.840	**-**

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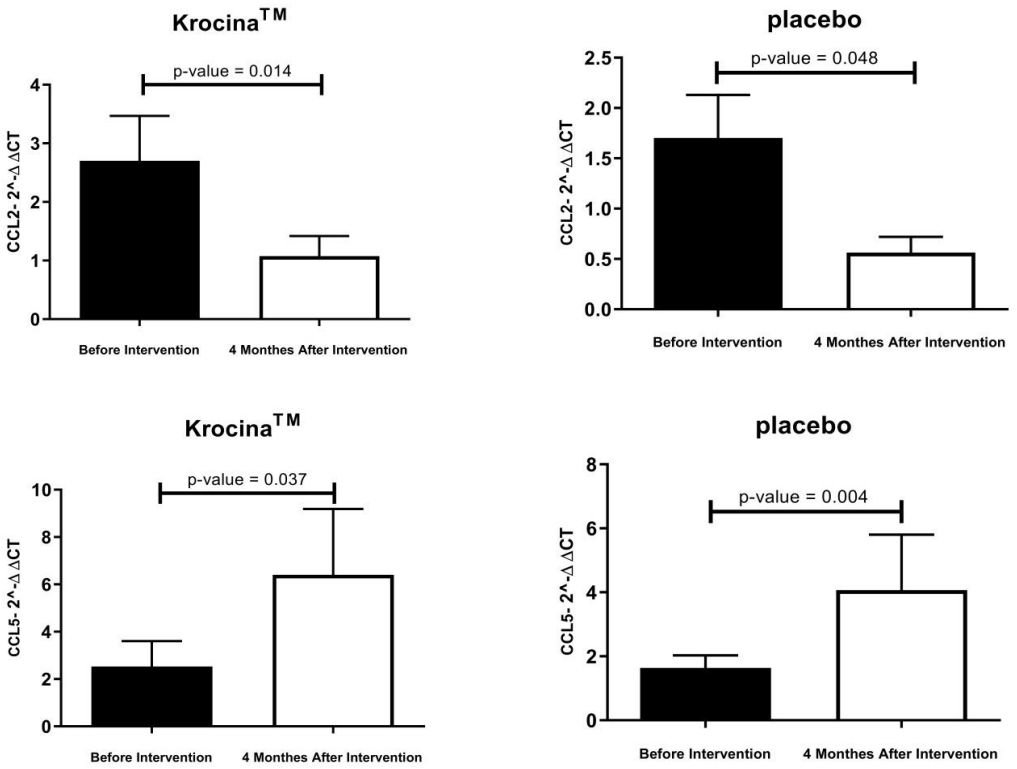

Fig. 1. SYBR-Green Real-Time RT PCR findings before and after prescription of Krocina™ and placebo. (A) CCL2 gene expression in Krocina™ group. (B) CCL2 gene expression in placebo group. (C) CCL5 gene expression in Krocina™ group. (D) CCL5 gene expression in placebo group. Data are provided as mean±SEM.

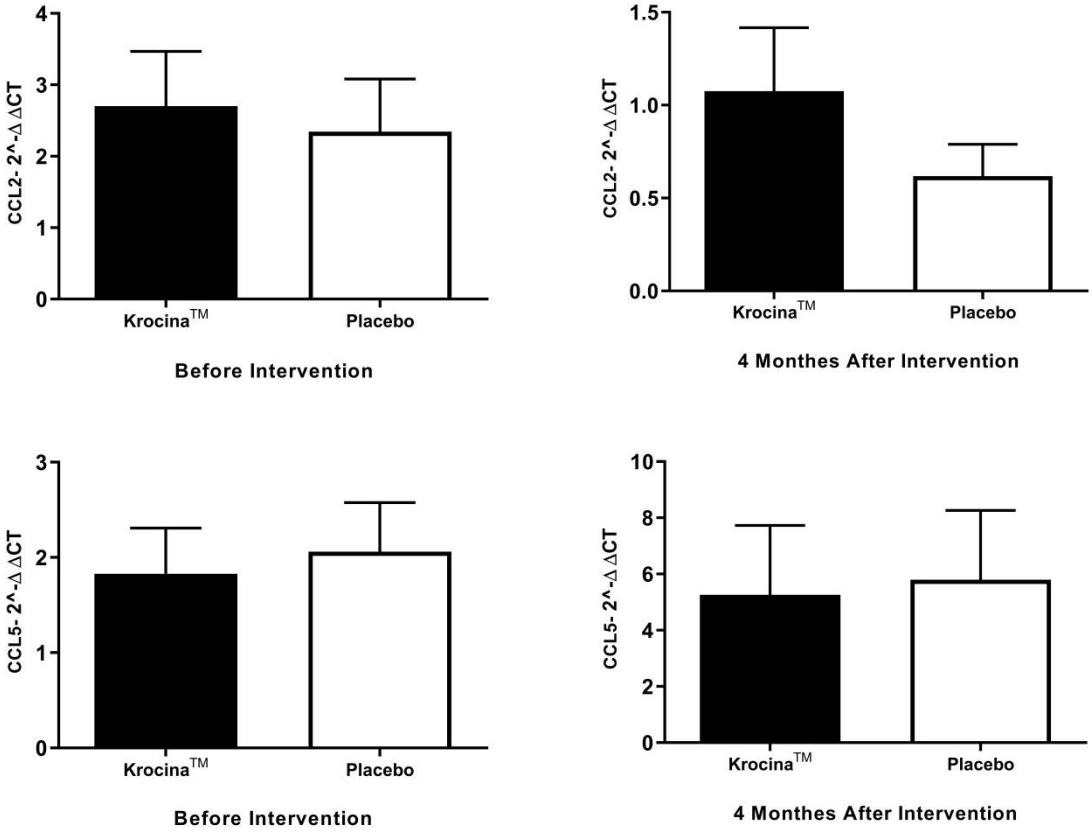

Fig. 2. SYBR-Green Real-Time RT PCR findings before and after administration of Krocina™ and placebo. (A) CCL2 gene expression before the intervention. (B) CCL2 gene expression after the intervention. (C) CCL5 gene expression before the intervention. (D) CCL5 gene expression after the intervention. Data are provided as mean±SEM.

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Plasma levels of CCL2 and CCL5 in patients with OA

The levels of CCL2 chemokine in the Krocina™ and placebo groups after the intervention was significantly lower than before the intervention (Table 3). In both groups, no significant change was observed in the levels of CCL5 after the intervention compared to before the intervention. Comparison The plasma levels of CCL2 and CCL5 chemokine between the Krocina™ and placebo groups before the intervention and also after the intervention was not significant (Table 3). Figures 3 and 4 show the average changes in plasma levels of these two chemokines.

Table 3. Comparison of Plasma levels of CCL2 and CCL5 of patients with knee osteoarthritis in the placebo and Krocina™ groups before the intervention and after the intervention.

Variables	Groups	Before the intervention (mean±SEM)	After the intervention (mean±SEM)	p- value
CCL2 plasma level (ρg/ml**)**	Placebo	155.5±15.99	102.2±11.57	**0.024**
	Krocina™	149.6±14.18	78.20±6.17	**0.002**
	p-value	0.786	0.180	-
CCL5 plasma level (ηg/ml**)**	Placebo	76.77±12.71	67.85±12.03	**0.689**
	Krocina™	88.42±10.30	104.8±16.59	**0.302**
	p-value	0.479	0.077	-

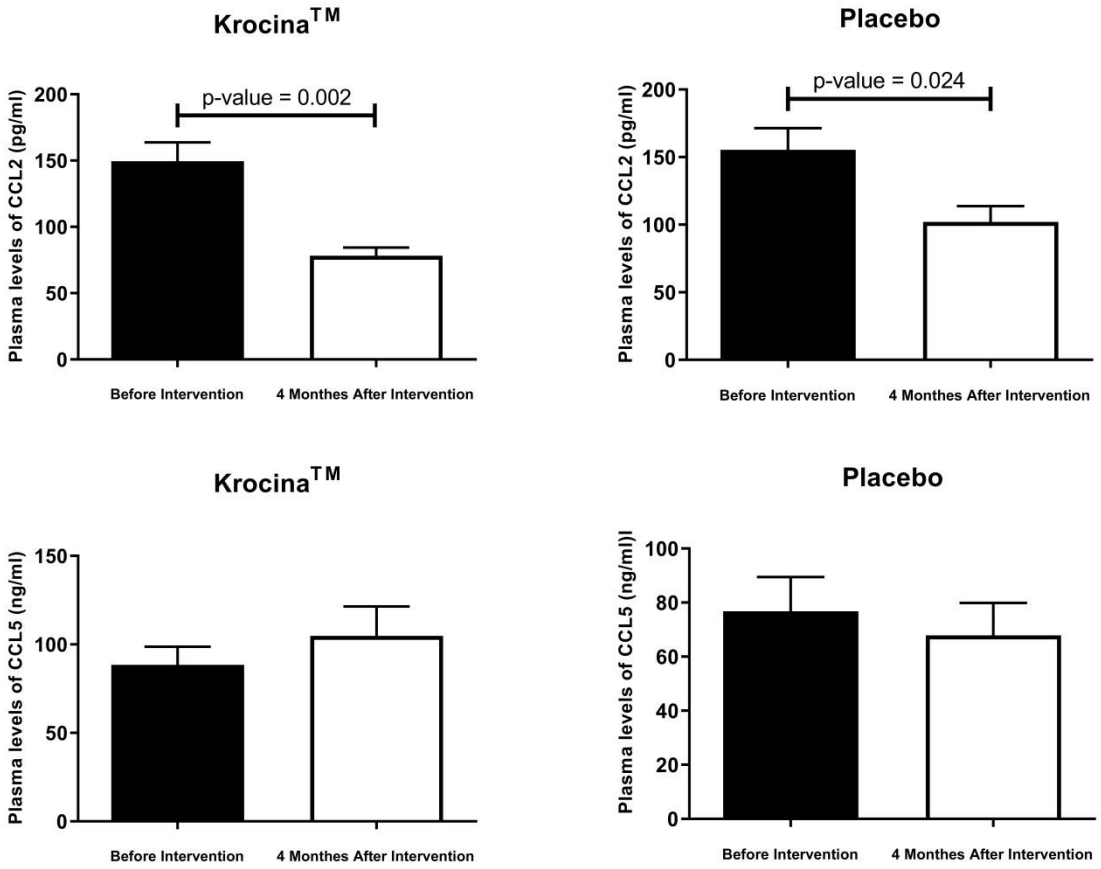

Fig. 3. ELISA findings before and after administration of Krocina™ and placebo. (A) CCL2 plasma levels in the Krocina™ group. (B) Plasma levels of CCL2 in the placebo group. (C) Plasma levels of CCL5 in the Krocina™ group. (D) CCL5 plasma levels in the placebo group. Data are provided as mean±SEM.

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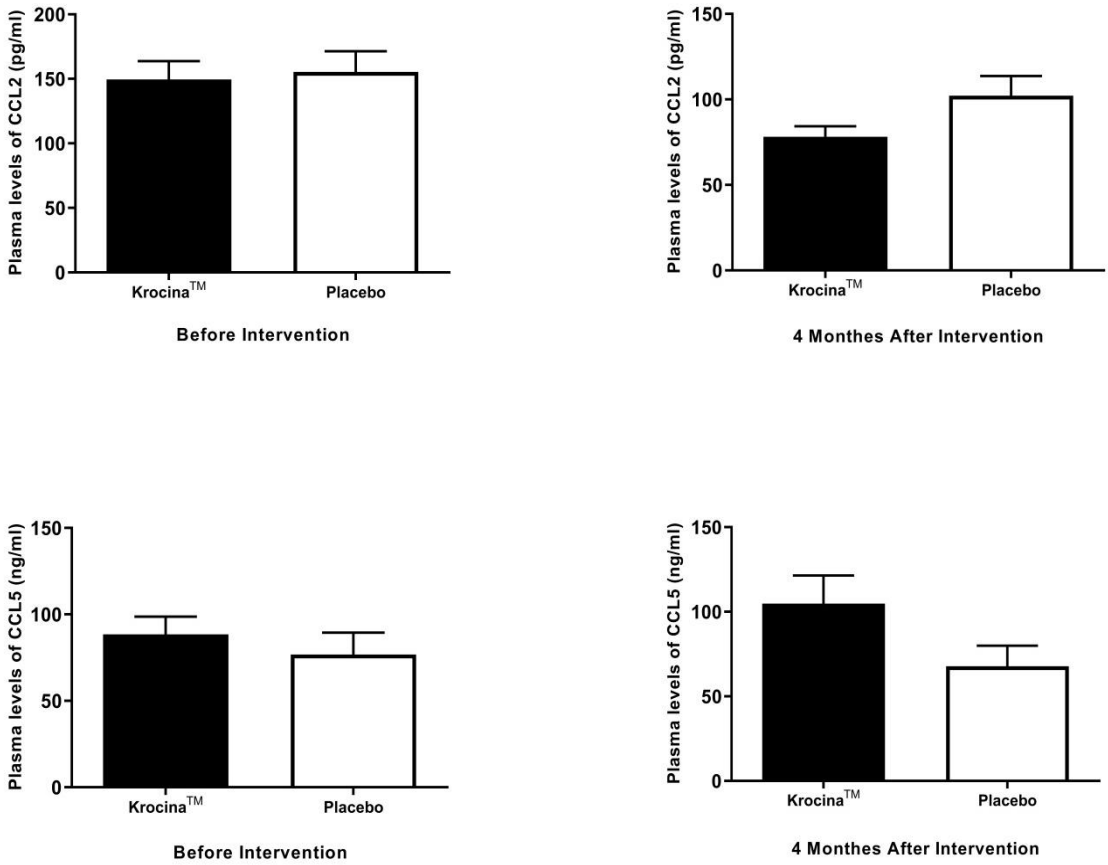

Fig. 4. ELISA findings before and after administration of Krocina™ and placebo. (A) Plasma levels of CCL2 before the intervention. (B) Plasma levels of CCL2, after the intervention. (C) Plasma levels of CCL5 before the intervention. (D) CCL5 plasma levels after the intervention. Data are presented as mean±SEM

complications and side effects

None of the 35 patients had any complications or side effects during or after the intervention.

Discussion

In this study that the effect of Krocina ™ as a herbal medicine containing crocin was examined on the gene expressions and plasma levels of CCL2 and CCL5 chemokines in patients with osteoarthritis; We found that treatment with Krocina™ could reduce the clinical signs of inflammation and CRP and VAS levels.

According to our recently published data (20) the VAS factor for pain index in the placebo and Krocina™ groups decreased significantly after the intervention although the statistical significance of this factor in the Krocina™ group was greater than the placebo group.

Given the previous studies, the placebo is particularly effective in pain reduction, stiffness and function reported by patients with OA, and factors such as the strength of the treatments, the severity of OA at the onset of the disease, the drug administration route and the sample size of studies, showed the impact on the placebo effects (22). The results of another meta-analysis, which were performed to evaluate the effect of placebo in clinical trials in the field of medical and psychotherapy, also indicates the strong effect of placebo (23). Accordingly, the reduction of VAS in the placebo group in our study might be attributed to the self-hypnosis effects of placebo or to the effects of non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, that are used routinely in these patients.

The results of our recently published article indicated a significant decrease in CRP levels in the Krocina ™-receiving group after the intervention. There was no crucial difference in the ESR in the group receiving the Krocina ™ before and after the intervention (20).

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Few studies have investigated the effect of saffron or its components on the amount of inflammatory markers of ESR and CRP. These studies have examined the effects of different doses of saffron's aqueous extract, crocetin, or crocin in animal and human models of diseases such as rheumatoid arthritis and schizophrenia or healthy volunteers (24–26). More investigations are required for a better conclusion on the effects of saffron and its ingredients on inflammatory markers.

Based on the search results in the databases, it seems that there have been no studies on the effect of crocin on the plasma level or gene expression of the chemokines in animal or human models of osteoarthritis. The impact of saffron components such as crocin or crocetin has been studied on gene expression of chemokines such as CCL2, CCL17 and CCL22, or secretion of these chemokines in the supernatant of various cells such as microglia BV-2, human umbilical vein endothelial cells (HUVEC), lung and skin tissues, whose results showed a decrease in gene expression and secretion of this chemokines (27–29).

Chemokines, by altering the cellular reaction patterns of chondrocytes, cause changes in bone tissue, as well as through destruction of the ECM by the MMPs and NAGs, leading to degradation of articular cartilage (5). CCL5 and CCL2 inhibit the synthesis of proteoglycans and also induce proteoglycan release from chondrocytes (11). Gene expression and serum level of inflammatory chemokines, including these two chemokines in OA patients, are higher than in healthy subjects (8–10).

Our results show a significant reduction in the gene expression and plasma levels of CCL2 in both the Krocina ™ and placebo groups after the intervention. Therefore, a greater decrease in the plasma level of this chemokine after the intervention in the Krocina ™ group compared to the placebo group can be considered as the effect of the Krocina™ . On the other hand, the significant reduction of this chemokine in the placebo group may be related to the effects of taking anti-inflammatory drugs such as diclofenac sodium in these patients or on the empathic effects of placebo. On the one hand, CCL5 gene expression had a significant increase in both the Krocina ™ and placebo groups at the end of the intervention. However, plasma levels of this chemokine in the studied groups did not change significantly after the intervention.

Based on previous studies, it has been shown that protein concentrations are often not proportional to the concentration of mRNA, and less than 40% of the protein content can be predicted by mRNA measurements. Also, the possibility of the effects of regulating micro RNAs and long noncoding RNAs (lncRNA) on the expression and translation of the genes may be suggested to justify the lack of concurrent results of the expression of the gene and plasma levels of CCL5 (30). MicroRNAs are non-coding single-strand RNAs with a length from 18 to 24 nucleotides that regulate the expression of genes (31). LncRNA is also a type of RNA molecule with more than 200 nucleotides and lacks an open reading frame with considerable length. LncRNAs are involved in various biological processes, including transcription and translation (32,33). Another possibility is that the post-transcriptional control mechanisms, affects the protein level. In addition to the copy number of the genes, other mechanisms, such as mRNA processing, mRNA localization, mRNA stability, translation regulation and protein degradation, can affect the expression levels of a protein (34).

To the best of our knowledge, our study is the first on the effect of crocin on CCL2 and CCL5 chemokines in patients with knee osteoarthritis. Here, we discovered that Krocina™ treatment can reduce the clinical signs of inflammation and the levels of CRP and VAS. In this study, gene expression and plasma levels of the chemokine CCL2 in Krocina™ and placebo groups decreased significantly after the intervention. The prominent role of Krocina™ on the significant reduction of this chemokine illustrates itself regarding the fact that the reduction of plasma

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levels of this chemokine in the Krocina™ group is more than the placebo group after the intervention**.** The gene expression of CCL5 chemokine significantly increased in both groups of Krocina™ and placebo after the intervention than before the intervention. However, there was no significant change in its plasma level within the groups, and it may be concluded that the Krocina ™ drug has an effect just on the plasma level of CCL2 chemokine. It is also likely that Krocina ™ has been effective in reducing inflammation of patients by affecting other chemokines and inflammatory mediators.

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