Effects of co-treatment with pioglitazone and methotrexate on experimentally induced rheumatoid arthritis in Wistar albino rats

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Abstract:
OBJECTIVES: Rheumatoid arthritis (RA) is a chronic inflammatory disease primarily affecting the synovial joints of the body. Methotrexate (MTX) is considered as a mainstay in the management of RA. However, monotherapy with MTX in RA is often limited by potential long-term toxicity. The present study was conducted to evaluate if MTX-pioglitazone combination therapy has an add-on benefit over monotherapy with MTX or pioglitazone on disease activity in male Wistar rats in adjuvant-induced arthritis model.

MATERIALS AND METHODS: Arthritis was induced by single subcutaneous injection of complete Freund’s adjuvant (CFA) in thirty male Wistar albino rats. They were then divided into five equal groups, which included two control groups (arthritic and nonarthritic), pioglitazone-treated (1.35 mg/kg daily), MTX-treated (0.225 mg/kg daily), and MTX + pioglitazone-treated. The disease-modifying action of the drugs was assessed by various physiological, hematological, and biochemical parameters along with histopathological and radiological analysis of affected joints. The experimental data were statistically assessed by one-way ANOVA.

RESULTS: There was a significant reduction of disease activity in the MTX monotherapy group when compared with disease control. However, pioglitazone monotherapy group failed to demonstrate any significant effect on disease activity. The MTX-pioglitazone combination group demonstrated greater suppression of disease activity as compared to MTX and pioglitazone monotherapy and disease control group (P < 0.05).

CONCLUSION: The present study demonstrates that the combination therapy of MTX with pioglitazone offers better control of disease activities in RA as compared to MTX or pioglitazone monotherapy.

Keywords: Complete Freund’s adjuvant, methotrexate, pioglitazone, rheumatoid arthritis, Wistar rats

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory disorder which primarily involves the synovial joints causing pain, immobility, and stiffness of the involved joint.[1] The clinical course of RA exhibits extreme variation, ranging from mild, self-limiting disease to rapidly progressive arthritis with extra-articular manifestations.[2] The current management of RA includes symptom-relieving nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs such as azathioprine, methotrexate (MTX), sulfasalazine, or hydroxychloroquine, biologicals such as tumor necrosis factor (TNF) inhibitors (etanercept, infliximab, and adalimumab), and interleukin-1 receptor antagonists (IL-1Ra; anakinra).[3] However, lack of optimum...
efficacy and potential safety concerns limit their long-term use as monotherapy in RA, and therefore, combination therapy is recommended.\cite{3,4} Nevertheless, there is a case for exploring newer therapies and regimes.

MTX constitutes a mainstay in the therapy of RA. The suggested mechanisms of MTX include (i) inhibition of T-cell proliferation by affecting purine and pyrimidine metabolism, (ii) inhibition of transmethylation reaction necessary for T-cell cytotoxicity prevention, (iii) alter the recruitment of monocytes to the inflamed joint by interfering with glutathione metabolism.\cite{4}

Pioglitazone is an oral antidiabetic agent, which acts as ligand for peroxisome proliferator-activated receptor gamma (PPAR-γ). PPAR-γ agonists suppress inflammatory cytokine\cite{5} and matrix metalloproteinases production\cite{6} and cause inhibition of proinflammatory gene expression.\cite{7} Further, they cause induction of apoptosis in macrophages and T lymphocytes which are important in perpetuating the RA disease process.\cite{8,9} Some experiments have shown the efficacy of pioglitazone in collagen-induced arthritis (CIA) induced RA disease process.\cite{10}

Since both MTX and pioglitazone exert anti-inflammatory actions in inflamed joints through different mechanisms at the nuclear level, we hypothesized that MTX and pioglitazone combination therapy might have additive efficacy in patients with RA.

Adjuvant-induced arthritis (AIA) is a commonly used model in rats denoted by chronic synovitis with inflammatory cell infiltration, pannus formation, bone erosion, and cartilage destruction, having more similar disease features with human RA than CIA. Further, as compared to the female rats, the male Wistar rats have been found to be more susceptible to RA, particularly in terms of onset and severity of the disease process.\cite{11}

In this backdrop, we conducted the present study to explore if MTX-pioglitazone combination therapy offers some add-on benefit over monotherapy with MTX or pioglitazone in male Wistar rats in AIA model.

**Materials and Methods**

**Materials**

Carboxymethylcellulose sodium (CMC-Na) was procured from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, Karnataka, India. 0.5% solution of CMC-Na was prepared in double-distilled water and was used as vehicle in the experiment. Commercially available MTX 2.5 mg tablets (Zenate® Dabur Pharmaceuticals Ltd.) and pioglitazone 15 mg tablets (Piolem® Alembic) were used. Both the drugs were dissolved in the vehicle for oral administration through gastric gavages. Complete Freund’s adjuvant (CFA) was procured from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, Karnataka, India. FCA used for the induction of arthritis contained paraffin oil, mannide monooleate and heat-killed mycobacteria (Mycobacterium tuberculosis). All the chemicals used in the study were of analytical grade.

**Grouping and dosing of animals**

Pathogen-free, 9-week-old male Wistar strain albino rats weighing 150–190 g were considered in this study. The animals were procured and housed in solid bottom polypropylene cages in the animal house maintained under standard hygienic conditions of temperature (20°C ± 2°C), humidity (50% ±10%), and light (12h light/12h dark cycle) with free access to food and water, which complied with the norms of the Institutional Animal Ethical Committee (IAEC-GCTS/13-14/04) as per Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines under optimum conditions and facilities for experimentation. Rats were fed pellet diet and water *ad libitum*. The animals were acclimatized to these laboratory conditions for 2 weeks before the experiments.

The animals were then divided into five groups with six rats in each group and named as follows:

1. **Normal control group (Group I):** Rats in this group were treated with 0.5% (w/v) CMC-Na solution per oral.
2. **Arthritic control group (Group II):** After induction of arthritis, no treatment provide for this group.
3. **Pioglitazone-treated group (Group III):** Arthritic animals received pioglitazone 1.35 mg/kg/d dissolved in 0.5% (w/v) CMC-Na solution per oral.
4. **MTX-treated group (Group IV):** Animals received MTX 0.225 mg/kg/d dissolved in 0.5% CMC-Na solution.
5. **Pioglitazone + MTX-treated group (Group V):** Animals were administered both drugs in above-mentioned doses dissolved in 0.5% CMC-Na solution.

Following the CFA injection in the animals of Group II to Group V, above-mentioned treatments were initiated from day 10 and continued up to day 21 (completion of the experiment).

**Induction of adjuvant-induced arthritis**

Arthritis induction was performed by injecting 0.1 ml (0.1% w/v) CFA suspension intradermally at base of the tail of each rat except those of Group I. Body
weight and ankle joint diameter on both of the hind limbs of individual rats of each group were measured before injection. These values were taken as baseline values for comparison. Post injection, it normally takes 9–12 days for disease progression and special care with normal diet was provided to all animals during this period.

**Assessment parameters**
For assessment of systemic inflammation associated with arthritis, the following parameters were used:

**Paw edema**
Digital Vernier caliper (Mitutoyo Digimatic caliper, Japan, Model CD-6 CSX) was used to measure the paw diameter of both hind limbs of each rat before giving CFA injection and on day 3, 6, 9, 12, 15, 18, and 21 following injection of CFA.

**Body weight and blood glucose level**
Body weights of each animal were measured using a precalibrated weighing balance (Mettler Toledo, Germany) before giving CFA injection and on the same time points as in case of paw diameter measurement. For blood glucose estimation, blood samples were obtained from tail vein of each animal. The blood glucose estimation was performed at same time points as that of body weight using a glucometer (Gluco-Chek sensor from Roche Diagnostic Corporation, USA) and presented as mean ± standard error mean (SEM) (mg/dl).

**Radiographic assessment of joint damage**
After the completion of study period (on day 22), the animals were anesthetized using sodium pentobarbital and placed on radiological plate. X-rays analysis of ankle joint of rats from each group was taken for assessing disease activity. X-ray apparatus (NE-X ray machine 100 MA, Japan operated at 220 V with a 54 V peak, 0.2 s exposure time) was used to take the radiographs.

**Histopathological analysis**
On day 22, rats were anesthetized and sacrificed by using chloroform. Cardiac puncture was done to obtain blood sample, and paws of rats were dissected out for histopathological examination. The sections were fixed in 1% formalin and were decalcified, sectioned, and finally stained with hematoxylin and eosin to study the histopathological changes in all groups during the experiment under light microscope.

**Biochemical and hematological parameters**
Blood samples collected by cardiac puncture from rats before sacrificing them was used for measurement of biochemical and hematological parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), albumin (ALB), erythrocyte sedimentation rate (ESR), and hemoglobin (Hb) levels. The SGOT, SGPT, and ALB were assessed with liquid enzyme kit (Accurex Biomedical pvt ltd. India) according to absorbance measured on ultraviolet-visible spectrophotometer (Beckman, USA) while ESR levels were measured using Westergren’s method and x (AE-11M, ER MAINC).

**Statistical analysis**
Data analysis was performed by statistical package SPSS (Statistical Product and Service Solutions, Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.). The study data were expressed as mean ± SEM. ANOVA followed by post hoc Tukey’s Honestly Significant Difference test was applied to compare each group differences. A two-tailed $P < 0.05$ was considered statistically significant.

**Results**
In this study, AIA model was developed using complete Freund’s adjuvant (CFA). Multiple joint swelling (mainly small joints) were observed in the animals of Groups II–V with evidence of joint cartilage erosion, bone destruction, and remodeling. In this experiment, each of the five groups had equal no of rats ($n = 6$) animals each, and the data are expressed as mean ± SEM.

**Paw edema**
Before CFA injection (day 0), paw diameter of both hind limbs of each rat from every group was measured and was taken as baseline values. These measurements were repeated on day 3, 6, 9, 12, 15, 18, and 21 [Table 1]. Paw diameters (expressed as mean ± SEM in centimeters) of CFA-treated rats in arthritic Groups II–V on day 9 increased up to $0.73 ± 0.016$, $0.747 ± 0.017$, $0.76 ± 0.015$, and $0.71 ± 0.014$, respectively, as compared with normal

| Table 1: Hind paw diameter (cm) value in mean±standard error mean of all groups |
|---------------------------------------------------------------|
| Group                        | Day 0             | Day 3             | Day 6             | Day 9             | Day 12            | Day 15            | Day 18            | Day 21            |
|-------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Normal control ($n=6$)       | 0.61±0.023        | 0.615±0.025       | 0.625±0.026       | 0.65±0.024        | 0.64±0.025        | 0.655±0.029       | 0.665±0.029       | 0.66±0.031        |
| Arthritis control ($n=6$)    | 0.64±0.0056       | 0.66±0.015        | 0.711±0.012**     | 0.73±0.016*       | 0.74±0.017*       | 0.765±0.019**     | 0.79±0.023*       | 0.79±0.020**      |
| Pioglitazone treated ($n=6$) | 0.64±0.004        | 0.65±0.0032       | 0.695±0.0066*     | 0.747±0.017*      | 0.74±0.017*       | 0.72±0.0094       | 0.71±0.0096       | 0.69±0.014*       |
| Methotrexate treated ($n=6$) | 0.63±0.001        | 0.64±0.005        | 0.69±0.012*       | 0.76±0.015*       | 0.75±0.015*       | 0.71±0.013        | 0.69±0.018        | 0.67±0.20*        |
| Pioglitazone + methotrexate treated ($n=6$) | 0.62±0.013         | 0.64±0.005        | 0.68±0.012        | 0.71±0.014        | 0.72±0.0078       | 0.70±0.012        | 0.68±0.012*       | 0.65±0.013**      |

*P<0.05, **P<0.01 compare with normal control group to group, ¥P<0.05, ¥¥P<0.05 compare with arthritic control group
Table 2: Variation in body weights and blood glucose level of all groups

| Group                          | Blood glucose | Parameter | Body weight |
|-------------------------------|---------------|-----------|-------------|
| Normal control (n=6)          |               | Day 0     | 175.8 ± 2.74 |
| Arthritis control (n=6)       |               | Day 9     | 183.3 ± 2.96 |
| Pioglitazone treated (n=6)    |               | Day 12    | 186.6 ± 1.52 |
| Methotrexate treated (n=6)    |               | Day 15    | 182.5 ± 2.82 |
| Pioglitazone + methotrexate treated (n=6) | | Day 18    | 181.6 ± 3.46 |
| Blood glucose                 |               | Day 21    | 182.5 ± 2.56 |

Body weight variation and blood glucose level

RA is characterized by reduction in body weight among individuals. Before adjuvant injection (day 0), initial body weights of each rat from every group were measured and taken as base line values. These measurements were repeated on same time points as that of paw diameter measurements [Table 2]. Body weight (expressed as mean ± SEM in grams) of CFA-treated rats in arthritic Groups II–V on day 9 were found to decrease up to 167.5 ± 2.56, 162.5 ± 3.28, 161.67 ± 3.46, and 158.34 ± 3.46 as compare with nonarthritic group I (184.166 ± 2.17). Further, following the drug treatments, body weight values on day 21 were found to have increased from day 9 values in Groups III–V (176.67 ± 1.92, 185 ± 1.67, and 179.167 ± 2.47) while body weight decreased over the same period in Group II (142.5 ± 2.82) with values of same in Group I being 189.167 ± 1.83. Intergroup comparative analysis of body weight variation over the entire study period was done, and statistically significant values were indicated [Table 2].

Biochemical and hematological estimation

SGOT levels measured on day 21 showed higher values in case of arthritic Group II (113.68 ± 5.31 U/L) and least in nonarthritic Group I (60.324 ± 3.93 U/L) with values (U/L) in drug-treated Group III–V being 79.21 ± 4.60, 76.37 ± 6.92 and 67.72 ± 2.19, respectively [Table 3]. Similar trend was observed in case of SGPT levels with values (U/L) of Group I–V being 29.51 ± 2.37, 91 ± 1.62 U/L, 63.44 ± 4.53, 52.2 ± 3.44, and 41.174 ± 1.58, respectively [Table 3]. However, serum ALB levels measured on the same day showed higher value in case of nonarthritic Group I (5.95 ± 0.73) and least in arthritic Group II (3.595 ± 0.26) with values (U/L) in drug-treated Group III–V being 5.66 ± 0.50, 4.26 ± 0.92 and 4.26 ± 0.67, respectively [Table 3].
rate in case of arthritic Group II (5.75 ± 0.34 mm/h) and least in nonarthritic Group I (1.95 ± 0.26 mm/h) with values (mm/h) in Group III-V being 4.91 ± 0.32, 3.575 ± 0.33, and 3.52 ± 0.27, respectively [Table 3]. However, Hb% levels measured on the same day showed higher value in case of nonarthritic Group I (11.86 ± 0.39 g%) and least in arthritic Group II (9.26 ± 0.50 g%) with values (g%) Group III-V being 10.2 ± 0.27, 10.13 ± 0.47, and 11.13 ± 0.39, respectively [Table 3].

Radiological analysis

Inflammation at joints associated with RA was seen in arthritic rats as compared to their normal counterparts [Figure 1a and b]. The animals of normal control rats (Group I) exhibit the characteristic normal structural design of phalangeal joint [Figure 1c] whereas images of arthritic control animals (Group II) present reduction in the joint area of metatarsals and phalanges besides phalangeal joint erosion. Further, deformative bending with changes in bone shape and inflammation of soft tissues was observed [Figure 1d]. Similar features were seen in case of pioglitazone-treated rats although to a lesser extent [Figure 1e]. However, in case of MTX-treated [Figure 1f] and combinational drug-treated group [Figure 1g], these changes were observed to have normalized and were similar to the radiographic images seen in animals of normal saline-treated group.

Histopathological parameters

The histopathological examination shows distinguishable differences between all groups as compared with normal
control group [Figure 2a]. The histology of ankle joints of arthritic arts exhibited massive presence of inflammatory conditions along with the blocking of the adjacent blood vessels. The proliferation of synovium and inflammation in subperiosteum region of joints was also observed [Figure 2b]. As compared to the normal group, various drug treatments showed variable histological responses. The degree of inflammation in periosteum region varied from mild to moderate in case of MTX-treated and pioglitazone-treated groups, respectively [Figure 2c and d]. However, surprisingly, the animal group with combined drug treatment showed near to normal architecture of synovial region [Figure 2e].

Discussion

Progression of RA is usually characterized macroscopically by paw edema, decrease in body weight, changes in hematological and skeletal changes besides being microscopically associated with the presence of inflammatory cells in synovium and granuloma leading to cartilage destruction and bone erosion.[1] AIA model bearing more similar cellular and molecular characteristics with human RA, it was chosen as a preferred model in this study to establish a useful correlation of events of pathogenesis of inflammation of joints associated with human RA. This model was particularly chosen as disease progression bore characteristic similarities with human RA than CIA.[12]

Paw edema determination serves not only as a simple marker of disease progression but also helps in the evaluation of therapeutic efficacy of drug therapy. In the present investigation, the animals of arthritic groups were found to have increased paw diameters after the advent of disease. Following drug treatments, a significant reduction in paw diameters in MTX-treated and combined drug-treated groups was observed with arthritic group showing steady increase in the same over the study period. However, pioglitazone-treated group showed no such significant decrease.

Characteristic to RA-affected patients, the rats of the arthritic group showed a marked soft tissue swelling around the ankle joints. These events may be attributed to ligamental and articular capsular edema, which is normally observed during the acute phase of human RA. During initial stages of the study, inflammation was found to have increased after which it neared constant level. Such conditions are usually observed in RA patients. This increase in paw diameter may be explained as follows: A time-dependent increase in granulocyte and monocyte levels has been normally associated with disease progression in RA. At the molecular level, this may be due to activated macrophages resulting in increased formation of proinflammatory cytokines which play a major role in propagation of this autoimmune disorder.[13,14] Among these factors, TNF-α and IL-1β have been implicated to be associated with the progressive inflammatory cascading.[13] Activation of such processes exhibits enhanced permeation and retention effect at the inflammation region thereby intensifying the disease conditions.[13,14] Prostaglandins under such conditions greatly increase exudation processes at the site by enhanced relaxation of blood vessel supplying the region and increased blood flow. In case of drug therapy, migration of leukocytes and generation of proinflammatory agents into the affected area is significantly reduced.[15]

Periodic changes in body weight observed in this study have been one of the major indicatives of disease progression and a measure of antirheumatoid drug treatment response. It was observed that with increase in severity of arthritis, body weight of all arthritic group rats decreased significantly as compared with their normal counterparts. However, with initiation of drug therapy, rats of all treatment groups showed further significant increase in body weight with values near to normal group rats over the study period. Such loss of body weight during arthritic disease conditions is consistent with the findings of earlier researchers.[16] Various studies have shown decrease in metabolic activities along with significantly reduced intestinal absorption of essential body weight regulating glucose and leucine to be associated with untreated RA. The reversal of decreasing body weight of animals of drug-treated arthritic groups after initiation of therapy may be attributed to recovery in intestinal absorption potential of nutrients leading to improvement in body weight.[17]

The blood glucose levels of various groups show a nonsignificant variation among the animals of drug-treated and saline-treated control. This indicates that despite using pioglitazone at 1.35 mg/kg/day, it may have been used at subdiabetic concentration leading to blood sugar levels being comparable to nondrug-treated animals. However, the arthritis control group shows significant reduction in blood glucose levels which may be associated to reduced absorption of glucose.[17]

RA is further associated with liver and kidney function impairment. The extent of tissue damage may be determined by measurement of serum enzyme activity. Under such conditions, increase in serum levels of aminotransferases (SGOT and SGPT) arises from cellular release of these enzymes from affected tissues. Similar results were obtained in this study with higher values of SGOT and SGPT being observed in arthritic group as compared to those of normal group while all other groups having intermediate values. The animals of
combined drug treatment group showed significantly lower levels of aminotransferases as compared to that of MTX-treated group thereby showing better improvement in the disease conditions of RA. Further, both the aminotransferases SGOT and SGPT have been indicated to have major roles in the generation of biologically active forms of chemical mediators of inflammation such as bradykinins, suggesting the importance of indicative assessment of their serum levels. Thus, observed levels of SGOT, SGPT in all arthritic groups provide a positive indication of the presence of inflammation and its degree of progression under different drug treatments.

Assessment of serum ALB serves as a negative acute phase indicator of arthritis both in rats and humans. Research has shown that reduced serum ALB levels correlated with increased degree of inflammation. It was observed that the serum ALB levels were highest in arthritic group with lowest values in normal group with all drug-treated groups having intermediate values. As compared to untreated arthritic group, MTX and combined drug treatments significantly inhibit the decrease level of serum ALB, thereby corresponding to the lower levels of inflammation.

ESR estimation may be used as a tool for the detection of inflammation in the body as higher values of sedimentation rates are usually associated with the presence of inflammation. Higher ESR levels observed in rats of arthritic group as compared to normal group indicate the presence of inflammation in them. Although ESR levels of drug-treated groups show high values as compared to normal group, yet they are lower than arthritic groups indicating their anti-inflammatory potential. The ESR level of combined drug therapy is comparable with that of MTX-treated group. Further, decreases in Hb levels are usually associated with the presence of inflammatory conditions. Experimental results showed similar trend with lower levels being observed in arthritic untreated group in comparison with other treated groups, these changes were observed to a higher extent in combinational drug-treated group than MTX-treated group indicating the agonistic effect of such a combination of drugs. However, no such observations were made in case of pioglitazone-treated rats indicating the absence of anti-inflammatory effect of same. Further, X-ray analysis and histopathological studies also provided conclusive evidence of the proper induction of arthritis in the rats following adjuvant injection.

Conclusion

The present study results suggest that a therapy of MTX with pioglitazone in combination offer better control of disease activities in RA as compared to MTX or pioglitazone monotherapy. However, further studies are needed to explore whether these findings in preclinical stage can be translated in clinical setups. There is a further need to ascertain the dose range of pioglitazone to be used in clinical setting.

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Conflicts of Interest

There are no conflicts of interest.

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