Rosuvastatin Attenuates Rheumatoid Arthritis-Associated Manifestations via Modulation of the Pro- and Anti-inflammatory Cytokine Network: A Combination of In Vitro and In Vivo Studies

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ABSTRACT: The current investigation employed rosuvastatin for evaluation as an antiarthritic agent by in vitro and in vivo studies. In vitro studies comprised egg albumin and bovine serum albumin protein denaturation assays along with membrane stabilization assays, while in vivo studies comprised formaldehyde and complete Freund’s adjuvant (CFA)-provoked arthritis. The antioxidant potential was estimated via DPPH free radical scavenging and ferric reducing assays. Rosuvastatin significantly inhibited heat-provoked protein denaturation of egg albumin and bovine serum in a concentration-dependent way with the highest inhibition of 1225 ± 9.83 and 82.80 ± 4.03 at 6400 μg/mL. The percentage protection of the RBC membrane from hypotonicity-prompted lysis was found to be 80.67 ± 2.7. Rosuvastatin promisingly subdued formaldehyde-provoked arthritis, with maximum reduction (65.47%) of the paw volume being observed at a dose of 40 mg/kg. Rosuvastatin also significantly (p < 0.001) attenuated arthritis induced by CFA injection by reducing the paw volume and arthritic index. The reduction in the body weight due to CFA injection was also preserved by rosuvastatin treatment. Hematological and biochemical changes due to arthritis induction by CFA injection were also maintained near normal values by rosuvastatin. The histopathological and radiographic investigation also revealed the protective effect of rosuvastatin on preventing structural changes. Gene expression of IL-1β, TNF-α, and IL-6 was reduced, while IL-4 and IL-10 levels were elevated by rosuvastatin in comparison to those for the disease control group. Concentration-dependent antioxidant potential was shown by rosuvastatin. Thus, rosuvastatin possesses a notable antiarthritic potential as evidenced via in vitro and in vivo studies.

1. INTRODUCTION

Rheumatoid arthritis (RA), a multifactorial autoimmune inflammatory disorder, has been associated with swelling, algesia, and synovial hyperplasia with pannus formation, leading to complete destruction of cartilage and bone, thus rendering physical immobility to patients. A precise mechanism conferring this autoimmune disorder remained to be an unsolved dilemma; however, many responsible factors with major roles in RA pathobiology have been unveiled. Cyclooxygenase enzymes have pivotal involvement in inflammatory response evoked in the body by downstream activation of immune cells and inflammatory cytokines. Furthermore, cytokines, especially IL-6, TNF-α, IL-1β, and NF-κβ, have a major contribution to RA development and progression. Treatment strategies available for RA comprise nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, and novel biologics such as TNF monoclonal antibodies and IL-1 receptor blockers. Despite advancement in RA treatment strategies, research is going on to discover new therapeutic options capable of preventing long-term joint damage and functional disability. Also, side effects associated with available treatment options and their high cost are another reason for finding an alternative antiarthritic agent with minimal side effects and low treatment cost. Joint dysfunction due to inflammation and severe pain are hallmarks of RA. Thereby, agents bearing anti-inflammatory and analgesic potential meet the criteria of serving as antiarthritic agents.

Statins, a class of lipid that lowers drug action via inhibition of HMG-CoA reductase, have gained much attention because of their pleiotropic nature. Among many factors responsible for nonlipid-related attributes of statins, the most important...
was thought to be the inhibition of isoprenylation of proteins. Statins inhibit this isoprenylation phenomenon by inhibiting mevalonic acid, which is a precursor molecule for the synthesis of isoprenoid intermediates. Isoprenylation of proteins is responsible for a variety of intracellular signaling mechanisms such as Ras, Rho, and Rac signaling pathways, which regulate cell proliferation, proinflammatory cytokine network, and ROS generation. The anti-inflammatory effect of statins has gained much attention in the past, and this property of statins has been proved by many preclinical and clinical studies. Statins exert their anti-inflammatory effects via numerous mechanisms including suppressed chemokine and proinflammatory cytokine synthesis, matrix metalloproteinase’s inhibition, reduced MHC-II expression induced by interferon γ, and reduced expression of CD40 on macrophages and other smooth muscle cells. Statins have gained much attention in the treatment of rheumatoid arthritis because of their proven immunomodulatory and anti-inflammatory properties. Statins have been tested by many animal studies and also clinically for their anti-inflammatory property. Atorvastatin and simvastatin showed reduced collagen-induced synovial inflammation in experimental animals. An epidemiological survey including 211,627 participants also concluded that the use of statins reduces the risk of RA development. Clinical data, especially conducted with atorvastatin, also supports the fact that the use of statins also helps in reducing disease severity and progression of RA. Despite numerous studies conducted on exploring the beneficial effect of statins on RA, there have been very few studies conducted on the role of rosuvastatin in RA; thereby, the current investigation was planned with an aim to explore the antiarthritic potential of rosuvastatin by different experimental strategies. Rosuvastatin is a commonly prescribed hydrophilic statin with a well-established safety profile. The anti-inflammatory effect of rosuvastatin has been established in various acute and chronic inflammatory models; thereby, this drug was selected for detailed antiarthritic investigation.

2. RESULTS

2.1. Suppressive Implication of Rosuvastatin on Thermally Induced Denaturation of Egg Albumin and Bovine Serum. Significant \( p < 0.001 \) suppression of thermally induced egg albumin and bovine serum albumin protein denaturation in a concentration gradient way was represented by rosuvastatin at the concentration of 100–6400 \( \mu \text{g/mL} \). The values of the highest percentage protection from protein denaturation observed for piroxicam and rosuvastatin at the highest concentration were 1088 ± 11.32 and 1225 ± 9.83 in the case of the egg albumin assay and 87.35 ± 2.53 and 82.80 ± 4.03 in the case of bovine serum protein denaturation. Based on the results obtained, it can be delineated that rosuvastatin possesses good potential for arresting protein denaturation, as depicted in Figure 1A,B.

2.2. Stabilization of HRBCs by Rosuvastatin against Hypotonicity-Induced Hemolysis. Rosuvastatin displayed significant concentration-dependent protection of the erythrocytic membrane of human red blood cells from lysis being provoked by a hypotonic solution, as shown in Figure 1C. The values of percentage stabilization from hypotonicity-prompted lysis of the erythrocytic membrane at the selected concentration of 100–6400 \( \mu \text{g/mL} \) revealed by rosuvastatin and piroxicam are 78.00 ± 1.42–47.63 ± 1.60% and 80.67 ± 2.7–50 ± 2.89%, respectively.

2.3. Antiedematogenic Implication of Rosuvastatin in Formaldehyde-Induced Arthritic Assay. Formaldehyde injection via the subplantar route into the left hind paw of rats in the disease control group revealed a continuous increase
in paw edema measured with the help of a plethysmometer. Rosuvastatin administered in three different doses (10, 20, and 40 mg/kg) displayed promising suppression of paw edema induced by formaldehyde, the maximum suppression being noticed at the highest dose of 40 mg/kg. Standard drug piroxicam (10 mg/kg) also displayed significant \((p < 0.001)\) protection from edema of the injected paw compared to the control group. Rosuvastatin (40 mg/kg) revealed a compelling reduction in edema of treated animals at the end of the study with 65.47% protection from paw edema, as depicted in Table 1.

2.4. Protective Attributes of Rosuvastatin on Different Parameters Assessed in the Complete Freund’s Adjuvant (CFA)-Induced Arthritis Model. After induction of arthritis via subplantar injection of CFA, different parameters including the paw volume, arthritic index, and body weight were assessed periodically in a 7 day interval for 28 days. It was evident from the outcomes of the study that the arthritic control group displayed continuously elevated paw volume assessed on different days, while a significant \((p < 0.001)\) reduction in the paw volume was delineated by piroxicam- and rosuvastatin-treated animals, which confirms the antiedematogenic attribute of rosuvastatin. The values of percentage protection from CFA-prompted swelling on the last day of the study displayed by piroxicam and rosuvastatin were (70.16%) and (67.45%), respectively, as shown in Table 2. Also, results obtained for arthritic index estimation were also in accordance with the results obtained for paw swelling evaluation. A perpetual elevation of the arthritic index was exhibited by the disease control group; the maximum increase observed on the 28th day was 15.33 \(\pm\) 0.42. Contrary to the disease control group, piroxicam- and rosuvastatin-treated animals displayed significantly reduced arthritic index, as evident in Figure 2A. Body weight assessment also supports the protective implication of rosuvastatin in CFA-provoked arthritis. Animals assigned to the disease control group presented continuously decreasing body weight, while the body weight was preserved significantly \((p < 0.001)\) in animals provided with piroxicam and rosuvastatin, as shown in Figure 2B.

2.5. Prevention of CFA-Associated Hematological and Biochemical Changes by Rosuvastatin. Hematological parameters, particularly the RBC count and Hb level, were found to be lowered in the disease control group, while WBCs, platelets, and ESR levels were elevated in animals included in the disease control group and these parameters were significantly preserved in piroxicam- and rosuvastatin-treated groups, as shown in Table 3. Biochemical parameters such as CRP, RF, AST, ALT, ALP, urea, and creatinine were found to be lowered in the disease control group, while WBCs, platelets, and ESR levels were elevated in animals included in all experimental groups compared to the control group. The values in parentheses represent percentage inhibition of paw edema compared to the control group calculated according to the above-mentioned formula.

2.6. Effect of Rosuvastatin on Radiographic and Histological Changes. Radiographic analysis of arthritic rats revealed joint deformities with narrowing of joint spaces and metatarsal joint inflammation along with swelling of the whole leg. These changes were promisingly suppressed by rosuvastatin and piroxicam, as shown in Figure 3. Histopathological assessment of all animals included in the disease control group manifested structural changes evident through pannus formation and cellular infiltration in comparison to the disease control group with no appearance of such characters, as shown in Table 3.
Table 2. Rosuvastatin Evoked Protection against CFA-Prompted Arthritis

| experimental groups                  | Paw volume changes (mL) |
|--------------------------------------|-------------------------|
|                                      | 0 day    | 7th day | 14th day | 21st day | 28th day |
| disease control (3 mL/kg distilled   | 1.22 ± 0.146 | 2.60 ± 0.046 | 3.21 ± 0.061 | 3.04 ± 0.034 | 2.95 ± 0.075 |
| water)                               |          |         |          |          |          |
| standard drug                        | 0.95 ± 0.055*** (41.53%) | 1.50 ± 0.063*** (53.27%) | 1.11 ± 0.078*** (63.48%) | 0.88 ± 0.112*** (70.16%) |          |
| Piroxicam (10 mg/kg)                 |          |         |          |          |          |
| Rosuvastatin (40 mg/kg)              | 0.95 ± 0.070*** (40.38%) | 1.54 ± 0.067*** (52.02%) | 1.21 ± 0.042*** (60.19%) | 0.96 ± 0.045*** (67.45%) |          |

*Data are represented as mean ± SEM, where n = 6, and analyzed by two-way ANOVA followed by the Bonferroni post-test, where ***p < 0.001, **p < 0.01, *p < 0.05, ns = not significant. The values in parentheses represent percentage inhibition of paw edema compared to the control group calculated according to the above-mentioned formula.

2.7. Rosuvastatin Effect on Gene Expression Level of Important Proinflammatory and Anti-Inflammatory Cytokines.

The reverse transcriptase polymerase chain reaction (RT-PCR)-estimated gene expression levels of some most important inflammatory mediators with pivotal roles in RA pathology revealed that the level of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) were markedly elevated in the negative control, while the levels of anti-inflammatory cytokines (IL-4 and IL-10) were suppressed in these animals in comparison to normal control animals, as shown in Figure 5. Fold changes observed for arthritic control animals were 6.49 ± 0.201 (IL-1β), 2.41 ± 0.13 (TNF-α), and 5.40 ± 0.51 (IL-6). Piroxicam- and rosuvastatin-treated animals strikingly suppressed the level of aforementioned proinflammatory cytokines with marked elevation of levels of anti-inflammatory (IL-4 and IL-10) cytokines.

2.8. Antioxidant Potential of Rosuvastatin in DPPH Free Radical Scavenging and Ferric Reducing Assays.

Significant concentration-dependent antioxidant potential was displayed by rosuvastatin in DPPH free radical scavenging and ferric reducing assays with percentage antioxidant potentials of 85.98 ± 0.83 and 1240.82 ± 4.07 at 6400 μg/mL in the case of DPPH free radical scavenging and ferric reducing assays, respectively. Ascorbic acid employed as the standard showed 91.28 ± 1.64% (DPPH free radical scavenging assay) and 1342.92 ± 6.54% (ferric reducing) antioxidant efficacy at the highest concentration of 6400 μg/mL, as shown in Figure 6A,B.

3. DISCUSSION

Rheumatoid arthritis, a synovial tissue-associated inflammation, results from an overactivated immune response directed against self-antigens and remains to be an unsolved dilemma with no cure until now. Available treatment modalities provide symptomatic relief only with associated side effects. Therefore, a new treatment option with maximum efficacy and minimal side effects is the need of hour. The current investigation was carried out in this context to identify a new antiarthritic agent from already available drugs used for other purposes. Rosuvastatin with a well-established anti-inflammatory profile was evaluated through in vitro and in vivo strategies to establish its role in RA.

Rosuvastatin has been described as a competitive inhibitor of HMG-CoA reductase enzyme selectively and reversibly. Rosuvastatin’s affinity for the active site of the enzyme is four times greater than the affinity of HMG-CoA for the enzyme, and this affinity is also greater as compared to other statins.
Rosuvastatin as compared to other statins is not metabolized by CYP enzymes and thereby is less likely to induced metabolic drug interactions as evident with other statins. Meta-analysis of clinical trials as well as marketing experience has shown rosuvastatin to be an efficacious statin as compared to atorvastatin and simvastatin. Also, a clinical trial conducted for estimating the efficacy of rosuvastatin and atorvastatin in cardiovascular disorders indicates that rosuvastatin proved to be more efficacious than atorvastatin and also possessed a greater safety profile as compared to other statins when administered at a dose of 10–40 mg daily.

Table 3. Impact of Rosuvastatin on Hematological and Biochemical Parameters

| parameters       | normal control | disease control (3 mL/kg distilled water) | piroxicam (10 mg/kg) | rosuvastatin (40 mg/kg) |
|------------------|----------------|------------------------------------------|----------------------|------------------------|
| CRP (mg/L)       | 3.5 ± 0.80***  | 38.83 ± 1.53                             | 10.35 ± 1.51***      | 11.15 ± 0.39***        |
| Hb (g/dL)        | 14.18 ± 0.65***| 8.81 ± 0.97                              | 13.41 ± 0.68***      | 12.15 ± 0.83***        |
| WBCs (×10⁶/μL)   | 8.1 ± 0.63***  | 14.01 ± 1.01                             | 9.23 ± 0.54***       | 9.49 ± 0.46***         |
| RBCs (×10⁶/μL)   | 8.78 ± 0.66*** | 5.83 ± 0.42                              | 7.88 ± 0.80***       | 7.73 ± 0.62***         |
| platelets (×10⁶/μL) | 840 ± 20.5***   | 1351 ± 18.65                             | 891 ± 32.12***       | 901 ± 19.43***         |
| ESR (MM/1st h)   | 3.55 ± 0.45*** | 8.65 ± 0.41                              | 4.11 ± 0.55***       | 4.38 ± 0.60***         |
| RF (IU/mL)       | 6.35 ± 0.50*** | 35.38 ± 0.29                             | 9.53 ± 0.91***       | 9.86 ± 0.78***         |
| AST (IU/L)       | 112.43 ± 5.10***| 149 ± 3.97                              | 119.02 ± 4.52***     | 120.01 ± 5.04***       |
| ALT (IU/L)       | 19.87 ± 5.38***| 50.21 ± 4.21                             | 21.43 ± 2.73***      | 22.30 ± 2.06***        |
| ALP (IU/L)       | 157.23 ± 3.85***| 273.11 ± 4.54                           | 165.56 ± 3.14***     | 167.31 ± 5.37***       |
| urea (mg/dL)     | 13.53 ± 1.65***| 31.83 ± 1.97                             | 16.74 ± 1.63***      | 17.80 ± 2.04***        |
| creatinine (mg/dL)| 0.38 ± 0.11***  | 0.97 ± 0.05                              | 0.51 ± 0.03***       | 0.56 ± 0.07***         |

“Data are represented as mean ± SEM, where n = 6, and analyzed by two-way ANOVA followed by the Bonferroni post-test, where ***p < 0.001, **p < 0.01, *p < 0.05, ns = not significant.”

Rosuvastatin as compared to other statins is not metabolized by CYP enzymes and thereby is less likely to induced metabolic drug interactions as evident with other statins. Meta-analysis of clinical trials as well as marketing experience has shown rosuvastatin to be an efficacious statin as compared to atorvastatin and simvastatin. Also, a clinical trial conducted for estimating the efficacy of rosuvastatin and atorvastatin in cardiovascular disorders indicates that rosuvastatin proved to be more efficacious than atorvastatin and also possessed a greater safety profile as compared to other statins when administered at a dose of 10–40 mg daily.

Protein denaturation as a result of any electrostatic, hydrophobic, hydrogen-bonding, and disulfide-bonding changes leads to the generation of autoantigens that are considered to be important factor in provoking an inflammatory response, leading to inflammatory disorders including arthritis. Generated autoantigens arouse a type III hypersensitivity reaction with the production of proinflammatory cytokines. Thereby, any drug bearing the potential to prevent this protein denaturation can act as an effective antiarthritic agent. In the current investigation, heating egg albumin and bovine serum leads to protein denaturation but rosuvastatin significantly inhibited this heat-induced protein denaturation in both egg albumin and BSA assays, which show that rosuvastatin can be considered as an effective anti-inflammatory and antiarthritic candidate.

As the inflammatory process continues, it triggers the bursting of the lysosomal membrane with subsequent release of degradative lysosomal enzymes, chemicals that lead to tissue damage, and phospholipase A2, which hydrolyzes phospholipids to produce inflammatory mediators. Thereby, the integrity of the lysosomal membrane is very essential to stop the associated damage to the synovial tissue. The erythrocytic membrane and lysosomal membrane share a lot of common features; thereby, an agent that can efficiently prevent the lysis of the RBC membrane can act as an effective antiarthritic agent. In this context, RBCs were exposed to a hypotonic medium, which instigates their bursting; however, our test drug rosuvastatin efficiently prevented this hypotonicity-provoked lysis of RBCs and further confirms its beneficial role in RA.

Formaldehyde-induced arthritis has been considered to be an important in vivo antiarthritic model because of its resemblance to human arthritis. Formaldehyde injection in the subplantar region of the experimental animal provokes pain and inflammation with denaturation of proteins at the injection site. These denatured proteins further initiate an immune response, leading to the generation of inflammatory mediators, further worsening the condition. The present study revealed the protective attribute of rosuvastatin against this formaldehyde-provoked damage to the injected paw, especially at the dose of 40 mg/kg. This protective attribute of rosuvastatin might be due to its potential to inhibit protein denaturation with subsequent inhibition of inflammatory mediator release.

CFA-instigated arthritis, a chronic inflammatory model, has been used widely to validate the immunomodulatory potential of any antiarthritic agent. CFA comprising a suspension of Mycobacterium tuberculosis in mineral oil initiates an immune response when injected along with subsequent generation of antibodies. CFA-instigated arthritis occurs in three phases with the first phase characterized by no inflammation, the second phase of synovial inflammation apparent after 3–5 days of injection, and the third phase of secondary synovitis apparent after 11–12 days of injection characterized by cartilage and bone destruction and also the appearance of inflammation at noninjected sites. In the present study, CFA provoked edema in the injected paw, which was described as a primary lesion, and also induced inflammation at the noninjected sites such as other limbs, face, and tail, which was described as a secondary lesion. Rosuvastatin promisingly suppressed these CFA-induced primary lesions as evident through a reduction in the paw volume and also inhibited the appearance of secondary lesions as demonstrated by a reduction in the arthritis index, thus proving its antiedematogenic and antiarthritic properties. Rheumatoid cachexia or muscle wasting is associated with RA and can result from a number of factors including lack of mobility due to pain, less food intake, and cytokine, especially TNF-α, induced augmented lipid and protein breakdown. In the current investigation, rats included in the disease control group revealed a prominent decline in their body weight estimated on weekly basis, while this CFA-provoked weight loss was successfully prevented by rosuvastatin, comparable to results obtained from standard drug piroxicam.

With the progression of RA, changes in numerous hematological and biochemical parameters were observed, and these parameters acted as a useful tool for estimating the antiarthritic ability of any test agent. Among many changes, anemia is commonly seen in RA patients and can occur because of a number of reasons including entrapment of iron in reticuloendothelial and synovial tissues, increased bleeding from GIT due to RA medications, premature destruction of
RBCs, and decreased erythropoietin response in the bone marrow. A similar anemic condition was observed in arthritic control rats with reduced levels of Hb and RBCs, while this condition was strikingly prevented in rosuvastatin-treated animals. The WBC level increased with the progression of RA and induced the augmented release of macrophages and granulocyte colony-stimulating factors. The WBC level was markedly preserved by rosuvastatin, thus preventing the exaggerated immune response. Also, the level of ESR increased in the arthritic control group, which leads to increased production of endogenous proteins having an active role in RA progression, while this level was significantly maintained to normal by rosuvastatin treatment.

Among many biochemical parameters, the C-reactive protein and rheumatoid factor acted as a useful tool in evaluating the antiarthritic activity as changes in their level play a prominent role in RA pathobiology. The levels of CRP and RF increased among arthritic control rats, while their levels were markedly suppressed in rosuvastatin- and piroxicam-treated animals, thereby establishing the antiarthritic potential of rosuvastatin. Hepatic enzymes including ALT, AST, and ALP are considered important inflammatory markers. Levels of these enzymes were significantly elevated in adjuvant-injected animals. The increased level of these enzymes has been linked with the release of some inflammatory mediators. These changes are further supported by the analysis of radiographic and histological changes in the CFA-induced arthritis model.
enzymes also have a role in bone erosion and osteopenia. In the current investigation, disease control animals presented a marked increase in levels of ALT, AST, and ALP, while rosuvastatin treatment significantly preserved the level of these enzymes to normal. Oxidative stress due to overproduction of reactive oxygen species and suppression of the antioxidant defense system also has an important role in the disease progression of RA. Current findings revealed strong antioxidant properties of rosuvastatin as evident by DPPH free radical scavenging and ferric reducing assays.

Radiological and histological assessments provides a useful way of assessing the structural changes that appear during the RA disease course. Hallmarks of RA, such as synovial infiltration, bone erosion, pannus formation with cartilage and joint erosion, are thought to be due to the activation of cytokines, especially IL-1β, TNF-α, and IL-6. Synovial infiltration, bone erosion, narrowing of the joint space, and cartilage destruction were observed among arthritic control rats, while these structural changes were promisingly suppressed by rosuvastatin and piroxicam treatments.

Proinflammatory cytokines, especially IL-1β, TNF-α, and IL-6, have a very critical contribution to the progression of RA, and these proinflammatory mediators are widely found in CFA-injected rats. These cytokines triggered local and later systemic inflammation and synthesis of matrix metalloproteinases along with osteoclast activation and thus contributed toward bone resorption, cartilage destruction, and extracellular matrix degradation. Synoviocytes act as the reservoir for these proinflammatory cytokines. Outcomes obtained from RT-PCR analysis revealed that the expression of proinflammatory cytokines IL-1β, TNF-α, and IL-6 was successfully inhibited by rosuvastatin; thus, rosuvastatin proved beneficial in preventing these cytokine-associated inflammatory and structural changes. RA has been considered as a Th1-mediated immune disorder, where activation of the Th1 pathway leads to the generation of Th1 cells, including TNF-α, IL-1, IFN-γ, and Th1, and the Th2 pathway has an opposite role, thereby suppressing each other’s effect. IL-4 and IL-10 are among the primary cytokines regulating Th2 functioning and promotes Th2 cell generation with the inhibition of Th1 response, thus providing beneficial effects in preventing autoimmune disorders. Current investigation’s findings displayed augmented levels of IL-4 and IL-10 by test drug rosuvastatin compared to the diseased control group, where a declined level of these anti-inflammatory mediators was evident. Thus, it can be inferred on the basis of RT-PCR analysis that rosuvastatin might exert its antiarthritic effect by suppressing the proinflammatory cytokine level along with upregulation of the anti-inflammatory cytokine level. Thus, on the basis of all of the outcomes of the current investigation, it can be delineated that rosuvastatin exhibits strong potential to act as an antiarthritic agent.

Figure 5. Modulation of the mRNA expression level of various proinflammatory and anti-inflammatory mediators by rosuvastatin. Data are represented as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Dunnett’s test, and p < 0.05 is considered significant compared to disease control, where ***p < 0.001, **p < 0.01, *p < 0.05, ns = not significant.
The test drug has a suppressive effect on arthritis, decreasing the paw edema in rats. Formaldehyde-induced denaturation stabilized human red blood cell membrane, and rosuvastatin showed strong antiarthritic potential. It inhibited protein upregulation of anti-inflammatory mediators such as TNF-α, IL-1β, and IL-6. Furthermore, it normalized hematological and biochemical parameters.

**5. MATERIALS AND METHODS**

5.1. Animals. For *in vivo* investigation of the antiarthritic property of rosuvastatin, Sprague–Dawley rats of either sex were used. Experimental rats were kept at a controlled temperature (24–26 °C) with a relative humidity of 55 ± 5% and a photoperiod of 12:12 h light/dark cycle. Standard commercial diet and tap water were provided ad libitum to these animals. Handling of rats was done according to the guidelines provided by the National Research Council. Prior approval was taken from the Institutional Animal Ethical Committee, College of Pharmacy, University of Sargodha, Pakistan (Approval no. 68B18 IAEC/UOS) before conducting any experiment involving animals.

5.2. *In Vitro* Antiarthritic Impact of Rosuvastatin on Egg Albumin Denaturation Assay. Reaction mixtures (5 mL) containing 2 mL of different concentrations (100–6400 μg/mL) of rosuvastatin and piroxicam, 0.2 mL of egg albumin, and 2.8 mL of phosphate buffer, pH 6.4, were prepared. The control solution comprised 0.2 mL of egg albumin and 2.8 mL of phosphate buffer as described above along with 2 mL of distilled water. A 15 min incubation at 37 °C followed by 5 min heating at 70 °C was done for all reaction mixtures. The absorbance was then recorded at 660 nm using distilled water as the blank. The percentage inhibition potential of rosuvastatin against heat-induced protein denaturation was estimated according to the following formula:

\[
\text{percentage inhibition} = \frac{A_{\text{test}} - A_{\text{control}}}{A_{\text{control}}} \times 100
\]

where \(A_{\text{test}}\) and \(A_{\text{control}}\) are the absorbances of the test sample and control, respectively.

5.3. *In Vitro* Antiarthritic Impact of Rosuvastatin on BSA Denaturation Assay. Reaction mixtures (0.5 mL) containing 0.45 mL of 5% BSA solution and 0.05 mL of different concentrations (100–6400 μg/mL) of rosuvastatin and piroxicam were prepared. The pH of the above-mentioned mixtures was maintained at 6.3. Incubation for 20 min at 37 °C followed by subsequent heating for 30 min at 57 °C was done for all reaction mixtures. Later, all samples were cooled down and phosphate buffer (2.5 mL) was added to each reaction mixture. The absorbance was recorded at 600 nm. Distilled water (0.05 mL) was taken as the test control, while product control comprised 0.2 mL of egg albumin, 2.8 mL of phosphate buffer, and 0.05 mL of different concentrations of drug solutions and distilled water without the addition of BSA. The percentage inhibition of BSA denaturation was estimated as follows:

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

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

5.4. *In Vitro* Antiarthritic Impact of Rosuvastatin on Human Red Blood Cell (HRBC) Membrane Stabilization Assay. Blood was drawn from a healthy person who had not taken NSAID at least two weeks before the study, and written informed consent was taken from the volunteer. Prior approval was taken from the Institutional Ethical Review Committee, College of Pharmacy, University of Sargodha, Pakistan (Approval no. 2884B17 IERC/UOS). Blood was combined with an equal volume of Alserv’s solution and centrifuged at 3000 rpm for 20 min. Packed cells were removed and washed three times with an isosoline solution (0.85% NaCl); then, 10% v/v HRBC suspension was prepared in the isosoline solution. The sample mixture was prepared by adding 1 mL of different drug concentrations (100–6400 μg/mL), 1 mL of phosphate buffer (0.15 M, pH 7.4), 2 mL of hyposaline solution (0.36%...
NaCl), and 0.5 mL of HRBC suspension (10% v/v). The control mixture was composed of phosphate buffer, HRBC suspension, and 2 mL of distilled water. Samples were prepared in triplicate. Piroxicam was used as the standard drug. All samples were incubated at 37 ± 2 °C for 30 min and then centrifuged at 3000 rpm for 20 min. Afterward, the absorbance of the supernatant was recorded at 560 nm. The percentage membrane stabilization was estimated by the formula:

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

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

### 5.5. Formaldehyde-Prompted Arthritis

Five groups with six animals allocated to each group were made to conduct this study. Only distilled water (3 mL/kg) was administered to group I, while 10 mg/kg piroxicam was provided to animals included in Group II. Test drug rosuvastatin (10, 20, and 40 mg/kg) was given to animals assigned to Groups III, IV, and V, respectively. All of the test solutions were administered orally. Formaldehyde (2%) injected in the left hind paw of rat, 30 min after providing the test and standard drugs to animals assigned to different groups, provoked acute, nonimmunological arthritis. The same procedure was repeated on the third day of the experiment. Animals were provided with the test sample and standard drug for 10 days. The antiarthritic potential of rosuvastatin was then appraised by estimating the paw volume with a plethysmometer. The percentage inhibition of formaldehyde-provoked paw edema by piroxicam and rosuvastatin was calculated on the basis of the below-mentioned formula:

\[
\text{percentage protection from paw edema} = \left[1 - \frac{\text{PE}_{\text{control}} - \text{PE}_{\text{treated}}}{\text{PE}_{\text{control}}}\right] \times 100
\]

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

### 5.6. Complete Freund’s Adjuvant (CFA)-Provoked Arthritis Model

The chronic inflammatory CFA model was adopted to reveal the immunomodulatory potential of rosuvastatin in RA. The best antiedematogenic dose of rosuvastatin (40 mg/kg) estimated from formaldehyde-provoked arthritis was used in this model. Four different groups with six animals allocated to each group were formed, where group I was designated as healthy rats with no induction with CFA, group II served as the disease control group, group III was provided with piroxicam (10 mg/(kg day)), and group four received rosuvastatin (40 mg/(kg day)) for 28 days. CFA (0.1 mL) was injected in the left hind paw of rats on day 1 only for provoking arthritis.

#### 5.6.1. Appraisal of Arthritis

##### 5.6.1.1. Changes in Paw Volume and Arthritic Score Estimation

Changes in the paw volume were assessed on a weekly basis by utilizing a digital plethysmometer for all treatment groups. The percentage protection from CFA-instigated arthritis was calculated according to the following formula:

\[
\text{percentage protection from CFA} = \left[1 - \frac{\text{PE}_{\text{control}} - \text{PE}_{\text{treated}}}{\text{PE}_{\text{control}}}\right] \times 100
\]

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

Macroscopic scoring criteria were used to assign the arthritic score to all animals assigned to different treatment groups by examining the animals on a weekly basis. Score 1 was assigned for erythema evident in toes only, score 2 was given when there were swelling and erythema of paws, score 3 for swelling evident in ankles, and score 4 was given if there was swelling of the whole leg.

#### 5.6.1.2. Changes in Body Weight

Changes in body weight were assessed for all animals in different treatment groups on a weekly basis.

#### 5.6.1.3. Changes in Hematological and Biochemical Parameters

Blood was collected via cardiac puncture after 28 days for the estimating hematological and biochemical parameters including WBCs, RBCs, platelets, ESR, Hb, C-reactive protein, RF, AST, ALP, ALT, urea, and creatinine.

#### 5.6.1.4. Radiographic and Histological Changes

After 28 days, ankle joints of all rats included in different treatment groups were dissected for radiographic analysis, and afterward, histological analysis was performed for these ankle joints. Inflamatory cell infiltration, bone erosion, and pannus formation were analyzed by a blinded histopathologist. Scores from 0 to 4 were given according to the following criterion: 0 for normal, 1 for minimal, 2 for mild, 3 for moderate, and 4 for severe changes.

#### 5.6.1.5. Estimation of mRNA Expression Levels of TNF-α, IL-1β, IL-6, IL-4, and IL-10

RNA was extracted according to the TRIzol method. cDNA was synthesized according to the instructions provided by the kit’s manufacturer (Thermo Scientific). The real-time polymerase chain reaction was executed using a Bio-Rad system to amplify and quantify the PCR product. Primer sequences for the above-mentioned genes and GAPDH were used from a previous study and were synthesized by a commercial manufacturer.

#### 5.7. DPPH Free Radical Scavenging Assay

The antioxidant capacity by estimating the radical scavenging potential against DPPH was examined according to a previously described method. A solution of rosuvastatin and ascorbic acid with different concentrations from 50 to 6400 μg/mL was prepared in methanol. Prepared solutions (2 mL) and 0.5 mL of 1 mM DPPH solution in methanol were added into test tubes. Afterward, incubation was performed at room temperature for 15 min. The absorbance was then measured at 517 nm. A blank tube was also prepared to comprise 2 mL of methanol and 0.5 mL of DPPH solution. All experiments were conducted in triplicate. The free radical scavenging potential of rosuvastatin was calculated according to the following formula:

\[
\text{DPPH scavenging effect (\%)} = \left[1 - \frac{A_{\text{blank}} - A_{\text{test}}}{A_{\text{blank}}}\right] \times 100
\]

where \(A_{\text{blank}}\) is the absorbance of the blank solution and \(A_{\text{test}}\) is the absorbance of the test drug solution.

#### 5.8. Ferric Reducing Assay

To evaluate the antioxidant potential of rosuvastatin, solutions of rosuvastatin and ascorbic acid with different concentrations from 50 to 6400 μg/mL were prepared and mixed with 0.2 M phosphate buffer of pH 6.6 along with 2.5 mL of 1% potassium ferricyanide followed by incubation of 20 min at 50 °C. Afterward, 2.5 mL of TCA trichloroacetic acid (10%) was added followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of solution was mixed to an equal volume of distilled water and 0.5 mL of ferric chloride solution (0.1%). Then the absorbance was measured at 700 nm.
measured at 700 nm. A blank was prepared without adding the drug. Finally, the reducing power was calculated using the following formula:

\[
\text{percentage increase in reducing power} = \left( \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{blank}}} \right) \times 100
\]

where \( A_{\text{test}} \) represents the absorbance of the test drug and \( A_{\text{blank}} \) represents the absorbance of the blank.

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### Notes

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group No. RG-1439-002.

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