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The Immunomodulatory Effects of Curcumin on Forkhead Box O1 and MicroRNA-873 in Patients with Osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is among the most prevalent articular disorders, whose incidence is directly related to aging. Due to the antiinflammatory potential of curcumin as the active component of turmeric, the present study evaluated the effects of curcumin on the expression of genes related to T helper 17 (Th17), including forkhead box p3 (*FOXP3*), forkhead box o1 (*FOXO1*), transforming growth factor-β (*TGFB1*) and microRNA-873, human (*HSA-MIR-873*), in OA patients.

Female patients with knee OA (n=30) were randomly categorized into 2 groups, including the intervention group who received curcumin (n=15) and the placebo (n=15) in a double-blind clinical trial for 3 months. The expression of *FOXO1*, *FOXP3*, *TGFB1*, and *HSA-MIR-873* genes was evaluated by SYBR Green real-time reverse transcription polymerase chain reaction.

In the curcumin group, *FOXO1* gene expression was significantly increased, while the increase in *FOXP3* gene expression was not significant. Moreover, the expression level of the *HSA-MIR-873* gene showed a significant increase in the curcumin group.

The modulatory effects of curcumin on Th17 function might be associated with the expression of *FOXO1* and HSA-MIR-873 genes.

Keywords: Curcumin; Forkhead box protein o1; Forkhead box protein p3; MicroRNA-873, human; Osteoarthritis; Transforming growth factor beta

INTRODUCTION

Osteoarthritis (OA) is considered the most common debilitating disorder in older people and is associated with high healthcare costs and significant disruption of routine activities, leading to reduced quality of life.[1] As a chronic painful disorder, OA causes local inflammatory responses in the synovial membrane, articular and periarticular structures, and the subchondral bone, resulting in severe pain and disability.[2-4] Risk factors associated with OA are categorized into: age, gender, and heredity as

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endogenous factors; trauma, obesity, and joint removal surgery as exogenous ones.[5]

The involvement of T cells is evident in many patients with OA, and different subtypes of T lymphocytes participate in the development and initiation of inflammatory responses through secreted cytokines.[6,7] Moradi et al, demonstrated the presence of T lymphocytes in the joints of OA patients and the relevance of their polarity towards T helper 1 (Th1) and Th17/regulatory T cells (Tregs) to disease severity and inflammation levels.[8] Multiple lines of evidence prove the presence of Th17 cells in the synovial membrane of OA patients.[7] The increased secretion of interleukin (IL)-17 in OA patients creates and aggravates joint inflammation and results in the overexpression of other inflammatory factors like IL-1β, IL-6, and IL-8, and these cytokines are also associated with disease progression.[7]

FOXO1, *FOXP3*, *human microRNA*-873 *(HSA-MIR-873)*, and *transforming growth factor-β (TGFB)* are the genes that were evaluated in this study. *FOXO1*, a crucial transcription factor, is highlighted for regulating various physiologic processes including apoptosis, autophagy, oxidative stress, and immune responses.[9] The expression of *FOXP3* and the secretion of antiinflammatory mediators, including IL-10 and *TGFB* as the signature of Tregs can participate in suppressing the function of $CD4^+$ and Th17 T cells, leading the immunological responses to an antiinflammatory state.[10] *TGFB* is an effective cytokine in osteoblast differentiation and induces tissue and bone regeneration.[11] The *HSA-MIR-873*, increases the proliferation and differentiation of Th17 lymphocytes via targeting the transcription factor forkhead box O1 (*FOXO1*) as a negative regulator on RAR-related orphan receptor-γt (RORγt).[12]

The goal of OA treatment is to relieve pain, reduce swelling, and improve joint mobility.[13] Curcuminoids are natural products that have attracted attention for prophylactic and therapeutic purposes in recent decades. They are lipophilic, so they quickly infuse into cellular membranes. They also have a polyphenolic structure and make up about 2% to 5% of the spice turmeric.[14,15] Curcumin and its derivatives are biologically active compounds of the *Curcuma longa* plant that exert antiinflammatory, antioxidant, antibacterial, antiviral, antiapoptotic, antitumor, chemopreventive, immunoregulatory, cardioprotective, neuroprotective, and antidiabetic potential; therefore, they are widely used in traditional medicine.[2,3,13,15-17]

In vivo and in vitro studies demonstrated the antiarthritic potentials of curcuminoids in OA and rheumatoid arthritis (RA) patients. It has been suggested that the antioxidant and antiinflammatory potentials of curcumin are the main protective effects of curcumin on OA patients.[13] In addition, curcumin potentially inhibits the production of inflammatory and catabolic mediators in chondrocytes.[18] Recent studies have shown the involvement of $CD4^+$ and $CD8^+$ T cells, Th17, Treg, and B lymphocytes in the OA pathogenesis, but most of these studies were conducted in animal models.[7,16,19] This study investigated the effects of curcumin on gene expression of *FOXO1*, *FOXP3*, *TGFB*, and *HSA-MIR-873*, in OA patients. Many studies have been conducted in relation to this disease, but due to the complexity of different interactions of immune cells and the effect of the responses of these cells on each other, as well as the different effects that curcumin can have in this field, it is necessary to conduct more studies on this disease seems quite obvious.

MATERIALS AND METHODS

Ethics and Study Design

This study was performed on biological samples archived in a −80°C freezer from a previously randomized, double-blind, placebo-controlled clinical trial study (IR.MUMS.MEDICAL.REC.1397.118). The Iranian Registry of Clinical Trials code for the study is IRCT20151028024760N4. All patients signed consent forms under the Declaration of Helsinki, which also emphasizes ethical principles in medical research.

Patient Enrollment

All patients with knee OA enrolled in this study were selected by an expert rheumatologist. Inclusion criteria included knee pain for more than 6 months, age between 40 and 55 years old,

Kellgren Lawrence (KL) grade 2 or 3, the visual analog scale (VAS) of 5 or higher in the knee joint, no history of joint surgery, no history of antiinflammatory drug administrations for long periods, no history of underlying diseases and body mass index (BMI) < 30. These participants also met the American College of Rheumatology (ACR) criteria and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) criteria. History of joint surgery, history of joint damage, history of underlying diseases, history of antiinflammatory drug administration for long periods, and BMI ≥ 30 were among the exclusion criteria. Since gender is one of the risk factors of OA, all patients were selected from women to avoid the effect of this factor on the results.

Finally, 30 female OA patients were included and randomly and equally categorized into 2 curcumin and placebo groups.

Blood Sampling

Six mL of blood was collected from all 30 patients with OA in tubes containing EDTA before and 3 months after the intervention. RNA extraction was performed using an RNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) based on the manufacturer's protocol. The microRNA sample was extracted using a kit specially designed for microRNA extraction (Bonyakhte Company, Tehran, Iran). The extracted RNA and microRNA were stored at −80°C. The cDNA was synthesized according to the manufacturer's instructions (Yekta Tajhiz Azma and Bonyakhte Company, Tehran, Iran).

SYBR Green Real-time PCR

Primers for *FOXO1*, *FOXP3*, *GAPDH*, and *TGFB* mRNA were designed in-house and purchased from Pishgam Company, Tehran, Iran. The sequence of forward and reverse primers is provided in supplementary materials. The sequence for *HSA-MIR-873* was patented by The Bonyakhteh Company. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression results.[20]

Statistical Analysis

The SPSS software version 16 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, USA) were applied for data analysis. The Kolmogorov-Smirnov (KS) test was used to assess data normality. The unpaired *t* test and Mann-Whitney test were applied to analyze normally and non-normally distributed data, respectively. $p<0.05$ was considered significant.

RESULTS

Patients' Demographic Characteristics

Thirty women with knee OA (curcumin group=15 and placebo group=15) ranging in age from 40 to 55 years were included in the study. All these patients completed a 3-month follow-up study. More details regarding demographic characteristics were previously published.[16]

The Gene Expression of *FOXO1*

The level of *FOXO1* gene expression significantly increased following intervention in the curcumin group ($p=0.0379$). The expression of this gene in patients receiving placebo showed a nonsignificant increase ($p=0.5555$) (Figure 1).

The Gene Expression of *FOXP3*

No significant changes were observed in the *FOXP3* gene expression following intervention in both groups receiving curcumin or placebo (Figure 2).

The gene expression of *TGFB*

The *TGFB* gene expression in both groups of patients did not show significant changes after the intervention compared to before (Figure 3).

The Gene Expression of miR-873

The expression of miR-873 showed a significant increase following curcumin consumption ($p<0.0001$). The expression of this gene in the placebo group showed a nonsignificant decrease ($p=0.9045$) (Figure 4).

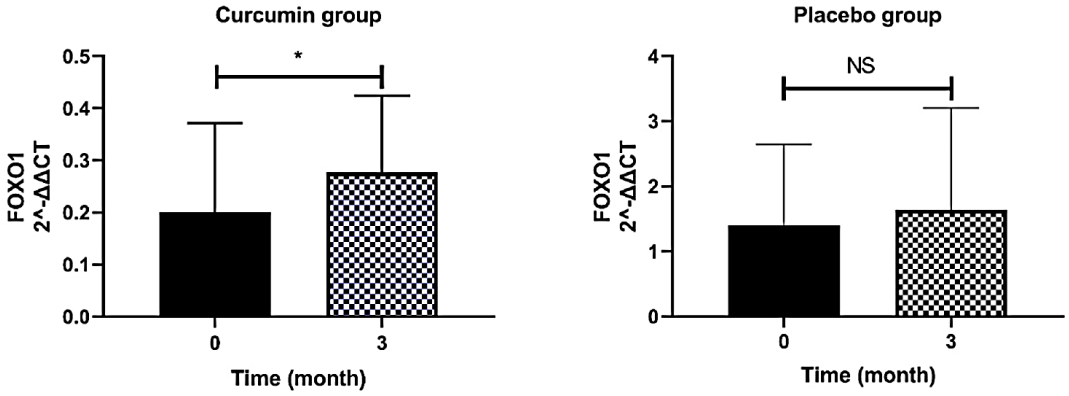

Figure 1. The gene expression level of *FOXO1* in osteoarthritis patients receiving curcumin and placebo before and 3 months after the intervention. (* $p<0.05$ and NS: $p>0.05$)

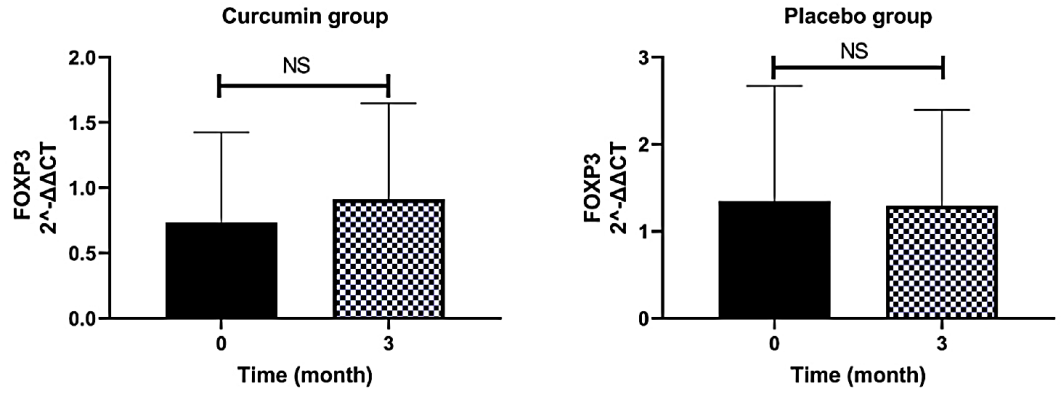

Figure 2. The gene expression level of *FOXP3* in osteoarthritis patients receiving curcumin and placebo before and 3 months after intervention. The *p* value in the curcumin group is 0.2226 and in the placebo group is 0.9158. (NS: $p>0.05$)

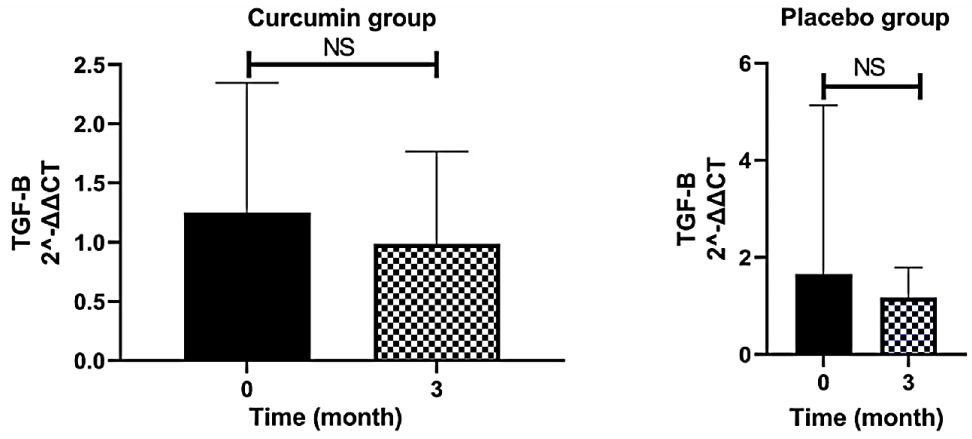

Figure 3. The gene expression level of transforming growth factor-beta (*TGFB1*) in osteoarthritis patients receiving curcumin and placebo before and 3 months after intervention. The *p* value in the curcumin group is 0.4839 and in the placebo group is 0.0507. (NS: $p>0.05$)

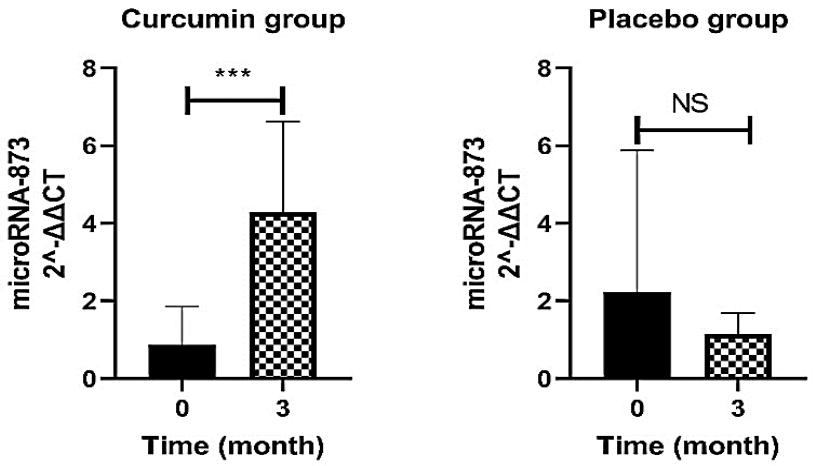

Figure 1. The gene expression level of miR-873 in osteoarthritis patients receiving curcumin and placebo before and 3 months after intervention. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS: p>0.05)

DISCUSSION

The current study evaluated the potential influence of curcumin on factors associated with Th17 cells in OA patients. OA, the most common form of arthritis,[21] is a disease that destroys joints and is the leading cause of the inability to perform daily activities.[22]

Previous reports have confirmed the role of $CD4^+$ T lymphocytes in OA pathogenesis.[23] The abundance of Th17 cells in the synovial membrane of patients with OA along with the increased secretion of cytokines such as IL-17, as well as exacerbating the inflammatory conditions of the joints, can cause the secretion of other inflammatory cytokines including IL-1, IL-6, and IL-8, and these cytokines have a direct relationship with the progression of the disease.[24-26] *FOXO1* regulates Th17 and Treg differentiation and function.[27] *FOXO1* deletion is associated with enhanced Th17 differentiation, suggesting a negative regulatory effect of this transcription factor on Th17 cells.[9] *FOXO1* has been shown to be essential for *FOXP3*.[28] *FOXO1* and *FOXO3* bind directly to the *FOXP3* promoter region and activate its promoter activity in a *FOXO1* binding sequence-specific manner.[29] Although the importance of *FOXP3* for inducing Treg differentiation has received much attention, studies on *FOXP3* knockout support the idea that a lack of *FOXP3* leads to maintaining Tregs in a Treg cell-like phenotype called "wannabe" Treg cells.[30-32] In a study by Laine et al, it was shown that *FOXO1* reduces Th17 cell proliferation and decreases IL-17 and IL-2 secretion by targeting the transcription factor RORγt. Thus, *FOXO1* is a direct antagonist in RORγt regulation. RORγt is a transcription factor that acts specifically on the activity of Th17 cells.[9]

Consistent with these findings, our study indicated a significant elevation in *FOXO1* expression in the curcumin group. However, *FOXO1* levels were not significantly increased in patients receiving placebo. According to the role of Th17 cells in the pathogenesis of OA and the results of our study, it may be concluded that curcumin can reduce the percentage of Th17 cells by increasing the expression of *FOXO1* and changing the immune responses towards antiinflammatory conditions in OA patients. Therefore, this result shows well the antiinflammatory and modulating effects of curcumin.

$CD4^+$ and $CD8^+$ T cells can influence inflammatory conditions in OA patients by secreting cytokines.[33] In a study conducted in rats, the frequency of $CD4^+CD25^+FOXP3^+$ cells in an OA model was significantly lower than that of controls. This decrease in Tregs resulted in a reduction in IL-4 levels and an elevation in IL-17 levels, thereby increasing the production of various enzymes, proinflammatory factors, and reactive oxygen species (ROS) that cause the development of joint damage.[34] In an in vitro study, Yu-Sen Chai et al. showed that curcumin can promote naïve $CD4^+$ T cell differentiation toward Treg and increase
IL-10 production. In other words, curcumin can suppress inflammation by increasing naïve $CD4^+$ T cell differentiation into $CD4^+CD25^+FOXP3^+$ Tregs.[35]

In our study, *FOXP3* expression exhibited an increase in the curcumin group and a decrease in the placebo group, neither of which were statistically

significant. From these results, it can be concluded that by increasing the sample size or by extending the time of patients receiving curcumin by patients to more than 3 months, there might be a possibility of a significant elevation in *FOXP3* expression due to curcumin's immune modulatory effects.

Type 1 regulatory T (Tr1) and Th3 cells were reported as regulatory T cells lacking *FOXP3* expression.[36,37] Tr1 cells are a suppressor subset of *FOXP3*$^-$ T lymphocytes that secrete high levels of IL-10 and are involved in regulating inflammation, graft-versus-host disease, and autoimmune conditions.[38] Th3 cells mainly produce *TGFB*.[39] The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling downstream of T-cell receptor activation is involved in Tr1 cell induction and maintenance.[40] FOXO1 phosphorylation has been reported to be decreased under conditions of repression of PI3K and AKT phosphorylation, resulting in impaired IL-27–associated Tr1 cell differentiation.[40] Niken Adiba Nadya et al conducted a study on BALB/c mice to evaluate the PI3K pathway's involvement in Tr1 cell differentiation. They observed a positive correlation with FOXO1 phosphorylation during IL-27-Tr1 cell differentiation. In other words, the PI3K-Akt-*FOXO1* axis may have a fundamental role in IL-27–associated Tr1 cell differentiation.[41] Although FOXO1 involvement in Tr1 cell differentiation has not been clearly explained, this molecule was characterized as an essential agent in IL-10, *TGFB*, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression in CD4$^+$ T cells in mice.[42,43] It has been reported that curcumin, as a natural antiinflammatory substance, can promote IL-10 production and increase its function in various tissues.[44] Yingzi Cong et al reported that curcumin treatment induced BM-derived dendritic cells (BMDC) to express aldehyde dehydrogenase 1A (ALDH1a) and IL-10. Curcumin-treated DCs also induce Treg differentiation in the gut, containing CD4$^+$CD25$^+$FOXP3$^+$ Treg cells and IL-10–producing Tr1 cells.[45]

It has been reported that *TGFB1* stimulates chondrocyte proliferation and loss of proteoglycans and cartilage degeneration results from *TGFB* type II receptor knockout or disruption of *TGFB* signaling.[46] In the cartilage of rat OA models, protective cytokines such as *TGFB1* are significantly reduced, and it has been observed that gene therapy with *TGFB* is effective in repairing cartilage damage.[11]

According to the current study, no significant difference in *TGFB* expression was observed after 3 months of curcumin consumption. Since curcumin did not cause a significant change in *FOXP3* expression, which is the main transcription factor of Tregs, and on the other hand, Treg cells are among the most important sources of *TGFB* cytokine production, it can be concluded that the lack of change in *TGFB* gene expression may be due to the lack of effect of curcumin on the differentiation of cells toward Tregs. If in future studies increasing the sample volume or increasing the time of receiving curcumin can lead to a significant increase in the expression of *FOXP3*, it may be expected that the expression of *TGFB* as a key cytokine of Tregs will also increase significantly.

MicroRNAs, a group of small noncoding RNAs, are posttranscriptional regulatory factors that cause mRNA degradation or inhibit the translation of mRNA into protein.[47] MicoRNAs are involved in various processes of cell metabolism such as proliferation, survival, apoptosis, and inflammation.[48] For example, in OA, it seems that the main function of microRNAs is to reduce the level of stability and translation of their target mRNA, which is involved in pathophysiological processes including inflammation, apoptosis, matrix synthesis, and chondrogenesis. MicroRNAs also appear to modulate extracellular matrix deposition and suppress or accelerate chondrocyte apoptosis.[49,50]

Recently, various studies have been conducted on the importance of miRNAs in OA pathogenesis. Altered levels of microRNAs in OA patients compared to healthy individuals provide a better understanding of the molecular pathways associated with OA pathophysiology.[51] The microRNAs appear to be involved in various pathophysiological processes of OA, such as apoptosis, inflammation, and proliferation.[51] The *HSA-MIR-195-5p* is one of the miRNAs that are overexpressed in patients with OA and increase both gene and protein expression of IL-1β, IL-6, and TNF-α cytokines.[52] Atabaki et al showed a significant decrease in *HSA-MIR-138, HSA-MIR-155, and HAS-MIR-16 expression in knee OA patients* after 3 months of curcumin treatment.[53] Furthermore, Mohebbi et al showed a significant decrease in miR-21 and a significant increase in *HSA-MIR-155* expression in OA patients receiving curcumin, respectively.[54] They also showed a nonstatistically significant decrease in miR-146 in OA patients who received curcumin. Veronica et al conducted a study in knee OA patients over 40 years

old and found that *HSA-MIR-335-5p* significantly elevated the expression of autophagy-related genes and suppressed inflammatory factors.[55] *HSA-MIR-182* has been shown to downregulate *FOXO1* while increasing *FOXP3* is required for Treg differentiation in Jurkat cells.[30] Soheilifar et al, reported an overexpressed miR-182 expression in breast cancer patients that downregulates *FOXO1* expression and protein levels while overexpressing *FOXP3*.[56]

Studies showed that the effects of miRNAs also vary based on disease severity and stage. Serum *HSA-MIR-22* and *HSA-MIR-103a* can anticipate the progression of RA in pre-RA patients who are categorized as susceptible individuals. While serum *HSA-MIR-16*, *HSA-MIR-24*, *HSA-MIR-125a*, and *HSA-MIR-223* are varied in early RA patients compared to established RA or healthy controls. For example, the serum levels of *HSA-MIR-16* and *HSA-MIR-223* in patients with early RA are lower than in healthy subjects and chronic RA patients.[57]

Xiaomei Liu et al, showed that *miR-873* overexpression in astrocytes elevated the levels of IL-6, TNF-α, macrophage inflammatory protein 2 (MIP-2), monocyte chemokine protein 1/5 (MCP-1/5), and p-NF-κB/p65, inflammatory mediators, and also decreased A20 protein expression following IL-17 induction. Conversely, *miR-873* knockdown using locked nucleic acid (LNA)-anti-miR-873 in mouse astrocytes effectively inverted the aforementioned changes.[58] Xiaobing Long et al showed that *miR-873a-5p* can effectively suppress proinflammatory and induce antiinflammatory factors released from microglia through inhibition of ERK phosphorylation and the NF-kB signaling pathway.[59] Jinhua Wu et al reported that the ratio of LC3II/LC3I protein expression in the *miR-873* or ATP-binding cassette transporter 1 (ABCA1)-transfected cells was significantly lower than that in the control group. Also, accumulation of the p62 protein was seen in these cells. It has also been reported that *miR-873* transfection or ABCA1 silencing led to elevated lysosomal cholesterol levels and α-synuclein accumulation in lysosomes and impaired autophagy.[60] It has been observed that *miR-873* as a p53-dependent tumor suppressor is correlated to better patient survival and is considered a main factor in regulating autophagy genes, including *BCN1*, *LC3*, *ATG7*, *ATG16L1*, and *ATG13* in triple-negative breast cancer. Using in silico data, miR-873 has been shown to directly repress these genes' expression by binding to the 3′-untranslated region (3′-UTR). It has also been observed that miR-873 regulates eukaryotic elongation factor 2 kinase (eEF2K) expression and inhibits starvation-induced autophagy via 3′-UTR binding. In other words, the p53/miR-873/eEF2K axis is a new autophagy regulator at the posttranscriptional level, and miR-873 acts as the main factor in the posttranscriptional regulation of the main autophagy genes directly and indirectly via eEF2K-dependent mechanisms.[61]

Compared to other assessment methods, such as radiographic imaging or magnetic resonance imaging, limited information is available on the impact of microRNAs as biomarkers for OA and their identification. Alterations in microRNA levels because of comorbidities are another reason to achieve a reasonable interpretation regarding the effects of microRNAs.

In the results of our study, the expression of the gene encoding *miR-873* increased significantly after 3 months of curcumin intake in patients with OA. The expression level of this microRNA in the placebo group showed a nonsignificant decrease after the study period. Since microRNAs play a role in the pathological processes associated with OA, *miR-873* may be another factor involved in the immunopathogenesis of OA and lead to noninflammatory conditions; But its mechanism of action needs more studies. To our knowledge, most of the studies related to microRNAs have focused on cell lines and cancers, so one of our limitations in drawing a better conclusion was the lack of enough resources to compare the results of our study with other clinical studies regarding the effects of curcumin on microRNAs.

In conclusion, our results showed modulatory effects of curcumin on *FOXO1* and *miR-873* expression that caused a shift in immune responses toward antiinflammatory conditions in OA patients. As previous studies showed that *FOXO1* and *miR-873* are important factors involved in Th17 function, for a better conclusion, we suggest investigating the effects of curcumin on the serum level of IL-17 and other microRNAs and transcription factors related to Th17 lymphocytes in patients with OA in future studies.

STATEMENT OF ETHICS

The Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran

(IR.MUMS.MEDICAL.REC.1398.112) approved this study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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