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RESEARCH COMMUNICATION

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Wogonin ameliorate complete Freund's adjuvant induced rheumatoid arthritis via targeting NF-κB/MAPK signaling pathway

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Abstract

Rheumatoid arthritis (RA) is a chronic and accelerated autoimmune illness with proliferative and damaging synovitis, resulting in joint death and cartilage and bone erosion. This study focused on the potential therapeutic effect of wogonin on complete Freund's adjuvant (CFA) induced RA in rats and the underlying mechanisms. Arthritis was experimentally caused in rats by subcutaneously injecting 0.1 mL of CFA into the subplantar area of the left hind paw under moderate anesthesia on day zero. The regular oral doses of indomethacin/wogonin began on day zero and proceeded after injection to day 35. Wogonin reduced arthritic score considerably, enhanced body weight, and reduced paw thickness. Wogonin also boosted red blood cell considerably along with hemoglobin and reduced white blood cell count and erythrocyte sedimentation rate. Wogonin substantially improved an altered level of oxidative stress markers, antioxidant proteins, and inflammatory cytokines in a dose-dependent way. Wogonin inhibited p38 phosphorylation triggered by CFA and p65 nuclear translocation.

KEYWORDS

complete Freund's adjuvant, indomethacin, MAPK, NF-κB, rheumatoid arthritis, wogonin

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and progressive autoimmune disease with proliferative and invasive synovitis, resulting in joint destruction and cartilage and bone erosion.[1] Moreover, RA has often been reported to be associated with periarticular or generalized osteoporosis, decreased muscle function, and low muscle mass. RA patients often have pain and reduced muscle work, limiting physical activity and diminishing quality of life.[2] It is projected to impact about 0.5–1% of the worldwide inhabitants, being about three times

Abbreviations: CAT, catalase; CFA, complete Freund's adjuvant; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; GSH, reduced glutathione; Hb, hemoglobin; IL-1, interleukin-1; IL-10, Interleukin 10; IL-1β, Interleukin 1 beta; MAPK, mitogen-activated protein kinases; MDA, malondialdehyde; MMP-3, matrix metalloproteinase-3; NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; RBC, red blood cell; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor alpha; WBC, white blood cell.

more prevalent in women than men. China's incidence frequency is revealed to range from 0.2 to 0.37%.[3–5] It is not yet fully grasped the etiology and pathogenesis of RA. Evidence suggests that RA includes a complicated interplay, including smoking and dust, between genotypes and environmental triggers. Autoantibodies development and subsequent chronic inflammation and articular devastation are usually thought to be the pathological foundation of RA.[6]

In RA, by secreting proinflammatory cytokines and mediators that drive angiogenesis, immune cell infiltration, and cartilage and bone destruction, macrophages are the key effector cells in the acute and chronic phases.[7] The amount of macrophages in the inflamed synovium increases owing to development of resident macrophages and infiltration through flowing monocytes.[8,9] Recent surveys indicate that mitogen-activated protein kinases (MAPKs) in macrophages are extremely activated and engaged in RA pathogenesis.[10] Many organizations have focused on blocking intracellular nuclear factor-κB (NF-κB) and p38 MAPK signaling pathways to discover a new generation of anti-inflammatory medicines for RA. NF-κB and MAPK signaling pathways engage macrophages in the release of cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), resulting in bone death and synovitis. NF-κB activation is noted in animal models arthritis and RA patient synovium.[11] It has been shown to reduce inflammatory reaction and joint devastation on collagen-induced arthritis in rodents by inhibiting the NF-κB signaling pathway.[12,13] In addition, RA pathogenesis has been revealed to be strongly correlated with oxidative stress, as reactive oxygen species (ROS) overproduction is accountable for tissue injury, high proinflammatory environment, and ultimately prolonged disease progression.

Wogonin (5,7-dihydroxy-8-methoxyflavone), a flavonoid contained in the Scutellaria Baicalensis Georgi root of the Chinese herb, is commonly used to cure some allergic and inflammatory diseases.[14] It has been engaged in many diseases incidence and therapy and has a broad range of pharmacological operations including anti-inflammation,[15] antiangiogenesis, antivirus,[16] antifibrosis, anticancer,[17] and cancer. Studies have shown chondroprotective impacts of wogonin generated in articular chondrocytes through its regulation of gene expression[18,19] and matrix metalloproteinase-3 (MMP-3) production, but wogonin has never been researched in RA.[20] Thus, through NF-κB/MAPK signaling pathway, we researched the protective function of wogonin in the complete Freund's adjuvant (CFA) induced RA.

2 | MATERIALS AND METHODS

Wogonin, indomethacin, bovine type II collagen (CII), and CFA were bought from Sigma Aldrich. TNF-α, IL-1β, and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were all bought from Biolegend, Inc. (San Diego), while superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) assay kits and malondialdehyde (MDA) assay kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Analytical grade was all other chemicals.

2.1 | Experimental animals

Wistar rats (male; 8 weeks of age) from the animal house of First Affiliated Hospital of Henan University of CM. All rats were left to acclimatization under experimental circumstances for a week. Rats were housed at a 12 h/12 h light/dark cycle under conventional laboratory conditions (room temperature: $22 \pm 2^{\circ}C$; comparative moisture: $55 \pm 5\%$) and free access to standard food and drinking water. All animal experiments and animal care processes were conducted as per University's Guide to Animal Use and Care First Affiliated Hospital of Henan University of CM.

2.2 | Induction of arthritis and administration of drugs

A sample of 30 Wistar rats (male) were randomly split into five groups ($n = 6$) to assess the amilorative impact of wogonin: normal control group; adverse control, rat with CFA; indomethacin group, CFA rats treated with indomethacin (standard drug, 2 mg/kg); wogonin,[25] CFA rats treated with 25 mg/kg wogonin dose; and wogonin,[50] CFA rats treated with 50 mg/kg wogonin dose.

Arthritis was experimentally induced in Wistar rats by injecting 0.1 mL of CFA subcutaneously on day zero under moderate anesthesia into the subplantar region of the left hind paw. The regular oral doses of indomethacin/wogonin began on day zero and proceeded after injection to day 35. Biophysical parameters (arthritic rating, body weight, and paw thickness), oxidative stress parameters, inflammatory cytokine assessment, expression of NF-κB/MAPK, and histopathological assessment were evaluated for the antiarthritic ability of wogonin.[21]

2.3 | Arthritic score

Expressive characteristics such as erythema, redness, and swelling were observed, and the following ranking of arthritis was given: 0 for normal paw; 1 for mild swelling; 2 for inflammation and digit erythema; 3 for severe

inflammation and erythema; and 4 for severe distortion and failure to use limb.[22]

2.4 | Body weight

Rat body weight was assessed on an electronic scale every 5 days beginning on day 5.

2.5 | Paw thickness

Paw density was assessed by electronic digital calipers every 5 days, beginning on day 5.

2.6 | Hematological parameter measurements

On the 35th day of the study, 0.5 mL of blood samples were collected from the rat's orbital plexus into ethylenediaminetetraacetic acid (EDTA) Eppendorf tubes. Rats were sacrificed by cervical decapitation, under light anesthesia with diethyl ether. To determine the quantity of leukocytes from each rat, a hematoanalyzer (UniCelDxH 600, Beckman, France) was used. Using Wintrobe method, the erythrocyte sedimentation rate (ESR) was determined. At the same time, to determine red blood cells (RBCs) and hemoglobin, frequent laboratory techniques were used.[23]

2.7 | Measurement of lipid peroxidation and antioxidant enzyme activities

From the blood sample withdrawn on the 35th day of the study, the various biological parameters, such as MDA, were estimated by the Liu method. CAT, GPx, and SOD activities were performed by method of Lawrence and Burk. The concentration of protein in tests was resolved by the strategy for lin et al.[24]

2.8 | Measurement of serum levels of IL-1β, IL-10, and TNF-α

Peripheral blood was gathered at the 35th day under general rat anesthesia by retro-orbital puncture. Blood was centrifuged for 25 min at 3000 rpm after staying for 1 hr. The serum was then separated and kept until assayed at −80°C. As directed by the manufacturer, the concentrations of IL-1β, IL-10, and TNF-α in rat blood were evaluated using ELISA kits. From the respective standard curves, the concentrations of IL-1β, IL-10, and TNF-α were calculated.[25]

2.9 | Histopathological studies

On day 36, all rats were sacrificed for histopathological examination after anesthesia and left hind paw was separated from rat ($n = 6$). Then, the tissues were fixed and decalcified in EDTA in 10% neutral buffered formalin. Tissue parts (3 μm dense) were colored with hematoxylin–eosin after the paraffin embedding method and regarded for histopathological modifications under a light microscope.[26]

2.10 | Western blot analysis

Protein specimens of rat synovial tissue (intended for serum and synovial tissue preparing) were packed with SDS-polyacrylamide gel 6–10% and divided by electrophoresis. The proteins were then transmitted to the difluoride membrane of polyvinylidene (Millipore, Bedford, Massachusetts). Nonspecific reactions at normal temperature were prevented with 5% skim water for 1 hr and membranes were then tested at 4°C overnight with the specified main antibiotics against p-p38 MAPK, p38 MAPK, NF-kB/p-p65, and NF-kB/p65. The membranes were incubated with secondary antibodies (Cell Signaling Technology) at room temperature for 1 hr after cleaning with TBST solution. The blotted bands of proteins were detected by an ECL Advanced kit.[27]

2.11 | Statistical analysis

All outcomes were presented as mean ± SEM, and all received individuals were statistically evaluated by comparison with the arthritic control. The entire data were analyzed using ANOVA two-way, followed by the test of Dunnett using the version of GraphPad Prism 5.0. The statistically values of $p < .05$ were considered significant.

3 | RESULTS

In this research, we used the model of arthritis induced by the CFA to observe the impact of wogonin. CFA has been commonly used as an animal model of RA in relation to other experimental designs of arthritis, as the CFA model shares a number of clinical, immunological and pathological characteristics with RA.

Figure 1 shows arthritic score outcomes in CFA-administered rats. CFA administration began to show

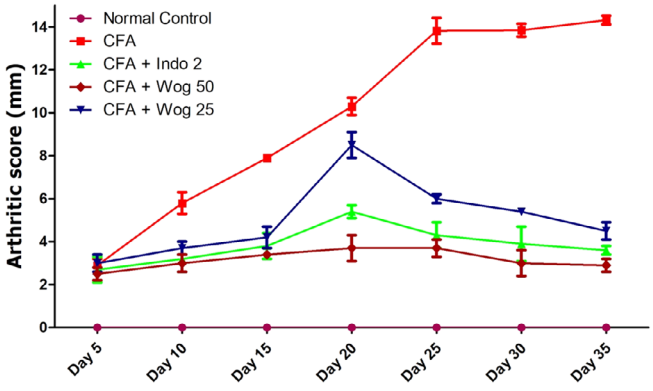

FIGURE 1 Effect of wogonin on arthritic score

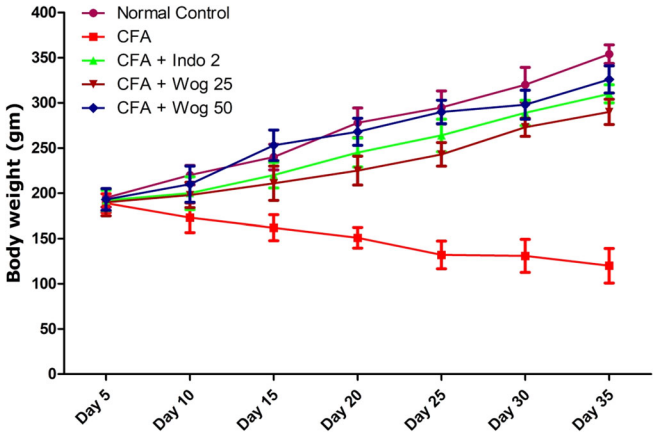

FIGURE 2 Effect of wogonin on body weight

signs and symptoms of inflammation in one or more hind claws. The arthritic score in arthritic control rats was considerably high ($p < .001$) relative to ordinary control rats from day 5 to day 35. Simultaneously, rats handled with Indo 2 mg/kg as a conventional medication reported a substantial reduction in arthritic score ($p < .001$) from day 20 to the end of the research relative to arthritic control rats. Similarly, Wogonin therapy in doses of 25 and 50 mg/kg showed a significant reduction in arthritic performance ($p < .05$, $p < .01$, and $p < .001$) from day 20 to day 35 relative to CFA arthritic rats.

Figure 2 shows a Wogonin impact on CFA rats' body weight. The body weight in arthritic control rats reduced considerably ($p < .001$) from day 5 to 35 relative to ordinary control rats. Indo 2 mg/kg rats handled as a standard medication and 25 and 50 mg/kg wogonin reported significant increases in body weight ($p < .05$, $p < .01$, and $p < .001$) from day 5 to 35 relative to dose-dependent arthritic control rats.

Figure 3 shows an impact of wogonin on paw thickness. The paw width in CFA arthritic rats improved considerably ($p < .001$) from day 5 to 35 relative to ordinary control rats. Rats handled as a conventional drug with Indo 2 mg/kg and wogonin at a dose of 50 mg/kg showed a significant reduction in paw density ($p < .01$ and $p < .001$) from day 20 to day 35 relative to rats under arthritic influence. Wogonin at a dose of 25 mg/kg was not shown to have an important impact on paw size decrease in rats given CFA.

Figure 4 shows an impact of wogonin on CFA administered rats hematological parameters. RBCs and hemoglobin (Hb) reduced considerably ($p < .001$), while significant rises were noted in white blood cells (WBCs) and ESR ($p < .01$) relative to ordinary control rats in CFA arthritic rats. Rats handled with Indo 2 mg/kg and 25 and 50 mg/kg wogonin found significant increases ($p < .05$, $p < .01$, and $p < .001$) in RBCs along with Hb, while demonstrating significant decreases ($p < .05$,

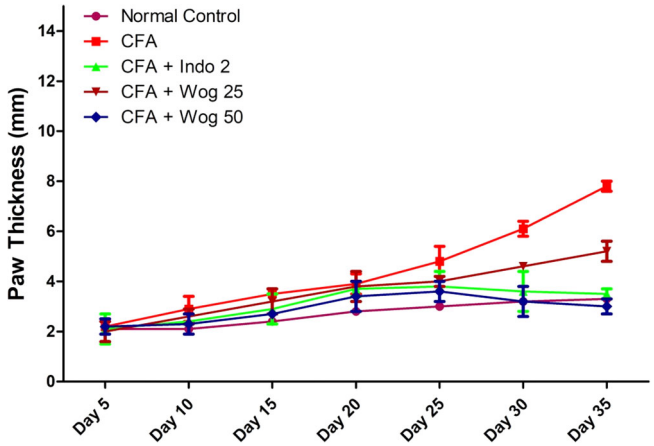

FIGURE 3 Effect of wogonin on paw thickness

$p < .01$, and $p < .001$) in WBC and ESR concentrations from day 20 to day 35 opposed to dose-dependent arthritic control rats.

Table 1 provides changes in MDA, SOD, CAT, and GSH. A substantial rise ($p < .001$) in MDA levels in rats administered by CFA. This outcome was followed by a substantial decrease ($p < .001$) in SOD, CAT, and GSH levels in rats administered by CFA compared to rat control group levels. Coadministration of Indo 2 mg/kg, Wog (25 and 50 mg/kg) considerably decreased MDA concentrations ($p < .05$, $p < .01$, and $p < .001$) and enhanced dose-dependent concentrations of SOD, CAT, and GSH relative to CFA rats.

Table 2 shows an impact of wogonin in rats administered by CFA on inflammatory cytokines. IL-1β, IL-6, and TNF-α have risen considerably ($p < .001$) relative to ordinary command in CFA rats. Treatment with Indo 2 mg/kg as a standard drug, Wog (25 and 50 mg/kg) in a dose-dependent manner significantly reduced ($p < .05$, $p < .01$, and $p < .001$) a level of various inflammatory

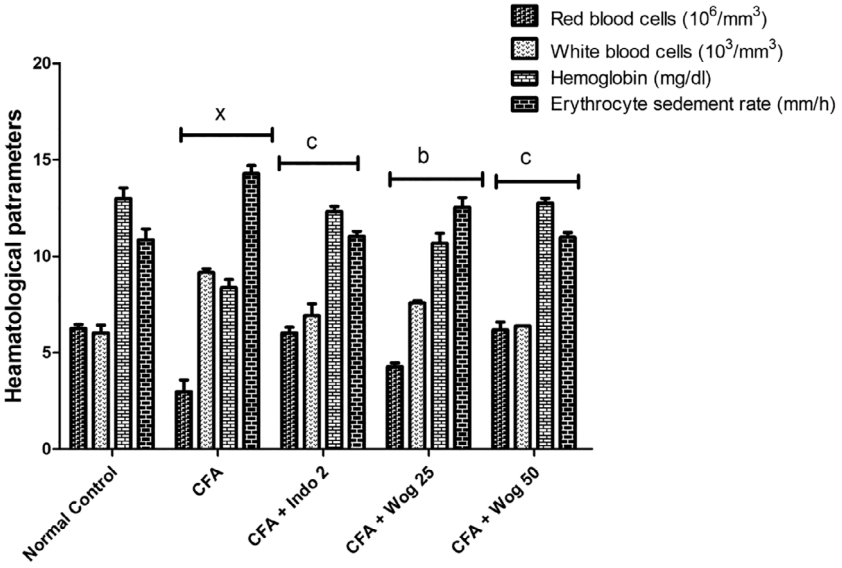

FIGURE 4 Effect of wogonin on hematological parameters

TABLE 1 Wogonin effect on oxidative stress parameters and antioxidant enzymes in CFA induced RA in rats

Treatment	MDA (nmol/mg protein)	SOD (units/mg protein)	CAT (activity/min)	GSH (nmol/mg protein)
Normal control	65.26 ± 3.29	37.27 ± 2.39	72.18 ± 4.28	174.27 ± 10.67
CFA	138.04 ± 8.91*	17.66 ± 1.89*	39.16 ± 2.19*	95.23 ± 7.52*
CFA + Indo 2	67.66 ± 3.06****	33.86 ± 2.10****	69.46 ± 4.05****	168.82 ± 10.51****
CFA + Wog 25	98.09 ± 5.73***	26.06 ± 2.05**	58.40 ± 4.27**	136.19 ± 9.93***
CFA + Wog 50	71.60 ± 4.61****	34.76 ± 3.02****	68.17 ± 4.92****	162.74 ± 10.83****

Note: All values are expressed mean ± SEM ($n = 6$).

Abbreviations: CAT, catalase; GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

*$p < .001$ compared to normal control group whereas **$p < .05$, ***$p < .01$, and ****$p < .001$ compared to CFA.

TABLE 2 Wogonin effect on inflammatory cytokines in CFA induced RA in rats

Treatment	IL-1β (pg/mg protein)	IL-10 (pg/mg protein)	TNF-α (pg/mg protein)
Normal control	274.18 ± 20.17	321.29 ± 22.18	54.26 ± 4.28
CFA	427.18 ± 36.28*	126.82 ± 19.27*	94.16 ± 2.19*
CFA + Indo 2	285.61 ± 21.83****	303.89 ± 22.96****	60.71 ± 4.05****
CFA + Wog 25	358.06 ± 27.56**	205.65 ± 22.91**	74.28 ± 4.27**
CFA + Wog 50	289.55 ± 20.78****	300.67 ± 22.18****	63.28 ± 4.92***

Note: All values are expressed mean ± SEM ($n = 6$).

Abbreviations: IL-1β, interleukin-1 beta; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha.

*$p < .001$ compared to normal control group whereas **$p < .05$, ***$p < .01$, and ****$p < .001$ compared to CFA.

cytokines such as IL-1β, IL-6, and TNF-α as compared to only CFA administered rats.

Additional proof to promote wogonin's inhibitory impacts on CFA was acquired through histopathology assessment of joint. The ordinary control rats showed ordinary joint architecture with ordinary cartilage covering appearance, joint space, and underlying bones without harmful synovial tissue infiltration (Figure 5a). The histological appearance of the joint in CFA administered rats (Figure 5b) was extremely abnormal, with pronounced synovial hyperplasia, infiltration of inflammatory cells. CFA rats treated with Indo 2 mg/kg (conventional drug) (Figure 5c) showed significant reduction in infiltration of inflammatory cells and devastation of cartilage. Wogonin-treated rats at a dose-dependent dose of 25 and 50 mg/kg considerably reduced the

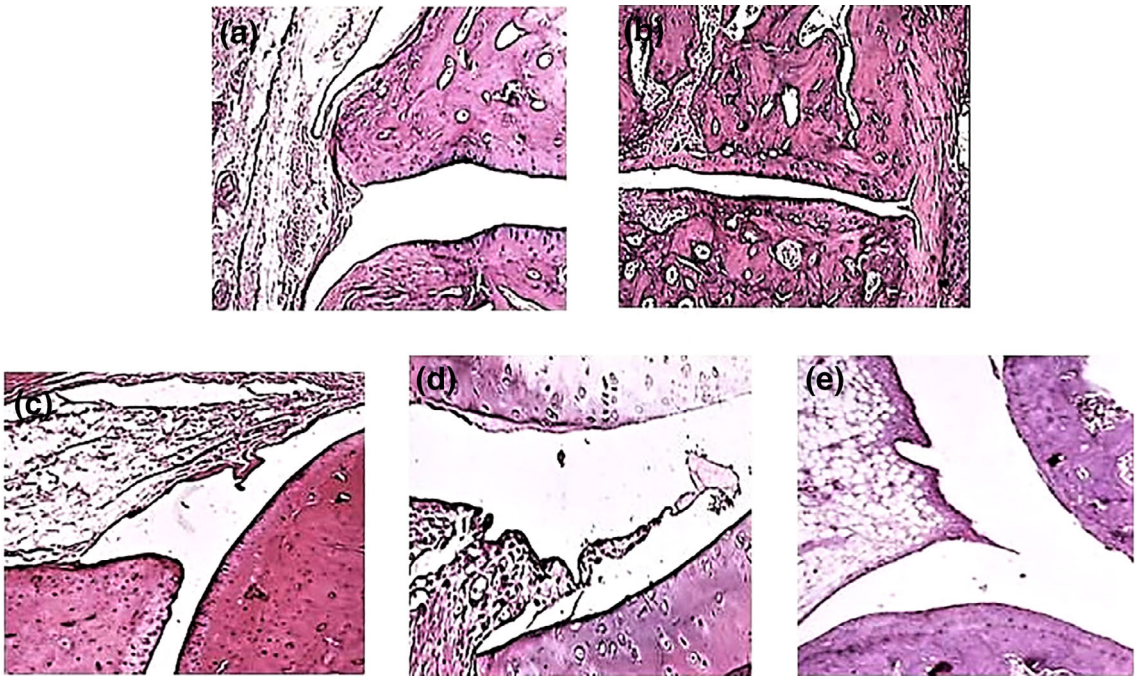

FIGURE 5 Wogonin effect on histopathology of rat ankle joint

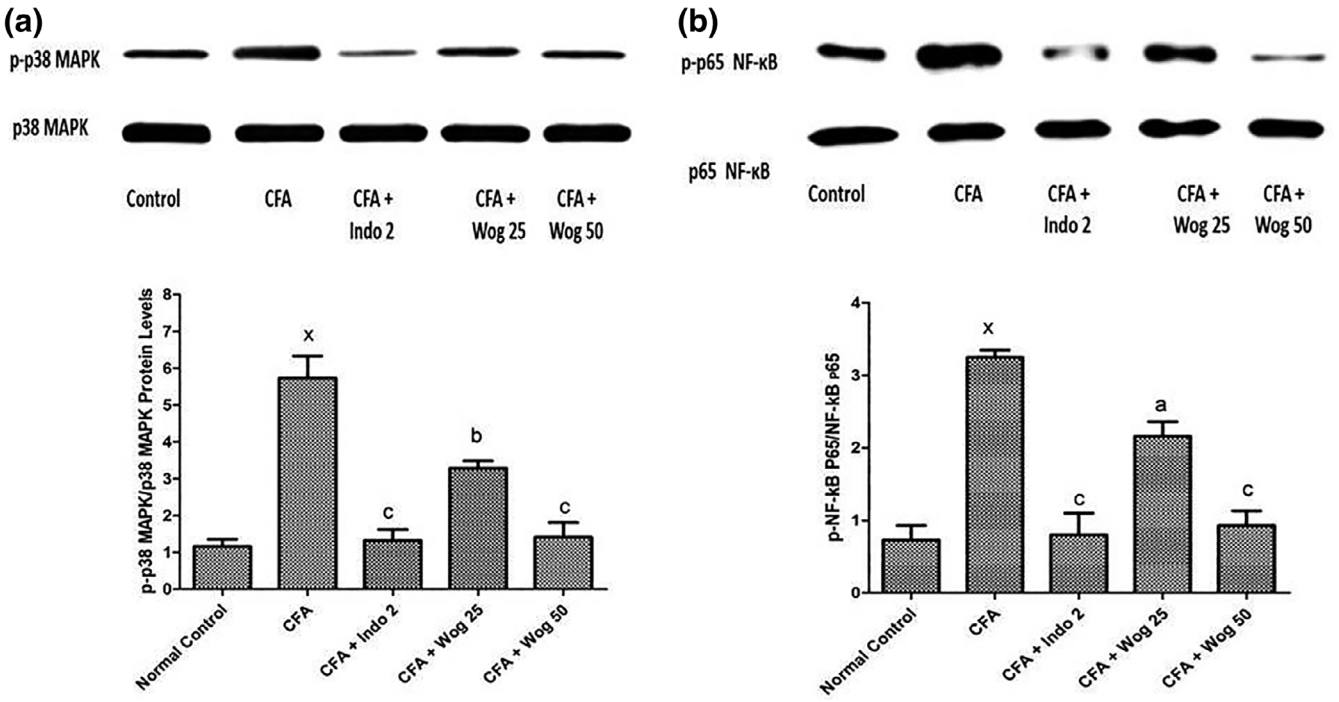

FIGURE 6 Wogonin effect on MAPK and NF-kB signaling pathway

development of pannus, bone erosion, cartilage devastation, and inflammatory cell infiltration (Figure 5d,e).

Figure 6 shows an impact of wogonin on the signaling mechanisms of MAPK/NF-kB on rats administered by CFA. As noted in the western blot analysis, CFA administration led in the acutation of MAPK. Although there was no change in the total protein of these isoforms, the phosphorylation levels of the MAPKs isoforms (p38) were increased in the CFA-treated group. Meanwhile, Indo 2 mg/kg (conventional drug), dose-dependent therapy with Wog 25 and 50 mg/kg reduced the phosphorylation of MAPKs relative to the CFA category (Figure 6a).

In the event of CFA administration, it was highly triggered with respect to the NF-kB signaling pathway. ELISA p65 detection (activated NF-kB subunit) showed a significant rise of NF-kB p65 relative to animal control (Figure 6B). Western blot analysis showed a noticeable increase of NF-kB (p-p65) phosphorylated subunit concentrations relative to the NF-kB activation control group. In contrast, Indo 2 mg/kg (conventional drug), Wog 25 and 50 mg/kg efficiently counteracted NF-kB activation and dose dependently reduced p65 phosphorylation.

4 | DISCUSSION

Given the biophysical parameters, wogonin reduced arthritic performance considerably, enhanced body weight, and reduced paw size. Wogonin also boosted RBC considerably along with Hb and reduced WBC count and ESR. The therapy of wogonin has noted significant recovery in RA by enhancing the parameters to a standard rate. The findings of the above research on wogonin obviously show its antiarthritis protective impacts. Because of its antioxidant characteristics, Wogonin is considered to produce its activity.[28,29]

Analysis of different biochemical parameters that are MDA, GSH, SOD, and CAT explored the antioxidant impact of wogonin. An imbalance between pro-oxidants and antioxidants is the creation of oxidative stress. MDA was increased during RA, suggesting that MDA was deemed a critical injury mechanism. A decrease in GSH could impair the clearance of H2O2 and support OH formation, enhancing the free radical load that leads to homeostasis disturbance.[30–32] In addition, many trials have shown that there is a lot of oxidative stress in RA patients and this oxidative stress can lead to overproduction of cytokines and harm to the joint tissue.[33,34] CFA rats have been shown to have reduced antioxidant enzyme activity in the plasma, such as SOD, CAT, and GSH, and some trials have shown that these antioxidant enzymes have RA impacts. The body therefore has antioxidant enzymatic mechanisms to safeguard tissues from oxidative stress.[35,36] Wogonin has been demonstrated to avoid oxidative stress and has powerful free-radical scavenging characteristics/antioxidant systems.[37,38] In this research, we discovered that the wogonin therapy group had considerably reduced lipid peroxides, greater GSH, and enhanced antioxidant enzyme activity such as SOD and CAT that could decrease or stop ROS from forming. Therefore, our findings stated that the suppression of the pathological state of arthritis by antioxidant could be one mechanism of Wogonin in CFA rats.[39–42]

Several investigations disclosed that inflammatory processes played a significant part in RA pathogenesis. An amount of cytokines were demonstrated, such as IL-1β, IL-10, and TNF-α during inflammatory procedures guided the mechanisms. Reducing bone resorption and seriousness of the disease could lead in these cytokines being blocked.[43,44] Otherwise, IL-10 has powerful anti-inflammatory impacts and suppresses RA pathology of cartilage and bone. In this research, we identified IL-1β, IL-10, and TNF-α gene concentrations in the group plasma. Interestingly, the findings indicated that the concentrations of IL-1β and TNF-α in the blood of CFA animals handled with wogonin were considerably reduced.[45,46] We could therefore infer that wogonin has a prospective anti-inflammatory role. These findings are consistent with earlier research that show wogonin's anti-inflammatory impacts by inhibiting proinflammatory cytokines.

In controlling progressive joint death in RA, MAPK mechanisms are critical. The MAPK family is made up of three major members of the subfamily, ERK, p38, and JNK. P38 MAPKs perform critical functions in controlling synovial infection, activated macrophage inflammatory cytokine secretion, and RA collagenase synthesis. Inhibitors targeting the p38 MAPK pathways are therefore suggested to have anti-inflammatory activity.[47,48] We identified inhibitory impacts of wogonin on the phosphorylation of MAPKs in rats induced by CFA in the current research. We discovered, in specific, that wogonin inhibited the p38 phosphorylation triggered by CFA.[49,50] These findings indicate that wogonin's impacts on inflammatory mediators and cytokines manufacturing are probable to be mediated by stopping the signaling pathways of p38. It is well founded that NF-kB signaling plays a crucial part in the advancement and growth of RA by controlling the expression in activated macrophages of proinflammatory mediators like TNF-α and IL-1β.[51,52] The activation of NF-kB and the translocation to the core of the p65 subunit is preceded by the phosphorylation, ubiquitination, and degradation of IkBα. In this research, we found that wogonin considerably attenuated the atomic translocation of p65 induced by CFA through the outcomes of the western blot for NF-kB p65.

The results of this study indicated that wogonin regulated the activity of the MAPK/NF-κB signaling pathway by inhibiting the phosphorylation of p38/MAPK and NF-κB/p65. Wogonin can inhibit the oxidative stress and invasion of inflammatory cytokines, and has a preventive effect on RA. However, further studies need to be performed to explore other mechanisms such as the influence of wogonin on the relationship of the NF-κB pathways and synovial cell proliferation and apoptosis.

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