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Immunoregulatory Effects of Krocina™, a Herbal Medicine Made of Crocin, on Osteoarthritis Patients: A Successful Clinical Trial in Iran

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ABSTRACT

Osteoarthritis (OA) is the major cause of joint pain and disability. This research was planned to examine the effects of Krocina™, aherbal medicine made of crocin, an ingredient of saffron, in patients with OA.

Forty patients suffering from OA were enrolled in our study and randomly divided into two groups, receiving Krocina™ and placebo, and the clinical trial continued for four months.Peripheral blood was taken from all patients and the percentage ofvarious subsets of T cells in addition to the levels of forkhead box protein P3 (FOXP3) and interleukin (IL)-17 were measured by flow cytometry technique.

The visualan alog scale (VAS) index analysis decreased significantly in both groups (krocina™ and placebo) (p<0.05). Assessment of the C-reactive protein (CRP) level in serum showed a significant decrease in the krocina™ group (p<0.05). Moreover, we found a meaningful increase in the percentage of regulatory T cells (Tregs)cellin samples gathered from Krocina™ group (P=0.02) patients. The mean percentages of T helper (Th) 17 cellsinthe Krocina™ group and CD8+ T cellsin the placebo group patients were also meaningfully reduced (p<0.05). The geometric mean fluorescence intensity (GMFI) for IL-17 showed a significant decrease and increase in Krocina™ and placebo groups, respectively (p<0.05). No noticeable difference was observed in the percentages of Th cells and GMFI-FOXP3 in either group. Treg/Th17 ratio was shifted towards Tregscell in Krocina™ group at the end of the intervention.

It is concluded that Krocina™ has immunoregulatory effects on patients with OA, ameliorating the disease.

Keywords: Clinical trial; Crocin; Immune regulation; Inflammation; Osteoarthritis; T helper cells

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INTRODUCTION

Osteoarthritis (OA) is considered as one of the most prevalent arthritis diseases and the most important cause of disability in the elderly presented with pain, motor limitation and knee joint involvement.[1]OA is classified as a non-inflammatory disease based on radiological findings, while available information has indicated that the inflammatory responses have influential roles in the development and progression of OA disease. [2]

Additionally, an extraordinary increase has been reported in the secretion of inflammatory cytokines and disintegration of cytokine equilibrium in OA.[3] Moreover inflammatory cytokines, and adaptive immune-related cytokines such as Interleukin (IL)-2 and interferon (IFN)-γ exist in arthritic joints, implying the presence of T helper (Th) cells.[4] In the pathogenesis of OA, the levels of cytokines such as tumor necrosis factor (TNF)-α and IFN-γ are increased, causing the activation of destructive enzymes such as matrix metalloproteinases (MMPs) in extracellular matrix (ECM) and an increase in the levels of C-reactive protein (CRP)[5]and erythrocyte sedimentation rate (ESR) as inflammatory biological symptoms.[6]

Currently, existing treatments mostly relieve the pain and its symptoms, but fail to halt the disease progression. So far, no effective drugs have been found able to affect the course of the disease.[7]

Nowadays, treatments are performed with acetaminophen, topical ointments, non-steroidal anti-inflammatory drugs (NSAIDs), or their concomitant prescription.[7] If the above treatments do not prove effective in relieving the pain, especially in case of joint swelling, intra-articular corticosteroids are recommended, possibly followed by surgery. The recent studies on the mechanisms of disease progression have influenced the joint condition by administering moderating drugs such as chondroitin sulfate and glucosamine.[8]

Krocina™ containing crocin (15 mg) is a water-soluble carotenoid made from the saffron extract and has anti-tumor,[9] anti-oxidant, and anti-inflammatory effects.[10] Moreover, Liu and colleagues reported the administration of crocin in the treatment of inflammatory diseases in animal models of arthritis. They reported a reduction in the synovitis and infiltration of inflammatory cells to synovial after administering crocin. Additionally, they showed that crocin inhibited cartilage erosion and postponed chondrocyte apoptosis. They also showed a reduction in serum levels of MMP-1, -3 and 13, TNF-α, IL-17, IL-6 and CXC motif chemokine ligand (CXCL)-8 (known as IL-8) following administration of crocin.[11]

For the first time, we have attempted at investigating the palliative effects of Krocina™ on the clinical and paraclinical parameters and percentage changes of The cells, regulatory T cells (Tregs), T helper 17 (Th 17) cells, CD8+ T cells and geometric mean fluorescence intensity (GMFI) of IL-17 and forkhead box protein P3 (FOXP3)y in OA patients.

MATERIALS AND METHODS

Patient Selection

This research is a randomized, double-blind study controlled by placebo. Forty participants living in Mashhad, capital of Khorasan Razavi province in Iran, referred to the Imamreza clinic and were diagnosed with Knee OA by a rheumatologist. Knee OA was identified according to the American college of rheumatology (ACR) criteria.[12]

Inclusion criteria for all subjects were (i) age range of 40-70, (ii) degenerative primary knee OA with grade 2 or 3 based on the Western Ontario McMaster University Osteoarthritis Index (WOMAC) scoring[13]and the Kellgren and Lawrence (KL) grading.[14]Visual analog scale (VAS) wasemployed to assess the pain severity of patients.[15] Twelve subjects were grade 2 and 23 were grade 3.

Patients were excluded if (i) they were not aged between 40 to 70, (ii) they were allergic to Krocina™ and placebo, and (iii) had a history of joint surgery for replacement over the past three months (iv) diabetic disorders, heart and kidney diseases, rheumatism, osteonecrosis and gout (v) a long history of anti-inflammatory drugs consumption (vi) challenging jobs, and (vii) a body mass index (BMI) of 30 or more.[16]

All subjects signed a written consent form before the study. Our research was executed in concurrence with the ethical consideration of the Declaration of Helsinki.

Our clinical trial was recorded in the Iranian Registry of Clinical Trials (IRCT) and ClinicalTrials.gov with the code of 2015021910507N2 and NCT03375814, respectively. Our clinical trial commenced in July 2016 and continued to June

2018 following the approval by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran, with a reference number of IR.MUMS.FM.REC.13940279. To consider the ethical issues, all patients who participated in our clinical trial took sodium diclofenac (50 mg/daily) as the routine pain killer treatment for OA.

Forty patients with OA were randomized into the two groups,20 patients in the Krocina™ group and 20 patients in the placebo group. During this period, two patients in the intervention group were excluded from the trial, one of which because of the risk of breast cancer and the other one for personal reasons. Three patients in the placebo group stopped the trial, one of which due to the risk of gastrointestinal disease and two for personal reasons. Ultimately 18 patients in the Krocina™ group and 17 in the placebo group finished the clinical trial for four months.

Assessment of Liver Aminotransferases Preparation of Krocina™ and Placebo

Krocina™ and Placebo were purchased from Samisaz Pharmaceutical Company (www.samisaz.com) with a patent certificate number of 83115 on 16 June 2014 from the Ministry of Health and Medical Education, Iran.

The administration dose of Krocina™ was 15 mg/daily for 4 months. All subjects in the intervention group received Krocina™ tablets once a day for 4 months, and the placebo group received placebo tablets on the same basis.

Randomization

Employing a computer-generated code, patients were randomly divided into 2 groups: (1) for intervention group who received Krocina™ tablets and (2) placebo group receiving placebo tablets. All patients with knee OA were monthly visited by a rheumatologist for checkup and monitoring of any side effects of Krocina™ or placebo.

Demographic and Anthropometric Data

For all patients with knee OA, anthropometric parameters including weight and height were recorded employing a standard protocol at the end of overnight fasting. The height and weight were calculated by specific scales with an accuracy of 0.1 cm and 0.1 kg, respectively; BMI was then gained through the following formula; BMI=(Weight(kg))/(Height(cm^2)) and data are shown in Table1.

Pain Scores

Assessment of pain was performed by the VAS index as patients assessed their pain severity from 0 to 10, where 0 indicates “no pain” and 10 is the maximum score.

Blood Sampling and Cell Culture

Blood sampling was done for all patients and transferred into tubes containing EDTA. Afterward, only peripheral blood mononuclear cells (PBMCs) were maintained in gradients of ficoll and cultivated incomplete tissue medium (CTM) containing RPMI+10% fetal bovine serum (FBS) plus penicillin/ streptomicinewith 5 µg/mL phytohemagglutinin (PHA, Bio west, USA) at 37°C in a humid incubator with 5% CO_2.

Para-clinical Methods for the Measurement of CRP and ESR

There are various methods (quantitative and qualitative) for the measurement of CRP concentration in serum. In this research, we used the nephelometry omit method here, which is a quantitative method. The ESR test was performed based on Westergren method, which is a standard and accepted technique insed of method for all patients. For men and women, the normal range of ESR was considered 0 to 15 mm per hour and 0 to 20 mm per hour, respectively.

Table1.Demographic and anthropometric data in patients with knee osteoarthritis (OA)

Grouping of patients with Knee OA	Number	Age (year) (Mean±SEM)	Weight (kg) (Mean±SEM)	Height (cm) (Mean±SEM)	BMI* (Mean±SEM)
Krocina™	18	58.70±1.51	71.5±2.28	166±0.02	26.04±0.8
Placebo	17	57.64±1.72	67.6±2	162±0.01	25.6±0.6

Abbreviation: *BMI: Body mass index

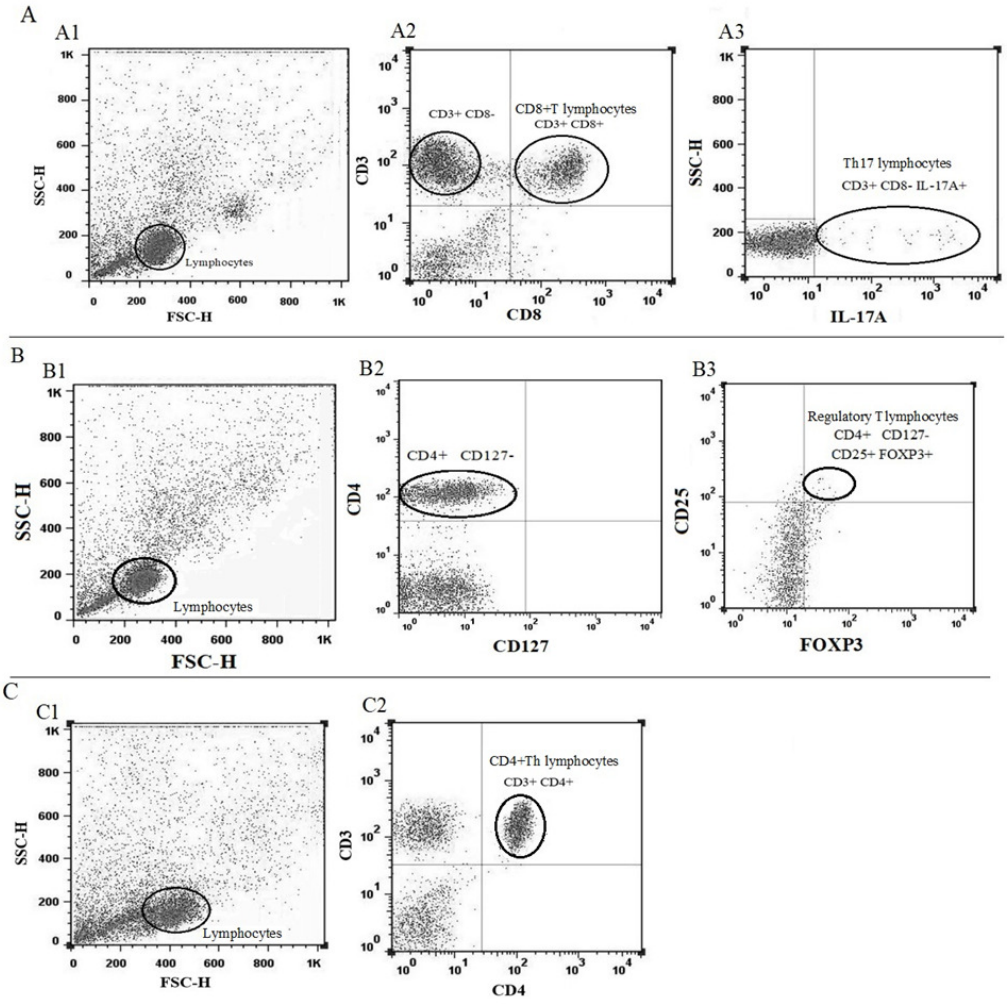

Figure 1. Flow cytometric analyzing procedure for various T cells. (A) For Th17-cells A1: at first total lymphocytes were gated according to the forward scatter (FSC) versus side scatter signals (SSC). Afterwards, A2: CD3+/CD8- T cells were selected and A3: CD3+/CD8-/IL-17+Th17 cells were evaluated. Also, in the A2 step: to identify CD8+T cells, the CD3+/CD8+ subset of T cells wasevaluated. (B) For Treg cells, B1: at first total cells were gated according to the FSC versus SSC signals. Next, B2: CD4+/CD127- T cells were selected and B3:aCD4+/CD127-/CD25+/FOXP3+ Treg cells were evaluated.
(C) For Th cells, C1: at first total lymphocytes were gated according to the FSC versus SSC signals. Then, C2: CD3+/CD4+ Th cells were evaluated.

Flow Cytometry Method

PBMCs from patients with OA were cultured in CTM containing PHA for 18 hours. Flow cytometry analysis of the various T cellsubsets was accomplished after the cell culture procedure. To detect the numbers of Treg-cells, Th17-cells, CD8+ T cells, and CD3+ CD4+ T cells , staining for specific clusters of differentiation (CD) and intracellular markers was performed via anti-human monoclonal antibodies purchased from Biolegend, USA.

Briefly, the staining of Tregcells for flow cytometry analysis was done by the incubation of 1×10^6 mononuclear cells by the use of anti-CD4, anti-CD25, anti-CD127 conjugated with FITC, PerCP/Cy5.5, and APC, respectively. Fixation and permeabilization steps for intracellular staining of mononuclear cells were performed with anti-FOXP3-conjugated by PE. To analyze Th17-cells, at first 1×10^6 mononuclear cells

were excited with Brefeldin cocktail A (Biolegend. the USA). Next, cells were placed in the vicinity of anti-CD3-conjugated by PerCP/Cy5.5 and also anti-CD8-conjugated by FITC. In addition, mononuclear cells were placed in the vicinity of anti-IL17A-PE following fixation and permeabilization.

To analyze the Th-cells, 1×10^6 mononuclear cells were placed in the vicinity of anti-CD3-conjugated by PerCP/Cy5.5 and anti-CD4-conjugated by-FITC. We used PE mouse IgG1 isotype control antibodyas negative controland unstained control for flow cytometric data analysis (Biolegend. the USA).

The fluorescence intensity of the stained cells was measured by the FACSCalibur flow cytometer (BD Biosciences, USA). Analysis of the flow cytometry data was performed via FlowJo software version 7.6.2 (TreeStar, USA), shown in Figure 1.

Procedures of Data Analysis

To analyze the data, IBM SPSS Statistics 21 (IBM Corp, USA) was employed. Mean differences and 95% confidence intervals (CI) were compared before and after the 4 months of intervention. The parametric data were further analyzed; using the Paired Sample t-Test, and nonparametric data were assessed via the Wilcoxon test. $p<0.05$ was considered as statistically meaningful.

RESULTS

Patients' Demograohic Data

Thirty-five patients with OA in both genders (women 66.7% and men 33.3%) aged 40-75 years including 18 patients in Krocina™ group (58.7±1.51) and 17 patients in the placebo group (57.64±1.72) were enrolled and completed the 4-month follow-up. Patients in both groups (Krocina™ and placebo) had non-meaningful differences in age and gender (the data has not been shown). Patients were randomized into two groups, Krocina™, comprised of 18 patients (11 women and 7 men) and placebo, consisting of 17 patients (15 women and 2 men).

VAS Pain Score, Levels of CRP and ESR in Patients with Knee OA before and after Intervention

The VAS pain score in patients receiving Krocina™ was 7.83±0.245 and 2.27±0.22 before and after the intervention, respectively. Our results showed a meaningful decline in VAS for pain in the Krocina™ group after four months of follow up ($p<0.001$). Moreover, in the placebo group, we observed a meaningful reduction in VAS pain score after the intervention (6.7±0.31) compared with before the intervention (8±0.17) ($p=0.002$). CRP values were in the normal levels for all patients with OA. However, the CRP levels were meaningfully reduced in Krocina™ group following the intervention (1.77±0.26 mg/l) in comparison to before the intervention (2.92±0.55mg/l) ($p=0.046$). On the other hand, measurements of CRP in the placebo groups showed no meaningful differences after (3.41±0.5) and before (3.37±0.42) the intervention, ($p>0.05$).

Results of ESR measurement in the Krocina™ group showed non-meaningful differences after the intervention (11.52±1.91) in comparison with before the intervention (12.00±1.22) ($p>0.05$). In addition, the difference between after the intervention (17.05±1.66) and before the intervention (15.11±2.34) was insignificant in the placebo group, ($p>0.05$).

Results of Flow Cytometric Analysis for Various Subsets of T Lymphocytes

Figure 2 shows the flow cytometry findings for various subsets of T lymphocytes before and 4 months after intervention with Krocina™ and placebo in patients with OA.

The Effect of Krocina™ and Placebo on Th17 Cells

The findings of FACS analysis of Th17 cells in OA patients showed a meaningful decline in Th17 cell percentage in Krocina™ group after the intervention (5.09±0.5) in comparison with before the intervention (6.93±0.6), ($p=0.026$). Moreover, Th17 cell percentage in the placebo group was increased, yet insignificantly attheend of the intervention (5.98±0.6) in comparison with before the intervention (5.67±0.7), ($p=0.67$) (Figure 2A).

The Effect of Krocina™ and Placebo on GMFI of IL-17

Measurement of GMFI-IL-17 in Krocina™ and placebo showed that the mean of GMFI-IL-17 in Krocina™ group was meaningfully reduced following the intervention (4.29±0.36) in comparison with before the intervention (5.73±0.48), ($p=0.03$). Moreover, GMFI-IL-17 in the placebo group was meaningfully increased following the intervention (6.14±0.52) in comparison with before the intervention (5.34±0.5), ($p= 0.001$) (Figure 2B).

The Effect of Krocina™ and Placebo on Treg Cells Percentage

Our research showed a meaningful increment in Tregcells percentage in Krocina™ group after the intervention (5.2±0.44) compared with before the intervention (2.99±0.23) (p=0.02). In the placebo group, no significant difference was seen in Tregcells percentage after the intervention (2.51±0.39) and before the intervention (3.82±0.51) (p>0.05) (Figure 2C).

The Effect of Krocina™ and Placebo on GMFI of FOXP3 in Treg Cells

Measurement of GMFI-FOXP3 in Krocina™ and placebo groups showed that the mean of GMFI-FOXP3 in Krocina™ group was increased, but non-meaningfully after the intervention (4.54±0.43) comparisons with before the intervention (4.36±0.41) (p>0.05). Further observed was an insignificant difference in GMFI-FOXP3 of the placebo group after the intervention (4.18±0.49) in comparison with before the intervention (4.45±0.33) (p>0.05). (Figure 2D).

The Effect of Krocina™ and Placebo on the Percentages of CD4+ and CD8+ T helper Cells

Our research showed a meaningful increment in CD4+Th cells percentage in Krocina™ group comparing after the intervention (40.08±0.93) with before the intervention (37.63±1.72) (P= 0.176). In addition, there was a decline in placebo group regarding CD4+Th cells percentage following the intervention (37.18±1.6) in comparison with before the intervention (38.01±1.4), which was non-meaningful (p=0.646) (Figure 2E) cell. There were no meaningful differences regarding CD8+ T cells percentage in Krocina™ group after the intervention (22.18±1.41) comparisons with before the intervention (23.8±1.03) (P>0.05). However, a meaningful decrease in CD8+ T

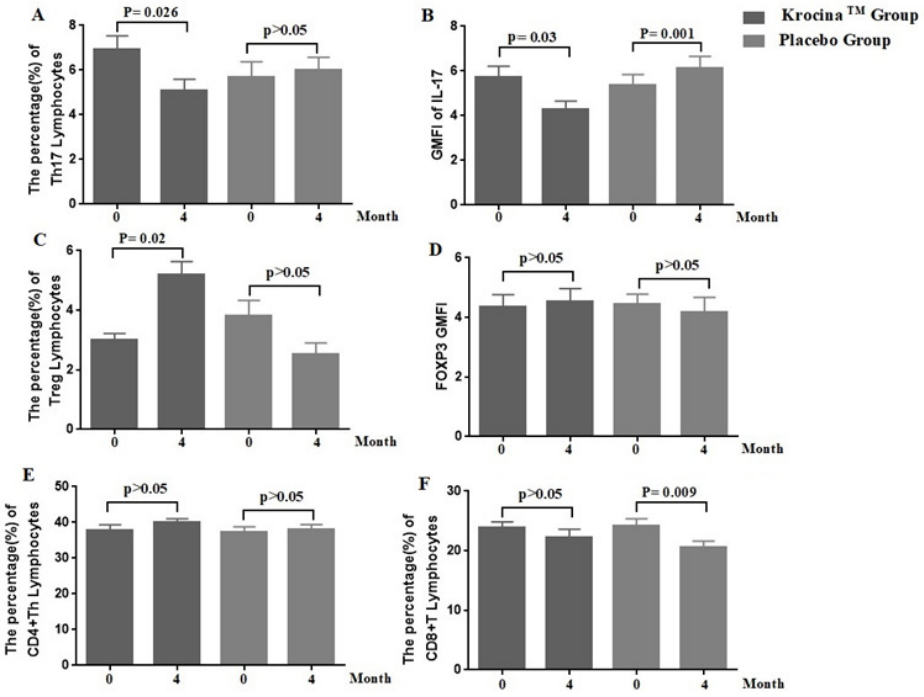

Figure 2. Flow cytometric analysis on various subsets of T lymphocytes before and 4 months after intervention with Krocina™ and placebo in patients with OA. (A) The percentage of Th17 cells. (B) Geometric Mean Fluorescence Intensity (GMFI)-IL-17. (C) The percentage of Tregcells. (D) GMFI-FOXP3. (E) The percentage of CD4+ Th cells. (F) The percentage of CD8+ T cells.The data are shown as Mean±SEM.

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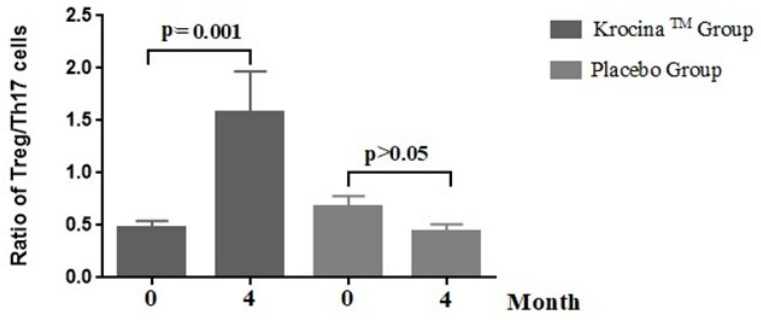

Figure 3. The ratio of Treg/Th17 cells in OA patients before and 4 months after intervention with Krocina™ and placebo

cells percentage in the placebo group was seen after the intervention (20.61±0.99), compared to before the intervention (24.05±1.28) (p=0.009) (Figure 2F).

The Effect of Krocina™ and Placebo on Treg/Th17 Cells Ratio in OA Patients

The Treg/Th17 cells ratio showed a meaningful increment in Krocina™ group after the intervention (1.58±0.39) in comparison with before the intervention (0.48±0.06) (p=0.001). Moreover, in the placebo group, a reduction in Treg/Th17 cells ratio was found following the intervention (0.44±0.07) in comparison with before the intervention (0.68±0.1), which was non-meaningful (p>0.05) (Figure 3).

DISCUSSION

The VAS index as a standard method is used to evaluate the pain of patients with OA suffering articular pains.[15] Our data showed that the VAS index was meaningfully reduced in patients receiving Krocina™ (p<0.05). Interestingly, the VAS index was meaningfully reduced in placebo after 4 months.

As far as the authors of this article are concerned, there are no published reports regarding the influence of saffron and its derivatives on the VAS index in OA. There are several studies on the anti-inflammatory and anti-depressant effects of saffron and its ingredients on neurological disorders,[17] with no measurement of the VAS index. Shakiba et al (2018) showed a non-meaningful difference in the VAS index of patients with fibromyalgia following the clinical trial with saffron.[18] In addition, Marenco et al (2000) and Pavelka (2012) reported that diclofenac consumption in patients with OA caused a meaningful reduction in the VAS index.[19] Therefore, the meaningful reduction of VAS in the placebo group in our trial might be related to the sodium diclofenac. It is to be noted that the meaningful decrease in this marker in Krocina™ group might be due to the Krocina™ or NSAIDs. Further studies would be helpful to get a better understanding ofthe influence of crocin in OA.

The serum level of CRP is considered as a diagnostic biomarker for OA.[20] Moreover, CRP and ESR levels are routinely detected for monitoring inflammatory and articular diseases such as OA, especially in the follow-up of patients.[21]

CRP values were in the normal levels for all patients with OA in our trial. However, the CRP levels in the present research were significantly reduced in Krocina™ group as compared with the placebo at the end of the four months of intervention (p<0.05). Additionally, our results showed non-meaningful changes in the ESR index in patients with OA, four months after intervention with Krocina™. ESR in the placebo group was increased after the 4-month follow-up, which was not meaningful (p>0.05).

Sharif et al (1997) reported that the serum concentration of CRP was a useful index for the prognosis of OA. The serum concentration of CRP is remarkably increased in patients with OA from the first year of diagnosis to 3-5 years later, an increase concomitant with the severity of deterioration in radiographic images and intensity of the articular

cartilage destruction.[22] Lopresti et al (2017) showed that CRP and pro-inflammatory cytokines were inhibited following the administration of curcumin alone or in combination with saffron in patients with depression.[17]

We did not find any clinical trials to compare with our data regarding the influence of saffron and/or its derivatives on the ESR levels.

Our flow cytometry findings showed that the cells percentage of Th lymphocytes in Krocina™ group was increased after 4 months of follow up compared with time point zero, which was not meaningful. The same analysis in the placebo group showed a negligible difference between the two-time points (p>0.05).

Zhang et al (2011) reported that the number of CD3+ CD4+ IFN-γ –secreting Th1 cells was not different between healthy individuals and patients with OA.[23] In another study performed by Vosooghi et al (2013), the saffron extract increased the total percentage of peripheral blood lymphocytes in mouse asthma model.[24]

Our findings showed the meaningful reduction of Th17 cells in the Krocina™ group four months after the intervention as compared with the time point zero (p<0.05). However, there were non-meaningful changes in the percentage of Th17 cells in placebo group comparing time point zero with the four months of follow-up (p>0.05). Zeng et al (2012) and Kagami et al (2010) showed that Th17 cells percentage was significantly increased in peripheral blood of type 2 diabetic patients and psoriasis, respectively, correlated with the intensity of inflammation in the disease.[25,26]

Our findings showed a meaningful increment in Tregcells percentage in Krocina™ group four months after the intervention as compared with time point zero (p<0.05). Nonetheless, there were no meaningful changes in the percentage of Tregcells in the placebo group, before and after the intervention.

Previous studies have reported that the number of human Tregcells with the phenotype of $CD4^+$, $CD25^+$, CD127- and $FOXP3^+$ in the peripheral blood of OA patients is similar to these cells in RA patients.[27]

Guo et al (2015) studied the mouse model of OA and showed that CD4+ CD25+ FOXP3+ Tregcells in the case group were meaningfully lower than healthy controls. Additionally, they showed a direct correlation between the deterioration of OA disease and the decline in Tregcells.[27]

Moreover, the results of a clinical trial carried out by Ponchel et al in 2015, showed that the reduction of Tregcells in peripheral blood of OA patients had a direct correlation with the severity of the OA disease.[28]

As far as the authors of this research are concerned, there are almost no published data regarding the effects of saffron and its derivatives on Tregcells in patients with a degenerative joint disease such as OA. There is only one study performed by Faridi et al in 2019 reporting that induced-autoimmune diabetes in the mice model was ameliorated by saffron. They further reported that the proliferation rate of lymphocytes in the mice model of autoimmune diabetes following treatment with saffron extract had a remarkable reduction as compared with the control group.[29]

Our results showed non-meaningful changes in CD8+ T cells subset between time point zero and four months after intervention with Krocina™ in patients with OA. However, the same analysis in the placebo group showed a meaningful reduction in the CD8+ T cells after the 4-month intervention (p<0.05).

Also, no publications and reports are showing the influence of saffron and its ingredients on the percentage of CD8+ T cells in clinical studies and/or animal models. However, there are just a few studies regarding the percentage of CD8+ T cells and their importance in patients with OA that are mentioned in the following. A clinical trial was performed by Hussein et al on the synovial and peripheral blood of OA patients and RA in 2008, specifying that the percentage of CD8+ T cells in the peripheral blood of OA was slightly reduced as compared with RA and normal individuals.[30]

Moreover, certain studies have shown that the majority of CD8+ T cells in the peripheral blood from OA patients are CD8+ memory cells.[27] Additionally, Ishii and colleagues (2002) showed that the number of CD8+ T cells and their related cytokines in the serum of OA patients might be a useful tool for the prognosis of the disease.[31]

Our results showed that GMFI-IL-17 was meaningfully decreased in Krocina™ group after the 4-month intervention, compared with time point zero (p<0.05).

On the contrary, a meaningful increment was observed in the placebo group at the end of four months as compared with the beginning of the clinical trial (p<0.05). So far, many studies have been performed on the inflammatory properties of OA and infiltration of CD4+Th1 and Th17 cells into synovial layers of

patients with OA.[32] Studies have shown that the presence of IL-6, IL-1β, and IL-23 as inflammatory cytokines along with TGF-β are very effective on the induction of Th17 cells differentiation.[33] Similar to IL-1β, IL-17 has been proved effective on the activation of cell destructive enzymes.[34] Moreover, fibroblasts and epithelial cells were activated by IL-17 to produce TNF-α, IL-6, IL-8, and IL-1β.[35] Some clinical studies have demonstrated that serum and synovial levels of IL-17 are significantly higher in OA patients' comparisons with normal individuals and are directly correlated with the severity of pain in the disease.[36] Moreover, saffron and its derivatives have been reported to be able to both decrease IL-17 levels and increase the generation of anti-inflammatory mediators, including IL-10 and transforming growth factor (TGF)-β.

Results of the present research with regards to GMFI-FOXP3 in patients with OA showed non-meaningful changes in both Krocina™ and placebo groups at the end of the 4-month intervention as compared with the time point zero ($p>0.05$).

Even though the changes in GMFI-FOXP3 were not significant in either group, there was an increasing and a decreasing trend in the production of FOXP3 in Krocina™ and placebo group, respectively. Regardless of the insignificant augment in GMFI-FOXP3 in Krocina™ group, such an increasing trend might be related to the effects of Krocina™. Raghavan et al (2009) assessed the intracellular expression of FOXP3 in rheumatoid arthritis (RA) patients and reported that the reduction of FOXP3 expression was associated with the intensity of inflammation in RA so that the increase in FOXP3 level and FOXP3+ Tregcells was a goal in the usage of anti-inflammatory drug.[37]

Ding et al (2015), showed that the regulatory properties of Tregcells caused a meaningful increment in the levels of Foxp3 in Tregcells following the administration of crocetin as active derivatives of saffron in animal models.[38] Our findings with regards to the Treg/Th17 ratio showed an increase in the percentage of Tregcells, concomitant with a decrease in the percentage of Th17 cells in the Krocina™ group between the two time-points. The same analysis in the placebo group showed a reduction in the ratio of Treg/Th17 cells.

The ontogenesis of Treg and Th17 cells was differentiated from naïve CD4+ T cells in the presence of TGF- β and inflammatory cytokines, respectively. Therefore, the maintenance of the homeostasis between these two cells plays a key role in inflammatory and autoimmune diseases.[39] Ferraro and colleagues (2011) showed that Tregcells and their activity were reduced when the cellular balance shifted towards Th17 cells in patients with type I diabetes.[39] Finally, Faridi et al showed that saffron extract conduced to maintaining Treg/Th17 cellular balance in the mouse model of autoimmune diabetes.[29]

This research demonstrates that Krocina™, an herbal medicine made of crocin, at a dose of 15 mg/daily, can meaningfully reduce CRP levels, percentage of Th17-cells and GMFI-IL-17, all of which have inflammatory properties. On the contrary, the percentage of Tregcells and GMFI-FOXP3 were meaningfully increased by the consumption of Krocina™ in patients with OA. The percentages of CD4+ Th cells and CD8+ T cells were not changed at the end of the clinical trial. Additionally, at the end of the intervention, the Treg/Th17 ratio showed an increase and a decrease in the percentage of Tregcells in the Krocina™ group and placebo group, respectively.

Taken together, it is concluded that Krocina™ has anti-inflammatory and immunoregulatory effects on patients with OA, ameliorating the disease. Regarding future research, it is recommended that a control group not receiving NSAIDs be considered to get a better conclusion regarding Krocina™ effects on VAS and other immunological factors in patients with OA.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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