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Appraisal of the Antiarthritic Potential of Prazosin via Inhibition of Proinflammatory Cytokine TNF-α: A Key Player in Rheumatoid Arthritis

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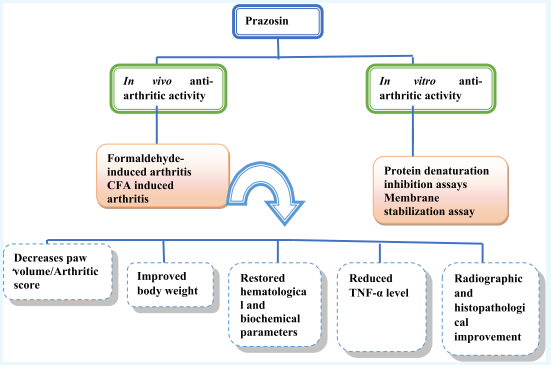
ABSTRACT: Prazosin, a selective α_1 adrenergic receptor antagonist, with documented anti-inflammatory potential, was evaluated for its antiarthritic efficacy by targeting specifically TNF-α. The antiarthritic attribute of prazosin validated through *in vitro* screening comprised thermally provoked denaturation of bovine serum albumin (BSA) and egg albumin along with membrane stabilization evaluation at a concentration of 100−6400 μg/mL, while *in vivo* screening comprised formaldehyde-instigated arthritis at the doses of 5, 10, and 20 mg/kg and complete Freund's adjuvant (CFA)-induced arthritis at 20 mg/kg dose. Paw swelling, body weight, arthritic score, hematological parameters, and histological and radiographic examination of ankle joints were assessed for a period of 28 days after CFA immunization. Moreover, the proinflammatory cytokine TNF-α level was also assessed through quantitative real-time polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Prazosin revealed significant antiarthritic effect evident through protein denaturation inhibition in the egg albumin and the BSA model, stabilization of red blood cell membrane in the membrane stabilizing assay, and reduction in paw volume in formaldehyde-induced arthritis. Likewise, prazosin exhibited propitious antiarthritic effects in the CFA-provoked arthritis model manifested by paw volume and arthritic score alleviation, substantial weight loss prevention, and preservation of the normal hematological and biochemical profile. Histological and X-ray investigation unveiled no substantive structural alterations in treated rat's ankle joints. The TNF-α expression level was also reduced. Thus, the current study is suggestive that prazosin exhibits a strong antiarthritic potential possibly through inhibition of TNF-α.



INTRODUCTION

Rheumatoid arthritis (RA), a chronic relapsing autoimmune malady affecting primarily the joints, has been circumscribed by synovial membrane inflammation, pain, and physical impairment.[1] Articular cartilage destruction observed in RA patients results in bone deformity with subsequent loss of joint function, thereby resulting in severe pain. Approximately, 1−2% of the worldwide population become a victim of this multisystem autoimmune disorder.[2] Disease development and progression have been linked to malfunctioning of cyclo-oxygenase enzymes and some inflammatory cytokines like TNF-α, IL-1β, and IL-6.[3] TNF-α, an essential cytokine associated with the pathogenesis of RA, has been found to be at biologically remarkable levels in RA synovial tissues and fluids. The levels of TNF-α in RA synovia seem to be parallel to the extent of both inflammation and bone erosion.[4] Biological outcomes associated with TNF-α stimulation result from direct actions on multiple target tissues along with induction of other cytokines, such as IL-1, IL-6, and IL-8, thus further worsening the disease condition.[5] Despite the availability of a number of treatment options for treating RA, up to 30% of the patients fail to respond to treatment.[6] However, besides their high cost, prolonged utilization of these treatments is associated with serious adverse attributes like peptic ulcers, complications of the small intestine and colon leading to colitis, perforation, bleeding, stricture, and chronic problems including anemia due to iron deficiency, protein loss, and toxicity. Because of associated toxic effects along with the greater expense of current therapies, there is an utmost need to develop an effective treatment strategy bearing the least side effect profile and being economical as well.[7]

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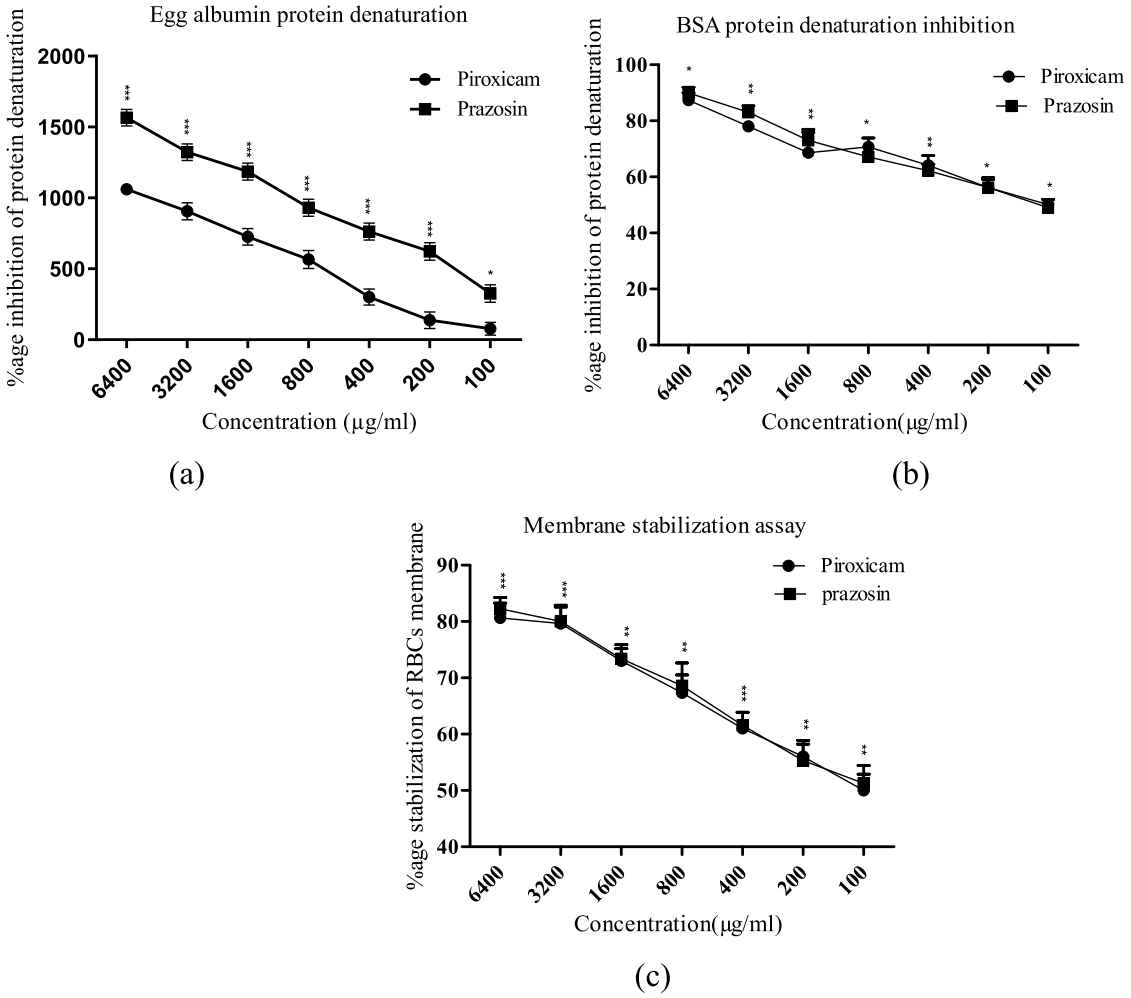

Figure 1. Impact of prazosin on *in vitro* antiarthritic models. (a) Egg albumin denaturation assay, (b) BSA denaturation assay, and (c) membrane stabilization assay. Values are expressed as mean ± standard error of the mean (SEM) ($n = 3$), by two-way analysis of variance (ANOVA) followed by the Bonferroni post-test used, and $p < 0.05$ was considered as significant compared to the arthritic control. Here, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant.

Repurposing drugs implies exploring novel drug−target interactions for established drug treatments, with an intention to utilize them for treatment of another disease. This strategy has gained popularity among pharmaceutical companies and academic scientists because of its ability to decrease investment, derisk clinical activities, and curtail the time required to market a drug for its new use. To date, a number of drugs have been repurposed successfully.[8]

Prazosin, a selective α_1 adrenergic receptor antagonist, has been employed extensively for the management of hypertension and benign prostate hyperplasia. Several studies have demonstrated the anti-inflammatory role of α adrenergic blockers but none of them postulated the mechanism responsible for anti-inflammatory action of these blockers.[9,10] After considerable literature evaluation, no appreciable research on anti-inflammatory and antiarthritic potential of prazosin was available; however, in 1990, a study conducted by Splengler suggested that stimulation of α adrenergic receptors can augment the production of macrophage-derived TNF-α, so it can be concluded from this study that inhibiting the α adrenergic receptor can prove to be a valuable target for suppressing TNF-α production, a proinflammatory cytokine responsible for many inflammatory disorders.[11] The α_1 adrenergic receptor also has a stimulatory effect on Rho/Rho kinase activation,[12] while this Rho/Rock signaling pathway has an important role in the release of proinflammatory cytokines, especially TNF-α;[13] thus, an agent that can block this Rho activation can be helpful in preventing TNF-α release. Also, a study conducted in 2009 by Perez concluded that α1 adrenergic receptor stimulation can activate IL-6 expression; thus, blocking the α1 adrenergic receptor can have an inhibitory effect on IL-6 expression,[14] which is also a major player in RA pathobiology. However, activation of IL-6 in RA is linked with activation of TNF-α; thus, on the basis of results of available scientific data, it was thought worthwhile to evaluate the potential of the α adrenergic antagonist prazosin as a strategy for treating RA with reference to TNF-α blockage.

RESULTS

Inhibition of Thermally Induced Denaturation of Egg Albumin and Bovine Serum Albumin (BSA) by Prazosin. Results of egg albumin and bovine serum albumin denaturation assay displayed the significant ($p < 0.001$) inhibitory potential of prazosin against protein denaturation in a concentration gradient manner (100−6400 μg/mL). Prazosin at the concentration of 100−6400 μg/mL provides 702−1860% percentage protection to egg albumin from denatura-

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ACS Omega 2021, 6, 2379−2388

tion, while 49−90% protection was provided by prazosin to BSA from heat-instigated denaturation. Thus, prazosin displayed a strong, concentration-dependent antidenaturation potential evident in both egg albumin and BSA assay as displayed in Figure 1a,b.

Stabilization of Human Red Blood Cell Membrane by Prazosin against Hypotonicity-Instigated Lysis. Prazosin significantly stabilized the membrane of erythrocytes exposed to a hypotonic saline medium in a concentration-dependent manner (100−6400 μg/mL) as displayed in Figure 1c. Prazosin and the standard drug piroxicam displayed percentage protections of 51.26−82.26 and 50−81%, respectively, to the RBC membrane from hypotonicity-prompted lysis.

Antiedematogenic Implication of Prazosin in Formaldehyde-Provoked Arthritis. Formaldehyde injected via the subplantar route into the hind paw of animals assigned to the disease control group elicited a continuously increasing paw edema of all animals throughout 10 days estimated via a plethysmometer. Contrarily, prazosin dose-dependently and significantly ($p < 0.001$) prevented this formaldehyde-induced augmentation of paw edema compared to the arthritic group. Also, piroxicam (10 mg/kg) significantly ($p < 0.001$) prevented formaldehyde-instigated paw edema. Maximum diminution of paw volume by prazosin at the highest dose of 20 mg/kg was observed on the 10th day with 74.25% paw edema inhibition, as depicted in Table 1. Based on the below-mentioned findings, the highest dose of prazosin was selected for further investigation of the antiarthritic mechanism of prazosin in the complete Freund's adjuvant (CFA)-instigated chronic immunological arthritic method.

Protective Implications of Prazosin on Clinical Attributes of CFA-Provoked Arthritis. All of the animals included in various treatment groups were assessed vigilantly on a weekly basis for establishing the antiarthritic impact of prazosin on disease severity using various clinical parameters such as paw volume determination via a plethysmometer, arthritic index estimation, and alterations in body weight. Animals assigned to the disease control group unveiled serious inflammation with associated erythema observed in both ipsilateral and contralateral paws including the ankle and interphalangeal and metatarsal joints that indicated primary (days 3−6) and secondary (days 14−28) arthritic lesions, which was confirmed by estimating the arthritic score. Arthritic score of greater than 4 confirmed that edema has spread from ipsilateral to contralateral limbs. Animals of the vehicle control group displayed no swelling. Animals provided with prazosin and piroxicam demonstrated unilateral inflammatory edema, which decreased gradually with the passage of time (Figure 2a). A significant ($p < 0.001$) alleviation of paw redness and volume was shown by animals treated with prazosin (20 mg/kg) with repression of edema spread from the ipsilateral to the contralateral hind limb, as evident through the arthritic score recorded on the 28th day (4 ± 0.289) compared to the disease control group (15.43 ± 0.522) as depicted in Figure 2a. Body weight changes observed on a weekly basis for animals of all treatment groups are shown in Figure 2b, which point out continuous declining body weights of animals included in the adjuvant-injected group provided with no treatment. Contrarily, a persistent increment in body weight was revealed by vehicle control animals. Moreover, prazosin-treated animals significantly ($p < 0.001$) prevented this CFA-provoked weight loss compared to disease control animals. Also, paw volume estimated via the plethysmometer on a weekly basis was also

Table 1. Prazosin Impact on Formaldehyde-Provoked Paw Edema[a]

	observed paw volume (mL)				
treatment groups	day 2	day 4	day 6	day 8	day 10
arthritic control (5 mL/kg)	1.55 ± 0.046	1.78 ± 0.026	1.72 ± 0.022	1.66 ± 0.055	1.67 ± 0.055
standard drug (piroxicam (10 mg/kg))	1.153 ± 0.032*** (25.61%)	0.990 ± 0.076*** (44.38%)	0.79 ± 0.052*** (54.06%)	0.657 ± 0.051*** (60.42%)	0.488 ± 0.031*** (70.77%)
prazosin (5 mg/kg)	1.130 ± 0.029** (27.09%)	1.308 ± 0.069** (26.51%)	0.990 ± 0.035** (42.44%)	0.900 ± 0.067** (45.78%)	0.688 ± 0.079*** (58.80%)
prazosin (10 mg/kg)	1.070 ± 0.039*** (30.96%)	1.240 ± 0.058*** (30.33%)	0.890 ± 0.071*** (48.25%)	0.840 ± 0.027*** (49.39%)	0.570 ± 0.019*** (65.86%)
prazosin (20 mg/kg)	0.950 ± 0.180*** (38.70%)	1.125 ± 0.239*** (36.79%)	0.770 ± 0.095*** (55.23%)	0.620 ± 0.080*** (62.65%)	0.430 ± 0.100*** (74.25%)

[a]Data illustrated in the form of mean ± SEM with $n = 6$ and analyzed by means of two-way ANOVA followed by the Bonferroni post-test, where $p < 0.05$ was considered as significant compared to the arthritic control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant.

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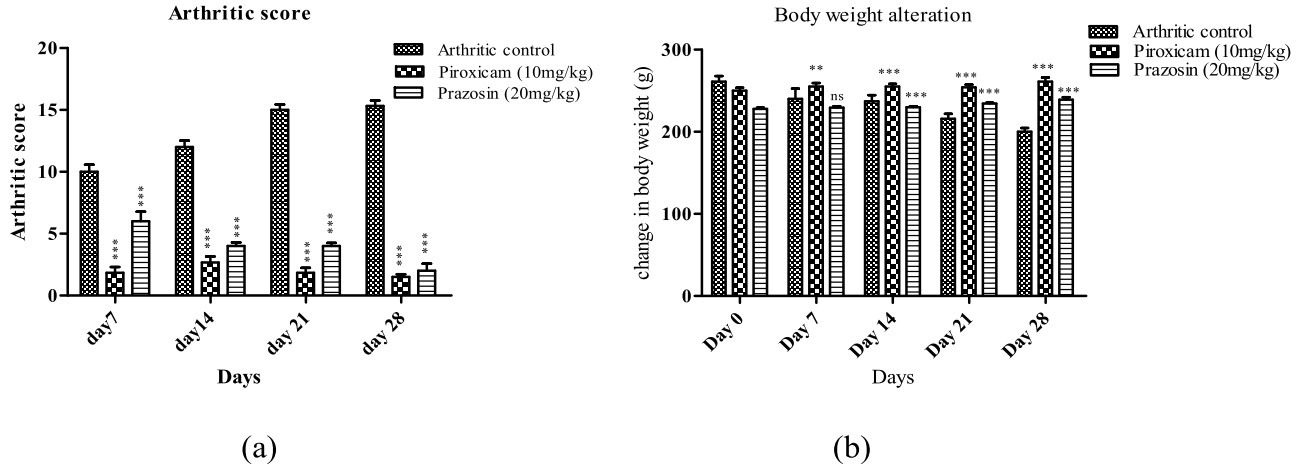

Figure 2. Impact of prazosin on clinical parameters after induction with CFA. (a) Arthritic score and (b) body weight alterations. Values are expressed as mean ± SEM (n = 6), by two-way ANOVA followed by the Bonferroni post-test, and $p < 0.05$ was considered as significant compared to the arthritic control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant.

Table 2. Prazosin Impact on CFA-Provoked Paw Edema[a]

	observed paw volume (mL)				
treatment groups	day 0	day 7	day 14	day 21	day 28
arthritic control (5 mL/kg)	1.22 ± 0.136	2.592 ± 0.056	3.171 ± 0.053	3.039 ± 0.055	2.941 ± 0.066
standard drug (piroxicam (10 mg/kg))	0.980 ± 0.110^{ns}	1.531 ± 0.099*** (40.94%)	1.491 ± 0.074*** (52.99%)	1.108 ± 0.059*** (63.56%)	0.891 ± 0.069*** (69.72%)
prazosin (20 mg/kg)	0.910 ± 0.143^{ns}	1.591 ± 0.125*** (38.63%)	1.541 ± 0.078*** (51.41%)	1.171 ± 0.043*** (61.48%)	0.861 ± 0.046*** (70.74%)

[a]Data illustrated in the form of mean ± SEM with n = 6 and analyzed by means of two-way ANOVA followed by the Bonferroni post-test where $p < 0.05$ was considered as significant compared to the arthritic control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant.

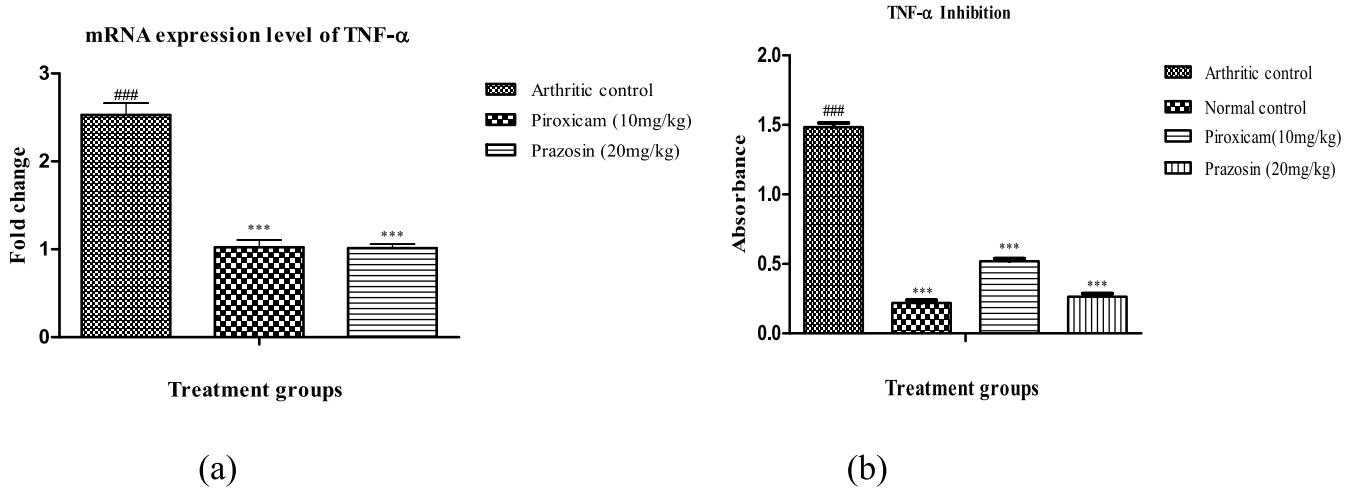

Figure 3. (a) Impact of prazosin on the mRNA expression level of TNF-α through quantitative RT-PCR. Values are expressed as mean ± SEM (n = 6) done by one-way ANOVA followed by Dunnet's test. (b) Inhibitory effect of prazosin on the TNF-α level estimated through the enzyme-linked immunosorbent assay (ELISA). Values are expressed as mean ± SEM (n = 6) done by one-way ANOVA followed by Dunnet's test where ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant represents comparison of the treatment group to the arthritic control group, while $^{###}p < 0.001$ represents comparison between the vehicle control and the arthritic control group.

significantly ($p < 0.001$) reduced by prazosin treatments shown in Table 2.

Suppression of Proinflammatory Cytokine TNF-α Gene Expression by Prazosin Using the Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Technique. Blood samples collected from all treatment groups were subjected to quantitative gene expression scrutinization of TNF-α by quantitative RT-PCR. Changes in the gene expression level of the TNF-α gene of all treatment-receiving groups along with the disease control group have been summarized in Figure 3a. A significantly high gene expression level was observed among animals of the disease control group compared to vehicle control group animals. On the contrary, highly significant decline in the fold change of TNF-α gene

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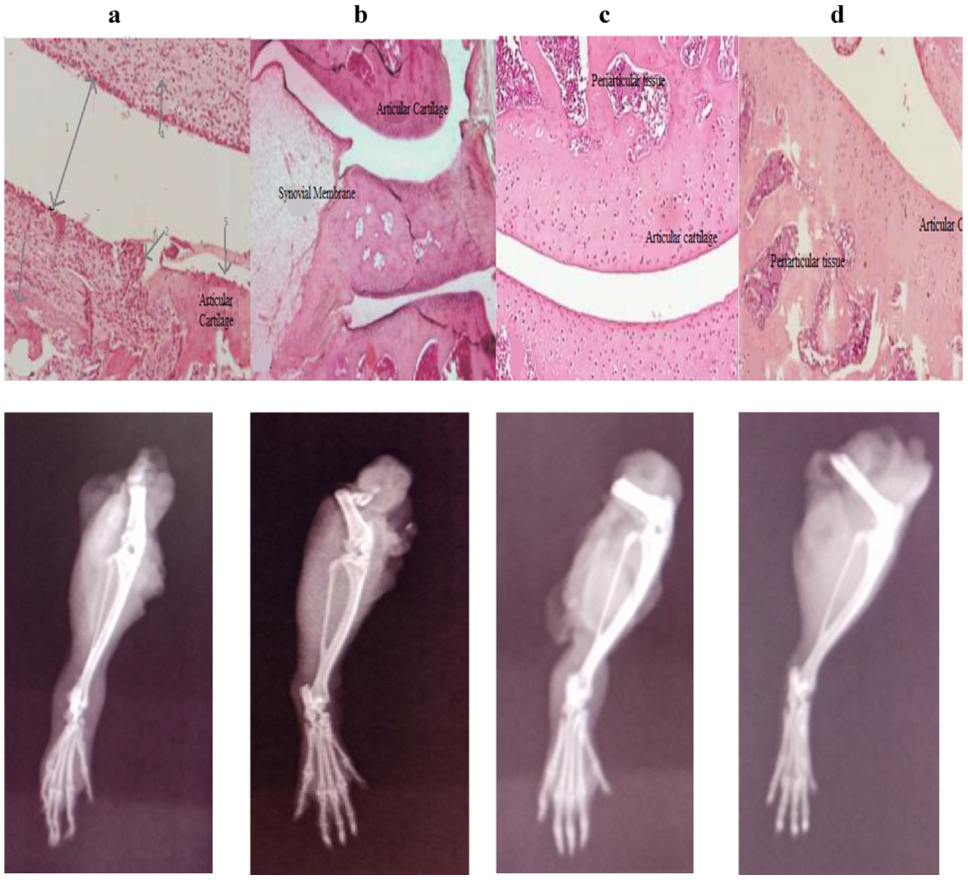

Figure 4. Effect of prazosin (20 mg/kg) on radiographic and histological alterations in the CFA-induced arthritic rat model. (a) Arthritic control, which displayed markedly enlarged joint spaces (1), prominent hyperplasia (2), granuloma formation (3), proliferation of inflammatory cells (4), and evident cartilage erosion (5); (b) normal control; (c) piroxicam-treated; and (d) prazosin-treated.

Table 3. Inhibitory Impact of Prazosin on CFA-Provoked Hematological and Biochemical Alterations[a]

markers	vehicle control	arthritic control	piroxicam (10 mg/kg)	prazosin (20 mg/kg)
WBCs ($\times 10^3/\mu$L)	7.4 ± 0.65***	13.6 ± 1.21$^{###}$	9.9 ± 0.73**	8.8 ± 0.7***
platelets ($\times 10^3/\mu$L)	822 ± 25.5***	1345 ± 19.07$^{##}$	902 ± 41.72***	892 ± 39.73***
ESR (MM/1st h)	3.6 ± 0.55***	8.36 ± 0.62$^{#}$	4.11 ± 0.55***	3.97 ± 0.51***
RBCs ($\times 10^3/\mu$L)	8.9 ± 0.66***	5.64 ± 0.64^{ns}	7.94 ± 0.91**	8.05 ± 0.72***
Hb (g/dL)	15.58 ± 0.75***	8.79 ± 0.78^{ns}	12.44 ± 0.68**	14.04 ± 0.80***
ALP (U/L)	157.23 ± 4.65***	294.01 ± 6.44$^{###}$	189.43 ± 5.84***	165.81 ± 7.87***
AST (U/L)	112.73 ± 5.35***	148 ± 4.68$^{##}$	123.13 ± 5.12**	120.41 ± 4.54**
ALT (U/L)	19.85 ± 6.68***	49.31 ± 5.41$^{###}$	30.53 ± 3.67**	25.52 ± 1.66***
urea (mg/dL)	13.52 ± 2.35***	31.42 ± 2.87$^{##}$	18.74 ± 3.53**	17.66 ± 3.44***
creatinine (mg/dL)	0.39 ± 0.016***	0.96 ± 0.06^{ns}	0.59 ± 0.03***	0.54 ± 0.15**
CRP (mg/L)	3.6 ± 0.97***	39.73 ± 1.84$^{###}$	12.34 ± 1.47***	11.07 ± 0.77***
RF (IU/mL)	6.6 ± 0.79***	35.7 ± 0.67$^{###}$	16.53 ± 0.98***	10.77 ± 0.88***

[a]Data illustrated in the form of mean ± SEM with $n = 6$ and analyzed by means of one-way ANOVA followed by Dunnett's post-test where $p < 0.05$ was considered as significant compared to the arthritic control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns is nonsignificant, while # represents comparison of the arthritic control group to the vehicle control group; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$, and ns is nonsignificant.

expression was displayed by the standard drug piroxicam (10 mg/kg) and prazosin (20 mg/kg) ($p < 0.0001$) when compared with animals in the disease control group.

Inhibitory Impact of Prazosin on the TNF-α Level Estimated via the Enzyme-Linked Immunosorbent Technique. Outcomes of the current study revealed strikingly ($p < 0.001$) attenuated TNF-α levels in animals' serum provided with prazosin (0.264 ± 0.022) and piroxicam (0.52 ± 0.017) when compared with arthritic control animals (1.48 ± 0.032) as presented in Figure 3b.

Protective Implication of Prazosin on CFA-Provoked Radiographic and Histological Alterations in Ankle Joints. Radiographic and histological alterations assessed in ankle/tibiotarsal joints of vehicle control and CFA-injected rats provided with different treatments are illustrated in Figure 4. Arthritic control rat's radiologic examination (Figure 4a) disclosed substantial phalangeal bone erosion, significant joint deformity, bone resorption, swelling in soft tissues, reduced joint spaces, and thickened as well as enlarged connective tissues. Contrarily, radiographs of normal control animals

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ACS Omega 2021, 6, 2379−2388

(Figure 4b) unveiled none of the aforementioned alterations. However, mild joint swelling, minute phalangeal bony erosion and resorption, minor alterations in joint space, and slightly enlarged connective tissue were manifested by animals treated with piroxicam (Figure 4c) and prazosin (Figure 4d). Similarly, results obtained from histological analysis are in correlation with those observed from radiological analysis. Histological examination of CFA control animals (Figure 4a) unveiled substantial synovial infiltration as well articular cartilage erosion. Contrarily, these histological alterations observed among disease control animals were significantly prevented by piroxicam (Figure 4c) and prazosin (Figure 4d) treatment.

Preservation of CFA-Instigated Hematological and Biochemical Alterations. At the end of the study protocol, blood samples were collected from all experimental animals and blood chemistry was analyzed. Significantly elevated levels of WBCs, platelets, and ESR level and attenuated RBCs and Hb level were observed among animals of the arthritic control group. However, significant protection from these abnormal alterations was provided by piroxicam and prazosin treatment, as outlined in Table 3. Moreover, serum levels of ALP, AST, ALT, urea, and creatinine were significantly increased in arthritic control animals in contrast to vehicle control rats. Nevertheless, these biochemical parameters were significantly preserved to near-normal values by piroxicam and prazosin treatment (Table 3). Also, the levels of CRP and RF were increased in arthritic rats, while these aforementioned markers are significantly preserved to near-normal values by piroxicam and prazosin treatment, as demonstrated in Table 3.

DISCUSSION

Rheumatoid arthritis, a multifactorial autoimmune disorder, has been characterized as a joint disability disease.[15,16] Among many responsible factors contributing to RA progression, TNF-α has been shown to be the key player in the pathobiology of RA. Patients suffering from RA present with a high level of TNF-α, and it plays a prominent role in joint destruction by producing inflammation. TNF-α exerts its arthritic manifestations via direct action on multiple tissues as well as induction of other cytokines, especially IL-1β, IL-8, and IL-6. Thereby, blockade of TNF-α not only prevents the devastating effects produced by TNF-α but also inhibits the functioning of other mediators of inflammation.[17−19] Therefore, keeping in mind the pivotal job of TNF-α in the advancement of disease severity, attention is diverted toward targeting this proinflammatory cytokine. Prazosin, with a documented anti-inflammatory potential, was selected to be screened as an antiarthritic agent via inhibiting TNF-α. This selected drug significantly produced anti-TNF-α effects; however, the exact mechanism that produced this blockade remains to be elucidated.

The antiarthritic attribute of prazosin was assessed by employing a number of *in vitro* and *in vivo* experimental models. Protein denaturation inhibition assays are commonly employed in *in vitro* assays to elucidate the antiarthritic potential of any test agent. Denatured proteins lead to the generation of autoantigens and provoke immune response, thus activating TNF-α production, which can then produce synovial infiltration.[20,21] Egg albumin and bovine serum albumin when heated caused denaturation of these proteins.[22] Thus, agents that possess the property of deterring this phenomenon of protein denaturation can serve as useful antiarthritic agents. Outcomes of the current investigation revealed the antidenaturation effect of prazosin, thereby proving its antiarthritic potential.

Human red blood cell membrane resembles the lysosomal membrane, so stability of the RBC membrane can be linked to the stability of the lysosomal membrane. Stability of the lysosomal membrane will prevent the release of degradative enzymes and also phospholipase A_2, which can cause tissue damage and production of inflammatory intermediaries.[15] Prazosin evidently subdued the hemolysis of the RBC membrane exposed to the hypotonic medium, thereby stabilizing the membrane of RBCs and thus is capable of exerting beneficial effects in RA by preventing degradative enzyme release from lysosomes.

Formaldehyde-provoked arthritis has gained wide popularity due to its close resemblance to arthritic manifestations observed in humans.[15] Formaldehyde when injected elicits a biphasic response; the first phase—termed the neurogenic phase—results in the release of histamine-, serotonin-, and kinin-like substances, while the later phase—characterized as tissue response—leads to liberation of prostaglandin-like substances. These released mediators result in vasodilatation, inflammatory edema, algesia, increased capillary permeability, and articular damage.[23] Findings obtained from the current investigation outlined the promising antiarthritic attributes of prazosin in formaldehyde-instigated arthritis.

Freund complete adjuvant-induced arthritis (FCAIA) has been documented as a well-established chronic inflammatory model of arthritis. An intense systemic inflammatory response being characterized by extreme swelling, joint remodeling, and permeation of the synovium has been displayed by animals inoculated with FCA. These symptoms are quite similar to those observed in human RA (characterized by paw edema, body weight loss, pain in joint, and cartilage destruction).[24,25] CFA injection in the hind paw activates the immune system with the resultant abnormal proliferation of leukocyte proliferation. Dendritic cells consider these adjuvant contents as foreign body, thus stimulating phagocytosis, marked CD4+ lymphocyte proliferation along with the release of some cytokines, particularly TNF-α. It has been documented that TNF-α has a central role in inflammation and synovial tissue damage, as is evident from its increased levels in RA patients.[26] Animals treated with the standard drug piroxicam and prazosin revealed pronounced alleviation of paw edema and the arthritic index. Thus, on the basis of our current finding, it can be concluded that prazosin reduced paw volume and the arthritic index by arresting TNF-α release. Furthermore, it can be postulated that TNF-α inhibition arrests the induction of IL-1β, IL-6, and IL-8, thus inhibiting the release of other inflammatory markers COX-2 and PGE2, which in turn inhibit inflammation and ultimately paw volume and the arthritic index.[27]

In addition to paw volume and the arthritic index, body weight measurement has been considered as an important parameter for estimation of the antiarthritic consequence against CFA-induced arthritis. Cachexia has been considered to be a hallmark of RA. Several factors contribute to the development of this muscle wasting associated with RA, but it has been well-documented that increased levels of TNF-α can lead to cachexia.[28] Energy consumption stimulation by TNF-α occurs via the action of TNF-α on protein and lipid metabolisms with the resultant reduction in energy intake by provoking anorexia, thus leading to cachexia.[29,30] Prazosin

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revealed a promising inhibitory impact on the expression of inflammatory cytokine TNF-α.

Aside from macroscopic parameters' evaluation associated with arthritis progression, histological and radiographic alterations specifically in the ankle joint were also assessed. A direct correlation was observed among alterations in clinical parameters and histological modifications observed after 28 days of CFA injection. TNF-α proved to be an inhibitor of osteoblast and a stimulator of osteoclastogenesis, thus leading to bone erosion demonstrated by RA patients.[31] TNF-α has also been known to accelerate cartilage destruction through induction of matrix metalloproteinases (MMPs), an enzyme involved in degradation of the cartilage extracellular matrix.[32] The outcomes obtained from the aforementioned study also revealed cartilage destruction and tissue erosion of the ankle joint of arthritic control animals with significant proliferation of inflammatory cells. Nevertheless, the histological investigation of the arthritic rats receiving the standard drug piroxicam and the investigated drug prazosin displayed moderate synovial infiltration and minor cartilage damage in comparison to the arthritic control animals. Thus, it might be suggestive in the light of the above-mentioned findings that the test drug inhibits bone erosion and cartilage destruction through modulation of the TNF-α effect.

Disturbances in blood parameters have been associated with RA pathobiology (CBC, liver, and kidney parameters) because of inflammatory and immunological attributes. Therefore, examining the impact of any test agent in rectifying such disturbances would be considered of great value when developing a new treatment strategy for RA. Among the extra-articular manifestations of RA, the most common is anemia, which ensues from decreased levels of erythropoietin, untimely erythrocyte destruction, diminished plasma iron, and abnormal loading up of iron in the reticuloendothelial system and synovial tissue; ultimately, the bone marrow fails to counter anemia.[33] Pathogenesis of anemia associated with RA is multifactorial, but inflammatory cytokines, particularly TNF-α, appear to play a prominent role. TNF-α has been documented to produce anemia via modulation of the macrophage iron metabolism. However, more recent evidence has suggested that inhibition of bone marrow erythropoiesis by TNF-α has been the major mechanism of RA-induced anemia.[34] The findings of our study revealed that prazosin considerably augmented Hb and RBC levels, thereby precluding anemia development by stimulating erythropoiesis, possibly by arresting TNF-α release from monocytes and macrophages. A significant decline in levels of ESR and RF (critical hematological markers)[35,36] has been displayed by prazosin in arthritic rats, especially a decline in the RF level observed in arthritic rat's serum depicts the protective role of prazosin in ameliorating symptoms associated with rheumatoid arthritis. Thus, it can be concluded that alterations in blood parameters due to arthritis have been shifted toward normal by prazosin treatment. Thus, on the basis of all experimental outcomes, it can be proposed that prazosin has good efficacy against RA; however, mechanistic studies are still required to sort out the major responsible signaling pathways.

CONCLUSIONS

Based on the aforementioned findings, it is conceivable that prazosin displayed promising antiarthritic attribute. A significant and concentration-dependent antiarthritic effect is revealed by prazosin by arresting thermally induced protein denaturation, thereby obstructing the formation of autoantigens liable for provoking autoimmunity. A noticeable HRBC membrane stabilization has been revealed, which is projected to be due to preservation of lysosomal membrane integrity, consequently counteracting the liberation of hydrolytic and proteolytic enzymes accountable for tissue impairment in RA. Correspondingly, prazosin significantly arrested paw edema/inflammation in formaldehyde-induced arthritis. Additionally, the antiarthritic potency in CFA-induced arthritis may be suggested owing to inhibiting the increase in paw edema, secondary lesions, body weight loss, cartilage impairment, and bone erosion perhaps by downregulating mRNA expression levels of TNF-α. Thus, it is noteworthy that the antiarthritic effect of prazosin might be due to its anti-inflammatory and immunomodulatory potential. Taken together, it can be concluded that prazosin might be a potential candidate for RA treatment. However, further detailed mechanistic screening should be done to get an insight into the exact mechanism of the action of this drug.

MATERIALS AND METHODS

Chemicals and Instruments Used. Rosuvastatin (Getz Pharma, Faisalabad), piroxicam (Sigma-Aldrich), disodium hydrogen phosphate (Sigma-Aldrich), sodium dihydrogen phosphate (Merck), potassium dihydrogen phosphate (Sigma-Aldrich), dextrose (Sigma-Aldrich), tri-sodium citrate (Merck), citric acid (Riedel-de Haën), sodium chloride (Sigma-Aldrich), potassium chloride (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich), hydrochloric acid (Sigma-Aldrich), formaldehyde (Merck), complete Freund's adjuvant (Sigma-Aldrich), cDNA synthesis kit, PCR loading dye (Thermoscientific), SYBER green master mix (RT- PCR) (Thermoscientific), agarose gel, RNase free water (Thermoscientific), trizole (Thermoscientific), DNA ladder (Thermoscientific), TNF-α ELISA kit (Abcam), real-time PCR (Bio-Rad), ELISA plate reader (BioTek), and digital plethysmometer (Panlab) were used.

Animals. Male and female healthy Sprague−Dawley rats weighing between 150 and 250 g were utilized in the study. All animals were provided with a controlled temperature of 24−26 °C, relative humidity of 55 ± 5%, and photoperiod of 12:12 h light:dark cycle. All animals received a standard commercial diet and tap water *ad libitum* and were handled in accordance with the guidelines provided by the National Research Council. All experiments were conducted with prior approval from the Institutional Animal Ethical Committee, College of Pharmacy, University of Sargodha (Approval no. 55B19 IEC/UOS).

***In Vitro* Egg Albumin Denaturation Inhibition Assay.** A 5 mL reaction mix was prepared by adding 0.2 mL of 90% egg white along with 2.8 mL of phosphate buffer (pH 6.5) and 2 mL of different concentrations (100−6400 μg/mL) of prazosin and piroxicam. The control solution was prepared using the above-mentioned procedure, except that 2 mL of distilled water was used instead of 2 mL of the test solution. After preparing all reaction mixtures, incubation of these mixtures was done at 37 °C for 15 min duration, followed by heating at 70 °C for 5 min. All reaction mixtures were then cooled down, and their absorbance was measured spectrophotometrically at 660 nm. Percent inhibition of protein denaturation was estimated according to the following formula:[37]

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ACS Omega 2021, 6, 2379−2388

$$\text{inhibition percentage} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right] \times 100$$

where A_{sample} and A_{control} are the absorbances of the sample and control, respectively.

***In Vitro* BSA Denaturation Inhibition Assay.** A 0.5 mL reaction mix was prepared comprising 5% BSA solution (0.45 mL) and 0.05 mL of different concentrations (100−6400 μg/mL) of prazosin and piroxicam. All reaction mixtures were incubated for 20 min at 37 °C following heating for 30 min at 58 °C. Then, 23.5 mL of phosphate buffer with pH 6.4 was added to each prepared mixture, and spectrophotometric absorbance was taken at 660 nm. Composition of the test control was the same as mentioned above except that 0.05 mL distilled water was added instead of the drug solution. Similarly, the product control was also made with the same composition except for the addition of BSA. The assay was conducted in triplicates, and percentage inhibition was calculated as follows:[38]

$$\text{inhibition percentage} = \left[1 - \frac{A_{\text{test solution}} - A_{\text{product control}}}{A_{\text{test control}}}\right] \times 100$$

where $A_{\text{test solution}}$, $A_{\text{product control}}$, and $A_{\text{test control}}$ are the absorbances of the test solution, product control, and test control, respectively.

***In Vitro* Human Red Blood Cell (HRBC) Membrane Stabilization Assay.** Blood was drawn from a healthy person who had not taken NSAID at least 2 weeks before the study, and a written informed consent was taken from a volunteer. Prior approval was taken from the Institutional Ethical Review Committee, College of Pharmacy, University of Sargodha, Pakistan (approval no. 2794B18 IERC/UOS). Blood was combined with an equal volume of Alsver's solution and centrifuged at 3000 rpm for 20 min. Packed cells were removed and washed three times with isosaline solution (0.85% NaCl), and then, 10% v/v HRBC suspension was prepared in an isosaline solution. The sample mixture was prepared by adding 1 mL of different drug concentrations (50−6400 μg/mL), 1 mL of phosphate buffer (0.15 M, pH 7.4), 2 mL of hypo saline solution (0.36% NaCl), and 0.5 mL of HRBC suspension (10% v/v). The control mixture comprised phosphate buffer, HRBC suspension, and 2 mL of distilled water. Samples were prepared in triplicate. Piroxicam was used as a standard drug. All samples were incubated at 37 ± 2 °C for 30 min and then centrifuged at 3000 rpm for 20 min. After that, absorbance of the supernatant was recorded at 560 nm. Percentage membrane stabilization was estimated by the following formula:[39]

$$\text{percentage protection} = [1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}] \times 100$$

where $\text{OD}_{\text{sample}}$ and $\text{OD}_{\text{control}}$ are the optical densities of the sample and control, respectively.

Formaldehyde-Prompted Arthritis. Different treatment groups were made with six animals allocated to each group. Group I served as the vehicle control group, while group II served as the standard-drug-treated group provided with 10 mg/kg piroxicam. Groups III, IV, and V were test-drug-treated groups and received 5, 10, and 20 mg/kg prazosin. All test solutions were administered orally. Arthritis was provoked via injection of formaldehyde 2% solution in the subplantar region of all animals except for those included in the vehicle control group. Formaldehyde was injected on day 1 of the study, 30 min after giving all treatments, and the same procedure was repeated on day 3 also. All treatments were provided for a duration of 10 days, and paw edema was measured by estimating changes in the paw volume with a digital plethysmometer. Protection percentage provided by each treatment was calculated as follows:[40]

$$\text{percentage protection from paw edema} = \frac{\text{PE}_{\text{control}} - \text{PE}_{\text{treated}}}{\text{PE}_{\text{control}}} \times 100$$

where $\text{PE}_{\text{control}}$ and $\text{PE}_{\text{treated}}$ are the amounts of paw edema for the control and treated animals, respectively.

Complete Freund's Adjuvant (CFA)-Induced Arthritis Model. After validating the antiarthritic potential of prazosin and screening the topmost antiarthritic dose, the CFA-induced arthritis model was employed to get a deep insight into the antiarthritic mechanism of prazosin. Animals were alienated into four groups with six rats in each group. Group I was kept as the vehicle-treated group, group II was the arthritic control group, group III received the standard drug piroxicam (10 mg/(kg day)), and group IV received the test drug prazosin (20 mg/(kg day)) for 28 days. CFA (0.1 mL) was injected via the subplantar route on day 1 only.

Clinical Evaluation of Arthritic Manifestations. Arthritic Score. All animals included in different treatment classes were carefully observed for arthritic manifestations. Rats inoculated with CFA developed arthritis manifestations after 4−5 days, as is evident from paw inflammation. Each paw was observed visually, and a score was given according to the criteria mentioned in Table 4. The maximum score for the arthritic index was 16/animal.[41]

Table 4. Scoring Criteria for Appraising the Severity of Paw Inflammation

score	manifestation
0	paw with no evident swelling and redness
1	swelling or redness in 1 digit
2	swelling or redness in 2 digits
3	swelling or redness in 3 digits
4	swelling or redness in the whole paw

Evaluation of Rat's Paw Volume. Paw volume was appraised on a weekly basis with a digital plethysmometer to elucidate the extent of paw edema provoked by CFA injection and suppressed by different treatments. Percentage inhibition of CFA-provoked arthritis was estimated according to the following formula:[41]

$$\text{percentage protection from paw edema} = \frac{V_{\text{control}} - V_{\text{treated}}}{V_{\text{control}}} \times 100$$

where V_c is the paw volume of the control group and V_t is the paw volume of the treatment-provided groups.

Prazosin Impact on CFA-Provoked Body Weight Alterations. Body weight was estimated before inducing arthritis and later weekly for all rats included in different treatment groups to assess the change in body weight induced by CFA.

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Body weight recorded on a specific day was subtracted from body weight of the same animal on day 0 to find out the mean change in body weight.

Appraisal of mRNA Expression Levels of TNF-α. RNA was extracted from blood samples of all animals included in different treatment groups according to the TRIZOL method. cDNA was then synthesized according to instructions provided along with the kit by the manufacturer (ThermoScientifc). Real-time polymerase chain reaction was executed using the Bio-Radsystem to amplify and quantify the PCR product. Primer sequences for GAPDH and TNF-α were used from a previously conducted study, and they were prepared by a local manufacturer.[42] The housekeeping gene GAPDH was utilized for normalization of the target gene, TNF-α.[43]

Enzyme-Linked Immunosorbent Assay for TNF-α. The TNF-α level was estimated by employing ELISA as per the instructions provided by the manufacturer (Abcam; ab101784—TNF-α Rat ELISA Kit). A total of 100 μL of the standard as well as sample solution was added in appropriate wells, followed by incubation for 2.5 h at room temperature. The solution was then discarded from each well and washed with 1× wash solution (300 μL) with 4 times repetition. Afterward, any residual wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. Then, 100 μL of 1× biotinylated TNF-α detection antibody was poured into each well and incubated for 1 hour at room temperature with gentle shaking. The solution was discarded, and the wash step was repeated again. Then, 100 μL of a 1× HRP-streptavidin solution was added to each well and heated in an incubator for 45 min at room temperature with gentle shaking. Again, the solution was discarded and washed. TMB one-step substrate reagent (100 μL) was added to each well, and the plate was incubated for 30 min at room temperature in the dark with gentle shaking. Subsequently, 50 μL of stop solution was added to each well and read at 450 nm immediately.[44]

Hematological and Biochemical Analyses. Hematological parameters including RBCs, WBCS, platelets, Hb, and ESR levels were estimated by an automated hemocytometer (Sysmex XT-1800i). Similarly, biochemical parameters such as ALT, AST, urea, and creatinine levels were estimated in the serum of sacrificed animals by employing an autoanalyzer (Humalyzer 3500). The manufacturer's protocol provided along with commercially obtainable kits was followed for analyzing the above-mentioned parameters (Analyticon Biotechnologies AG, Germany). Furthermore, serum RF and CRP level were also measured using commercially available kits.

Radiography and Histopathology. Rats ankle joints excised after the completion of the study were taken for radiographic investigation and later for histological assessment.[41,43] Radiographic examination was done with a computerized radiographic system (Toshiba 630 MA, DS-TA-5A). For histopathological assessment, ankle joints were placed in 10% formalin for fixation; later on, after five days, they were decalcified using a decalcification solution. The tissues were then processed for paraffin embedding. Afterward, tissue slices of about 5 μm thickness were prepared using microtome and then stained with hematoxylin and eosin (H&E). Bone erosion, inflammatory cell infiltration, and pannus formation were examined by a blinded histopathologist.[40]

Statistical Analysis. Results were presented as mean ± SEM. Two-way analysis of variance (ANOVA) test, followed by the Bonferroni post-test and one-way analysis of variance (ANOVA) test followed by Dunnett's test was employed for statistical analysis using Graph Pad Prism 5. The value of significant difference was considered at $p < 0.05$

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Notes

The authors declare no competing financial interest.

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