Regular Article

Olmesartan Prevented Intra-articular Inflammation Induced by Zymosan in Rats

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The objective of this study was to study the effect of olmesartan medoxomil (OLM), an antihypertensive drug, on intra-articular inflammation induced by zymosan (Zy) in Wistar rats. Intra-articular inflammation was induced in the right knees of rats by 1 mg Zy dissolved in saline. The animals were divided into the following groups: saline only (oral saline and intra-articular saline); Zy only (intra-articular Zy and oral saline), and intra-articular Zy and oral OLM (5, 15, or 30 mg/kg) or diclofenac sodium (SD; 100 mg/kg). Twenty-four hours after Zy injection, synovial fluid was collected for total leukocyte counts, blood was collected for biochemical measurements, and synovial tissue was collected for histopathology, immunohistochemistry, immunofluorescence and myeloperoxidase (MPO), malonaldehyde (MDA), and non-protein sulphhydryl (NPSH) assays. OLM doses of 15 and 30 mg/kg had protective effects, as evidenced by improved histopathological parameters of synovium, reduced total leukocyte counts, reduced MPO and MDA levels, and increased NPSH group levels compared with the Zy group. OLM reduced immunostaining for cyclooxygenase 2, tumour necrosis factor and interleukin 17 and increased immunostaining for superoxide dismutase and glutathione peroxidase. SD produced similar results. The drugs studied caused no change in biochemical parameters of the animals. OLM showed protective effects in this model of Zy-induced intra-articular inflammation.

Key words olmesartan; zymosan; intra-articular inflammation

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis accompanied by the destruction of articular cartilage and bone. Articular inflammation is a limiting step in disease development and for structural damage.¹⁾ Zymosan (Zy), a derivative of *Saccharomyces cerevisiae* yeast, induces joint inflammation mediated by toll-like receptor 2 (TLR2).²⁾ Zy-induced articular inflammation is an experimental model of RA described in the literature, and a useful tool for the study of substances' anti-inflammatory activities.³⁾

The acute phase of Zy-induced RA is characterised by increased vascular permeability, oedema, and inflammatory infiltration, which peaks in the 6th hour with a polymorphonuclear predominance. Increasing mononuclear cell infiltration and fibroblast activation occurs, similar to that in chronic rheumatoid synovitis, in which the synovial tissue grows to cover the cartilage (rheumatoid pannus).³⁾

The pathogenesis of RA is complex and incompletely understood. Oxidative stress plays an important role.⁴⁾ Reactive oxygen species (ROS) are produced by cellular processes and oxidative phosphorylation. An excess of ROS leads to lipid peroxidation of the cell membrane and abuse of tissue proteins, carbohydrates, and DNA. Patients with RA have high

oxidative stress levels and higher malonaldehyde (MDA) levels than do normal patients.⁵⁾ Data suggest that an antioxidant diet can reduce RA incidence.⁶⁾

RA is considered to be a systemic disease that primarily affects the joints, but inflammatory mediators involved in the pathogenesis of the disease can affect other organs, such as the heart. Patients with RA have higher incidence of and mortality from cardiovascular disease (CVD), including myocardial infarction, cerebrovascular events, and heart failure. Proinflammatory cytokines, immune complexes, acute phase reactants, and modified lipid particles increase endothelial cell activation and potentially atherosclerotic plaque instability.⁷⁾

RA has been treated with anti-inflammatory,⁸⁾ immunosuppressive,⁹⁾ and biologic agents such as infliximab and interleukin-35.^{10,11)} Clinical results remain limited, and research on new therapies is needed.

Olmesartan medoxomil (OLM) is an angiotensin II type 1 receptor blocker (ARB), used clinically for the treatment of primary essential hypertension. This drug has an anti-inflammatory effect on the atherosclerotic process and improves cardiovascular dysfunction.¹²⁾ The anti-inflammatory activities of other ARBs have been described in the literature. Valsar-

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tan had a protective effect in an experimental model of acute pancreatitis.¹³ Irbesartan attenuated tumor necrosis factor (TNF)- α -induced intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin expression levels in an *in vitro* model.¹⁴ Losartan attenuated lipopolysaccharide-induced lung injury.¹⁵ Telmisartan reduced the inflammatory response in an experimental model of periodontitis,¹⁶ and azilsartan prevented oral mucositis induced by 5-fluorouracil.¹⁷ In the renin-angiotensin system, angiotensin II participates in the pathogenesis of atherosclerosis through effects on endothelial function, inflammation, ROS production, and proliferation/migration of vascular cells.¹⁸ In this study, we investigated the effect of OLM on Zy-induced acute articular inflammation in a rat model.

MATERIALS AND METHODS

Animals and Zymosan (Zy)-Induced Articular Inflammation Model Male Wistar rats weighing 180–220 g were used in the experimental model. The animals were housed in polypropylene cages (2 animals per cage), with food and water provided *ad libitum*, in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12/12-h light/dark cycle. The Committee on Ethics in Animal Experiments of the Federal University of Rio Grande do Norte approved the study protocol (permit number 016-2013).

For the experimental model of Zy-induced joint inflammation, rats were anaesthetised with ketamine (70 mg/kg) and xylazine (10 mg/kg), followed by intra-articular injection into the right knee of a 0.05-mL solution of 1 mg Zy dissolved in saline. The animals were divided into experimental groups of five animals each ($n=5$). Normal rats received intra-articular and oral (*via* gavage) saline. Animals in the Zy group received intra-articular Zy and oral saline. The treated groups received intra-articular Zy and oral OLM at different doses (5, 15, or 30 mg/kg)¹⁹ or oral diclofenac sodium (SD) at a dose of 100 mg/kg.²⁰ OLM and SD were administered orally 30 min before Zy injection. The saline was used to dissolve olmesartan and diclofenac sodium. The anti-inflammatory effect of OLM was compared to that of SD, a nonsteroidal anti-inflammatory drug used in clinical practice.

The rats were euthanized with anaesthetic thiopental (80 mg/kg) 24 h after induction of inflammation. Synovial fluid was used for total leukocyte counts, and synovial membranes were collected for histopathology and immunohistochemistry. The synovium was frozen at -80°C and processed for the analysis of myeloperoxidase (MPO) activity, non-protein sulphhydryl (NPSH) groups, and MDA level. Blood collection was performed for the analysis of serum urea, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels.

Synovial Fluid Collection and Total Leukocyte Count Synovial fluid collection was performed with the administration of two intra-articular injections of sodium phosphate buffer (0.15 M, pH 7.4) and ethylenediaminetetraacetic acid (EDTA) (0.01 M), followed by aspiration. Synovial fluid (20 μL) was diluted with 380 μL Turk solution for leukocyte counts.²¹

Histopathology The synovium was fixed in 10% buffered formaldehyde for 24 h and processed for staining with hematoxylin and eosin according to a previously described technique.²² Microscopic analysis was performed to evaluate

aspects such as inflammatory cell infiltration, fibrosis, and granuloma.

MPO Activity MPO activity in synovia was assessed using a modified version of the method described by Bradley *et al.*²³ Samples were incubated in a 0.5% hexadecyltrimethylammonium bromide solution, homogenised, and centrifuged. MPO activity was determined by a colorimetric method, with absorbance at 460 nm.

Glutathione (GSH) Assay GSH concentrations were determined by testing for NPSH groups.²⁴ Samples of synovium were triturated with 0.02 M EDTA, and 50% trichloroacetic acid was used to precipitate the protein. Absorbance was determined at 412 nm immediately after the addition of 5,5-dithiobis(2-nitrobenzoic acid).

Malondialdehyde Assay We use a previously described method with a high degree of specificity to detect MDA.²⁵ Samples of synovium were homogenised with 20 mM Tris-HCl and centrifuged. MDA reacts with the chromogen 1-methyl-2-phenylindole, with maximum absorbance at 586 nm.

Immunohistochemistry for the Analysis of Cyclooxygenase-2 (COX-2), Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), TNF and Interleukin 17 (IL 17) Synovial tissue was analysed immunohistochemically to detect COX-2, SOD, GSH-Px, TNF and IL 17. The biotin-streptavidin-peroxidase technique as used.²⁶ The samples were incubated with primary antibody, and then with biotinylated secondary antibody. Peroxidase-streptavidin binds to the biotin

Fig. 1. Effects of Olmesartan on Intra-articular Inflammation Induced by Zymosan

(a) Total leukocyte counts in synovial fluid. (b) Myeloperoxidase activity in synovia. Rats received intra-articular saline alone (saline), intra-articular zymosan alone (Zy), and intra-articular zymosan and olmesartan (OLM; 5, 15, or 30 mg/kg) or diclofenac sodium (SD; 100 mg/kg). OLM and SD were administered orally 30 min before zymosan injection. Results are presented as the mean $\pm$ S.E.M. ($n=5$). [#] $p < 0.05$ vs. saline, * $p < 0.05$ vs. Zy (ANOVA with Tukey's post-test).

molecule attached to the secondary antibody. The chromogen (DAB) reacts with the peroxidase linked to streptavidin, emitting colour.

Immunofluorescence for the Analysis of TNF and IL 17
Synovial tissue was analysed by immunofluorescence to detect TNF and IL 17, according to a previously described technique.²⁷⁾

The samples (five samples per group) were incubated overnight with primary antibody, then washed three times in PBS/0.2% Triton X-100 for 5 min and incubated with Alexa Fluor 488-conjugated secondary antibody and 4'-6-diamidino-2-phenylindole (DAPI) (Sigma Chemicals, U.S.A.). Tissue reactivity in groups was assessed by computerized densitometry.

Fig. 2. (a) Histopathological Examination of the Synovial Tissue of Rats (Hematoxylin and Eosin Staining, 40×); (b) Histopathological Scores

Synovium was collected 24 h after zymosan administration. Synovium of animals given saline alone showed no change (A–C). Synovium of rats given zymosan alone showed fibrosis (arrow), inflammatory infiltrate (arrowhead), and granuloma (larger circles; D–F). Synovium of animals that received zymosan and were treated with 5 mg/kg olmesartan showed fibrosis (arrow) and inflammatory infiltrate (arrowhead; G). Synovium of animals treated with OLM 15 mg/kg (H) and 30 mg/kg (I) olmesartan or diclofenac sodium (100 mg/kg; J–L) showed improvement in histopathological parameters. Scores are expressed as the median ± S.E.M. ($n=5$). # $p<0.05$ vs. saline, * $p<0.05$ vs. zymosan (Kruskal–Wallis test and Dunn's multiple comparison test).

etry analysis using digital images captured by an immunofluorescence microscope (Zeiss Imager M.2 ApoTome2). Average densitometric values were obtained using ImageJ software (<http://rsb.info.nih.gov/ij/>). Contrast index measurements were obtained from the selected area $\times$ 100/total area after removing the background positioned across the regions of interest (five samples per animal).²⁷⁾

Analysis of Urea, Creatinine, ALT, and AST Levels

Blood collection was performed by cardiac puncture following anaesthesia with thiopental. Biochemical measurements were made from serum samples, using standard LABTEST kits and a spectrophotometer.²⁸⁾ The colorimetric method was used to measure urea and creatinine concentrations. ALT and AST levels were determined by kinetic assay.

Statistical Analysis Data were expressed as the mean with standard errors of the mean (S.E.M.) or the medians. ANOVA with *post hoc* Tukey's tests was used to compare mean values among groups. Median values were compared using the Kruskal–Wallis test and Dunn's multiple comparison test (GraphPad Prism 5.0 Software, La Jolla, CA, U.S.A.). A *p*-value <0.05 indicated a statistically significant difference.

RESULTS

Total Leukocyte Count and Myeloperoxidase (MPO) Activity

Zy promoted joint inflammation, as evidenced by the increased number of total leukocytes in the synovial fluid and increased MPO activity in the synovia of rats compared with those in animals that received only intra-articular saline (Figs. 1A, B). OLM at doses of 15 and 30mg/kg and SD improved these inflammation parameters in relation to rats that received Zy alone ($p<0.05$). Treatment with OLM at a dose of 5mg/kg had no significant effect (Figs. 1A, B).

Histopathology of the Synovium The synovium of untreated rats that received intra-articular injections of Zy showed histopathological changes, characterised by fibrosis, inflammatory infiltrate, and granuloma, with higher histopathological scores than rats that received saline (Figs. 2A, B). The synovium of rats treated with 5mg/kg OLM showed fibrosis and inflammatory infiltrate, with no reduction in histopathological score compared with rats that received Zy alone (Figs. 2A, B). However, 15 and 30mg/kg OLM improved inflammatory influx and eliminated fibrosis and granuloma, with a significant reduction in histopathological scores ($p<0.05$; Figs. 2A, B). SD also promoted improvement in histopathological parameters ($p<0.05$; Figs. 2A, B).

MDA and GSH Levels Twenty-four hours after the induction of joint inflammation with Zy, MDA levels were increased and GSH levels were reduced compared with saline-treated rats (Figs. 3A, B). OLM doses of 15 and 30mg/kg and SD reduced MDA levels and restored GSH levels ($p<0.05$), but 5mg/kg OLM did not.

Immunohistochemical Parameters Synovia of rats with untreated Zy-induced joint inflammation showed intense immunostaining for COX-2 (Fig. 4B), TNF (Fig. 5B) and IL 17 (Fig. 5F) compared with saline-treated animals. OLM and SD reduced immunostaining for COX-2 (Figs. 4C–E), TNF (Figs. 5C, D) and IL 17 (Figs. 5G, H). Immunostaining for SOD and GSH-Px was more pronounced in the groups treated with 15 and 30mg/kg OLM than in animals that received Zy alone (Figs. 4H, I, M, N).

Fig. 3. Malondialdehyde (MDA) Levels (a) and Glutathione (GSH) Levels, Determined by Testing Non-protein Sulphydryl (NPSH) Groups (b)

The assays were performed on the synovia of rats collected 24h after zymosan induction. Rats received intra-articular saline alone (saline), intra-articular zymosan alone (Zy), and intra-articular zymosan and olmesartan (OLM; 5, 15, or 30mg/kg) or diclofenac sodium (SD; 100mg/kg). OLM and SD were administered orally 30min before zymosan injection. Results are presented as the mean $\pm$ S.E.M. ($n=5$). # $p<0.05$ vs. saline, * $p<0.05$ vs. Zy (ANOVA with Tukey's post-test).

Immunofluorescence Parameters Synovia of rats with untreated Zy-induced joint inflammation showed intense diffuse (green) in the cells for TNF (Figs. 6a, B), and IL 17 (Figs. 6a, F) compared with saline-treated animals (Figs. 6a, A, E). OLM and SD showed moderately diffuse (green) in all synovial layers for TNF (Figs. 6a, C, D) and IL 17 (Figs. 6G, H). Densitometric analysis confirmed a significant reduction in TNF and IL 17 immunoreactivity in olmesartan and diclofenac sodium groups ($p<0.05$; Figs. 6b, c).

Biochemical Parameters Treatment of animals with OLM (5, 15 and 30mg/kg) caused no change in biochemical parameters, as evidenced by normal serum levels of urea, creatinine, AST, and ALT (Figs. 7a–d). SD promoted increases in the serum urea level, but not the serum creatinine, AST, or ALT level, in animals with joint inflammation ($p<0.05$; Fig. 7).

DISCUSSION

Zy induces intra-articular inflammation by binding to TLR2 expressed in synovial fibroblasts, which signals the inflammatory response.²⁾ In the present study, OLM had a protective effect on Zy-induced intra-articular inflammation, reducing inflammation parameters and oxidative stress.

In this study, OLM reduced the total leukocyte count, especially the number of neutrophils, as shown by the analysis of MPO activity and histopathological parameters of the synovium. Neutrophils are considered to be primary cells of the innate immune defence system that participate actively in

Fig. 4. Representative Immunohistochemical Results for Cyclooxygenase-2 (COX-2; A–E), Superoxide Dismutase (SOD; F–J), and Glutathione Peroxidase (GSH-Px; K–O) in Synovial Tissue of Rats, Obtained 24h after Zymosan Administration

The experimental groups were saline only, zymosan only (Zy), olmesartan (OLM) 15 and 30 mg/kg, and diclofenac sodium (SD) 100 mg/kg. Synovium of Zy rats showed intense immunostaining for COX-2, which was reduced by OLM and SD. Immunostaining for SOD and GSH-Px was more pronounced in OLM groups than in the Zy group.

the phagocytosis of pathogens. Recently, some authors have suggested that the formation of neutrophil extracellular traps (NETs) has the advantage of cancelling large bio-structures, such that fungi cannot be internalised by phagocytes.²⁹⁾ NETs participate in the pathogenesis of autoimmune diseases; in patients with RA, NET formation stimulates synovial fibroblasts to produce pro-inflammatory mediators that participate in joint injury.³⁰⁾

In this study and in other experimental models of inflammation, OLM reduced the expression of COX-2, which could result in tissue protection.^{19,31)} COX-2 is an enzyme induced in inflammatory processes; it is involved in the synthesis of prostaglandins and inflammatory cytokines, which promote local inflammation and tissue damage. Some authors have suggested that COX-2 expression in the synovial fibroblasts of patients with RA contributes to increased expression of inflammatory cytokines in cartilage matrix.³²⁾ According to the results of immunohistochemistry and immunofluorescence, treatment with 30 mg/kg OLM or 100 mg/kg SD reduced TNF and IL 17 levels. OLM protected against intra-articular inflammation by down regulating immunostaining of TNF and

IL 17 in synovial tissue. These cytokines regulate the inflammatory response and activities related to joint destruction, thereby participating in RA pathogenesis.^{33,34)}

We observed that SD, a non-steroidal anti-inflammatory agent, improved the inflammation parameters analysed. These results are similar to those observed for OLM. OLM did not promote changes in serum liver or kidney markers. SD did not cause liver abnormalities, but it did increase the serum urea level. The ability of SD to inhibit cyclooxygenases 1 and 2 in non-selective media explains this adverse effect on the renal system,³⁵⁾ as well as gastrointestinal effects described previously.³⁶⁾

OLM is considered to be a cardioprotective drug, due to its anti-inflammatory activity (*via* inhibition of the AT1 receptor of angiotensin II) and antioxidant properties.^{37,38)} Oxidative stress is involved in the pathogenesis of RA.⁵⁾ ROS degrade proteoglycans, activate metalloproteinases, and induce apoptosis of chondrocytes, contributing to the damage to cartilage and bone tissue.³⁹⁾ We demonstrated that OLM reduced MDA levels in the synovium of rats, reflecting an improvement in lipid peroxidation, and increased levels of GSH, an impor-

Fig. 5. Representative Immunohistochemical Results for Tumour Necrosis Factor (TNF; A–D) and Interleukin 17 (IL 17; E–H) in Synovial Tissue of Rats, Obtained 24h after Zymosan Administration

The experimental groups were saline only, zymosan only (Zy), olmesartan (OLM) 30mg/kg, and diclofenac sodium (SD) 100mg/kg. Synovium of Zy rats showed intense immunostaining for TNF and IL 17, which was reduced by OLM and SD.

Fig. 6. Representative Immunofluorescence Results (a) and Contrast Index (b, c) for Tumour Necrosis Factor (TNF; A–D) and Interleukin 17 (IL 17; E–H) in Synovial Tissue of Rats, Obtained 24h after Zymosan Administration

The experimental groups were saline only, zymosan only (Zy), olmesartan (OLM) 30mg/kg, and diclofenac sodium (SD) 100mg/kg. Synovium of Zy rats showed intense immunoreactivity for TNF and IL 17, which was reduced by OLM and SD. [#] $p < 0.05$ vs. saline, ^{*} $p < 0.05$ vs. zymosan (Kruskal–Wallis test and Dunn's multiple comparison test).

tant antioxidant, which contributed to the protective effect on intra-articular inflammation. Other authors have described the antioxidant activity of OLM.^{40,41)}

In this study, OLM treatment increased immunostaining for antioxidant enzymes, such as SOD and GSH-Px, strengthening the evidence for this drug's protective effect on oxidative

Fig. 7. Biochemical Levels of Urea (a), Creatinine (b), Alanine Aminotransferase (ALT; c), and Aspartate Aminotransferase (AST; d)

Biochemical measurements were made from serum samples of rats obtained 24h after zymosan induction. Rats received intra-articular saline alone (saline), intra-articular zymosan alone (Zy), and intra-articular zymosan and olmesartan (OLM; 5, 15, or 30mg/kg) or diclofenac sodium (SD; 100mg/kg). OLM and SD were administered orally 30min before zymosan injection. Results are presented as the mean $\pm$ S.E.M. ($n=5$). [#] $p<0.05$ vs. saline, * $p<0.05$ vs. Zy (ANOVA with Tukey's post-test).

stress. Corroborating our results, OLM increased levels of SOD in diabetic nephropathy in streptozotocin-induced diabetes in rats⁴²⁾ and restored the activity of GSH-Px in daunorubicin-induced nephrotoxicity in rats.⁴³⁾

Angiotensin II plays a key role in inflammation; it amplifies the inflammatory response mediated by AT1R *via* the activation of nuclear factor kappa B and regulates the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules in living cells, contributing to the migration of inflammatory cells to sites of tissue injury.^{44,45)}

No previous report has described the effect of OLM on Zy-induced joint inflammation. The effect of OLM in RA has been demonstrated in an experimental model of collagen-induced arthritis in mice.⁴⁶⁾ Additional studies have confirmed the anti-inflammatory activity of OLM in other models of inflammation.^{19,31,47)}

Our results are of importance, given that RA is associated with an increased risk of CVD, and that hypertension is a prevalent risk factor for CVD in patients with RA. Thus, the use of OLM in patients with RA can bring dual benefits, due to the drug's anti-inflammatory and antihypertensive properties. Although the blocking of angiotensin II probably will not replace anti-rheumatic drugs, such as methotrexate, non-steroidal anti-inflammatory agents, and biological agents, ARB may be an antihypertensive that benefits patients with RA. We conclude that OLM had a protective effect on intra-articular inflammation and deserves further study in humans.

Conflict of Interest The authors declare no conflict of interest.

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