

## Leflunomide with Meloxicam on Progression of Rheumatoid Arthritis and its Associated Depression in AIA Rats

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### Abstract

**Background:** Rheumatoid arthritis is an autoimmune disorder in which patients not only suffer from joint misery but also face its associated depression.

**Objectives:** To evaluate the anti-arthritic effects of leflunomide (5 mg.kg<sup>-1</sup>.day<sup>-1</sup>) and meloxicam (5 mg.kg<sup>-1</sup>.day<sup>-1</sup>) when given together on progression of rheumatoid arthritis and its associated depression in Adjuvant-Induced Arthritic (AIA) rats.

**Methodology:** AIA was induced in female Sprague-Dawley rats. Paw volumes was measured to evaluate arthritic progression while brain indolamines (tryptophan, serotonin and its metabolite 5-hydroxyindoleacetic acid) were estimated by HPLC-EC method to determine associated depression. Leflunomide and meloxicam were given orally and intraperitoneally throughout the experiment.

**Results:** Leflunomide and meloxicam inhibited RA progression significantly (p<0.005), in terms of joint erythema and limb swelling, when given alone but fail to do so in combination in contrast to untreated or saline-treated arthritic rats. Significant reduction (p<0.005) in all brain indolamines levels was found in all arthritic rats when compared with normal. Furthermore, treatment with leflunomide and meloxicam alone or mutually significantly decrease (p<0.005) brain indolamines level in comparison with untreated or saline-treated arthritic rats.

**Conclusion:** Leflunomide and meloxicam though reduces RA progression when given alone but in combined therapy produce severe adverse effect. Depression is prominent with RA and therapy with leflunomide and meloxicam exaggerate the conditions.

**Keywords:** Rheumatoid arthritis; Depression; Leflunomide; Meloxicam

### Introduction

Among chronic pains, rheumatoid arthritis (RA) holds the predominant position [1]. It is a chronic symmetrical polyarthritic progressive inflammatory disorder [2] in which patient has to face long-term pain, stiffness and fatigue due to hyper activity of immune system mainly T and B-lymphocytes [3,4]. In addition to this during inflammatory states of RA, synthesis of prostaglandins (PGs), thromboxane, prostacyclin and interleukins also increases by cyclooxygenase and lipoxygenase pathways [5,6].

Over and above increase levels of pain, the prevalence of depression in this group is about twice than healthy one [7]. Alliance of depression in this group is unified with agitated activity of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) [8-10] which impinge on a number of mechanisms in brain as reduce brain monoamines activity [8], activation of stress-induced or mitogen-induced protein kinases, Janus kinase or signal transducers and activators of transcription [7,11-14]. It was reported earlier that level of tryptophan, which is the precursor of serotonin, in RA patient decreases due to its increase catabolism by indoleamine 2,3-dioxygenase enzyme released from interferon- $\gamma$  in inflammatory cell. Therefore its availability in brain decreases which ultimately decreases serotonin synthesis in brain. Since serotonin is the neurotransmitter which is involved in mood, consciousness and sleep therefore decrease concentration of serotonin makes the arthritic body depressed [11-16]. Thus determination of these biologic amines in RA patients and animal models of RA help to understand neurological disorder as anxiety and depression related to this pathology [17].

Management of RA entails stepwise approach towards the use

of accessible therapeutic agents [5]. In the middle of them, disease modifying anti rheumatic drug (DMARDs) all along with NSAIDs (prostaglandin inhibitors) are used [4,18] in the instigation of remedy within three months of diagnosis to eradicate or diminish soreness, irritation, joint damage and to sustain function [19]. Among leading (DMARDs), leflunomide (lef) is currently in use that decreases pro-inflammatory cytokines level by inhibiting denovo synthesis of pyrimidine nucleotide and prevent T-lymphocyte proliferation [20, 21].

There are some studies that highlight the problem of depression and anxiety in patients receiving leflunomide [22,23]. Similarly, it was also reported that in some patients depression was associated with the use of NSAIDs and as NSAIDs were withdrawn from their regimen they showed good recovery from anxiety and depression [24-26]. Therefore the combined effect of these DMARDs and NSAIDs on RA should not be limited upto the joint misery but their effects on neurobiological disorder as depression and anxiety in such cases should also be monitored.

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Previously many *in-vivo* studies were conducted to observe the effect of leflunomide either in comparison or along with other DMARDs and NSAIDs. Emery et al. [26] and Pfeil et al. [27] compared the anti-arthritis effect of leflunomide with methotrexate on human. Kraan et al. [28] analyzed the ability of these drugs on cytokine production. Flendrie et al. [29] studied leflunomide influence on the efficacy and safety of infliximab. Altogether, these studies were performed either to compare or to check leflunomide efficacy with other DMARDs and NSAIDs focusing only towards joint related arthritic disorder. But the effect of these drugs on neurobiological disorder as depression and anxiety in such patients should also be monitored.

Present research work aims to study the consequence of leflunomide in conjunction with meloxicam (melox) when given simultaneously. Meloxicam is a potent NSAID that exert its effect by inhibiting cyclooxygenase enzyme in prostaglandin synthesis via arachidonic acid pathway. Thus slows down production of inflammatory prostaglandins. RA patients are likely to receive DMARDs after diagnosis, rather than with disease induction. As experiments investigating the effect of leflunomide combined with meloxicam after induction of disease make it clinically more relevant, the effect was compared with leflunomide and meloxicam given alone (Table 1). The principle aim of this work was to study not only the combined effect of leflunomide and meloxicam on progression of RA but also on its associated depression. For this purpose adjuvant induced arthritic (AIA) rats were used as animal model that have similar pathological features as RA in human [30,31].

## Materials and Methods

The study followed Ethical Guidelines approved by “Board of Advanced Studies and Research” (BASR), University of Karachi. The study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving animals.

### Animals

Female Sprague–Dawley rats, weighing 215-230 g (8-10 weeks), kept at 21 ± 2°C on a 12-hour light/dark cycle with free access to standard laboratory rat food pellets and water, were used for this study under the ethical guidelines of International Association for the Study of Pain in conscious animals. Rats were randomized and grouped into non-treated healthy (normal), non-treated arthritic rats (AIA control), normal saline treated arthritic rats (AIA saline), leflunomide treated arthritic rats (AIA lef), meloxicam treated arthritic rats (AIA melox) and leflunomide along with meloxicam treated arthritic (AIA lef+melox) groups.

Groups	Brain Indolamines (ng gm <sup>-1</sup> )		
	Tryptophan	Serotonin	5-HIAA
Normal	173.2 ± 1.27	1246.8 ± 37.5	235.9 ± 8.81
AIA control	111.7 ± 1.25	*974.8 ± 18.9	*138.8 ± 5.7
AIA saline	**109.9 ± 1.53	**980.58 ± 4.4	**148.6 ± 4
AIA lef	+++14.34 ± 0.48	+++125.9 ± 4.56	+++26.09 ± 0.28
AIA melox	**35.64 ± 0.29	+*307.9 ± 3.57	+*47.31 ± 1.37
AIA lef+melox	+++11.79 ± 0.55	+++102.6 ± 3.44	+++22.1 ± 0.39
One Way ANOVA (df= 5)	F=4358.559 P<0.005	F=12322.432 P<0.005	F=38.45 P<0.005

Values are means ± S.D. Significant difference by multiple comparisons Bonferroni test \*p<0.05, \*\*p<0.01 from normal control and +p<0.05, ++ p<0.01 from AIA control.

Table 1: Brain indolamines levels in AIA rats.

### Induction of arthritis

For this study, chronic model of arthritis i.e., arthritis induced by adjuvant was selected. Arthritis was induced by intradermal injection of 0.1 ml suspension of 1 mg of fresh lyophilized *Mycobacterium tuberculosis* H37Ra (MT H37Ra; DIFCO Laboratories, Detroit, MI, USA) in liquid paraffin oil into the tail base of all the rats except normal group, using a sterile hypodermic needle [1].

### Treatment protocol

Reference standard of leflunomide and meloxicam were gifted by Hilton Pharmaceutical Pakistan. Treatment of the respective groups was initiated on the same day of arthritis induction, which was considered as day zero and was continued for 3 weeks. Leflunomide and meloxicam were given at a dose of 5 mg kg<sup>-1</sup> through oral and intraperitoneal (i.p) route, respectively. Both drugs were given as suspension in saline (NaCl 0.9% w/v in water).

### Clinical assessment on progression of adjuvant induced arthritis

Measurement of rat’s hind paw volume was used to evaluate arthritic severity produced by the adjuvant administration. It was determined by quantitating the change in their paw volume on alternate days throughout the experiment by water displacement procedure with the help of plethysmometer (model 7140; Ugo Basile, Varese, Italy) which has the capability to measure paw tibiotarsal joint three in dimensions. Thus any variability of the pattern of swelling of individual limbs can be monitored. Rat’s body weights were also measured on alternate days during whole experiment.

### Evaluation of depression

To study the effect of treatment on depression associated with adjuvant induced arthritis, level of brain indolamines (tryptophan, serotonin and 5-hydroxyindole acetic acid i.e. 5-HIAA) were estimated.

### Brain dissection technique

Rat’s brain samples were collected to estimate brain indolamines levels. For this purpose, on final day of experiment rats were chopped and brains were quickly excised (within one minute) from cranial cavity removing duramater. The brain extending upto frontal cortex rostrally and medulla oblongata caudally was dipped in chilled saline and stored at -80°C until assay of indolamines were conducted.

### Extraction of indoleamines from brain

The brain indoleamines from the frozen rat’s brain were extracted by electric homogenizer using 0.4 M perchlorate as an extraction medium. Homogenates were then allowed to stand for 10-15 minutes for the precipitation of proteins. Supernatant was decanted in a separate eppendorf tube and centrifuged at 12,000 rpm for five minutes. After centrifugation supernatant was separated and used for the estimation of tryptophan, serotonin (5-HT) and 5-HIAA.

### Estimation of brain indolamines

Concentration of these indolamines is very low in brain sample that can typically be estimated by RP-HPLC method coupled with electrochemical detector. Their levels were estimated as previously described [32] to study the influence of treatment on RA associated depression. Briefly, a 5II Shim-Pack ODS separation column (4.0 mm×150 mm) was used and separation was accomplished by a mobile phase containing methanol (14%), Octyl sodium sulfate (0.023%) and EDTA (0.0035%) in 0.1 M phosphate buffer at pH 2.9 at an opening

pressure of 2000-3000 psi on Shimadzu LEC 6A detector at an operating potential of 0.8V for biogenic amines and 1.0V for tryptophan reference electrode was +0.8–1.0 volt.

### Statistical analysis

Data was analyzed by one-way analysis of variance using SPSS INC. software. Bonferroni's post-hoc test was conducted to determine inter group means differences taking significant level  $p < 0.05$ .

## Results

### Drugs effect on progression of adjuvant-induced arthritis

During experiment evidence of clinical tenderness was instigated to observe from day 10, showing erythema in joints especially in ankle, metatarsal and interphalangeal joints in AIA control and AIA saline which became significant ( $p = 0.032$ ) from day 12 in contrast to normal. However arthritic groups receiving leflunomide and meloxicam showed non-significant difference ( $p > 0.05$ ) throughout the whole experiment when compared with normal. In case of the arthritic groups receiving leflunomide along with meloxicam, no significant difference existed up to day '18' when compared with normal healthy rats but from day '20' and onwards the difference became significant ( $p < 0.05$ ) (Figures 1 and 2). The body weights of animals in all groups were not significantly different throughout the experiment.

### Drugs effect on brain indolamines (tryptophan, serotonin and 5-HIAA)

In the entire arthritic groups, brain indolamines levels decreased significantly ( $p < 0.005$ ) when judged against normal (Table 1). The Bonferroni's post-hoc tests for inter group means differences illustrate that this difference was also significant ( $p < 0.005$ ) in all treated arthritic groups when compared with AIA control and AIA saline. Indolamines level in AIA lef showed significant decrease ( $p < 0.05$ ) in contrast to AIA melox while non-significant in AIA lef+melox.

## Discussion

Previous studies showed that treatment of RA with DMARDs decreased symptom of rigorousness, impediment sequence, or

sustained a relapsing-remitting pattern of ailment in concern with joint improvement [19] but their effects on neurobiological disorder as depression and anxiety in such patients should also be monitored. The principle aim of this work was to study not only the combined effect of leflunomide and meloxicam on progression of RA but also on its associated depression. For this purpose experiment was performed on adjuvant induce arthritic (AIA) rats which represents chronic model of inflammation.

It has been reported earlier that leflunomide and meloxicam showed good anti-arthritic property by inhibiting proinflammatory cytokines through DHODH (dihydroorotate dehydrogenase) inhibition [33] and inflammatory PGs [34] levels through cyclooxygenase enzymes (COX-I and COX-II) inhibition respectively. Present results also showed the same consistency that leflunomide ( $5 \text{ mg Kg}^{-1} \text{ day}^{-1}$ ) and meloxicam ( $5 \text{ mg Kg}^{-1} \text{ day}^{-1}$ ) inhibited RA progression significantly ( $p < 0.005$ ), in terms of joint erythema and limb swelling, when given alone. While with the same doses, when these drugs were given mutually as indicated in AIA lef+melox group, initially they produced synergistic effect but after some time (day 20) a strong negative effect was observed since they showed erythema in ankle, metatarsal and interphalangeal joints which was also significantly high ( $p < 0.05$ ) to the arthritic group receiving no treatment and almost similar to the arthritic group treated with saline only. Then after day 22 this group showed more significant ( $p < 0.05$ ) edema when compared to AIA control and AIA saline.

Previous studies also showed the existence of neurological disorders as depression and anxiety in RA patient which is also associated with hyperactivity of pro-inflammatory cytokines during inflammation [8]. It has also been reported that tryptophan is an essential amino acid and being the precursor of serotonin is involved in mood, consciousness and sleep, it is mainly metabolized in liver by kynurenine (Kyn) pathway through tryptophan pyrolyase enzyme while remaining in brain [35]. In addition to normal metabolism of tryptophan in RA patient, it has also been reported that levels of plasma tryptophan further decreases due to its increased additional catabolism by indoleamine 2,3-dioxygenase enzyme released from interferon- $\gamma$  in inflammatory cell during arthritis. This extra increase in tryptophan catabolism in RA patients may effect tryptophan transportation into brain via common carrier system located on blood brain barrier [36]. Therefore decrease tryptophan availability in the brain may contribute to the decreased serotonin synthesis in brain [37] which might be one of the important reasons to make the arthritic body depressed [38]. As previous research indicated that depletion of brain serotonin results in severe depression and anxiety [38,39].

With regard of the RA treatment, there are some studies that highlighted the problem of depression and anxiety in patients receiving leflunomide [22,23]. Similarly, it was also reported that in some patients severe depression was associated with the use of NSAIDs and as they were withdrawn from their regimen they showed good recovery from anxiety and depression [23,24].

In the present work, brain indolamines levels (tryptophan, serotonin and 5-hydroxyindole acetic acid i.e. 5-HIAA) decreased significantly ( $p < 0.005$ ) in the entire arthritic groups when judged against normal healthy rats which clarified depression existence in all arthritic groups as indicated in Table 1. This experiment produced same result as reported earlier that in RA body level of tryptophan decreases [9,10,16]. Furthermore, this study also indicated that treatment of RA in AIA rats also produced marked and highly significant ( $p < 0.005$ ) effect on brain tryptophan ( $F = 4358.559$ ,  $p < 0.005$ ) and serotonin ( $F = 12322.432$ ,  $p < 0.005$ ), and 5-HIAA ( $F = 38.499$ ,  $p < 0.005$ ), levels. The Bonferroni's



Figure 1: Paw edema.

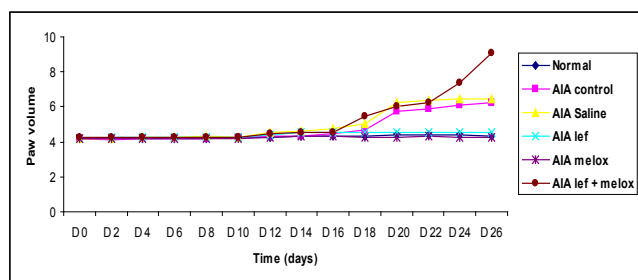


Figure 2: Paw edema assay in AIA rats.

post-hoc tests for inter group means differences illustrated that this depression exaggerated significantly ( $p < 0.005$ ) when these arthritic rats were treated with leflunomide and meloxicam alone or mutually in comparison with untreated or saline-treated arthritic rats. Moreover, brain indolamines level in arthritic rats treated with leflunomide showed significant decrease ( $p < 0.05$ ) in contrast to arthritic rats treated with meloxicam while non-significant ( $p > 0.05$ ) results were observed in arthritic rats treated with leflunomide along with meloxicam.

The possible explanation of the results is the side effect associated with these drugs as present in literature that leflunomide and meloxicam [23] both have strong ability to produce severe depression. In the present study, severe reduction in brain indoleamine (tryptophan, serotonin and 5-hydroxy indole acetic acid i.e. 5-HIAA) concentration after the treatment of AIA rats by leflunomide and meloxicam alone or mutually may be one of the most important reason of the exaggeration of this neurobiological disorder in AIA rats. Hence, this portion of research highlighted that both the drugs, leflunomide and meloxicam potentiates depression in AIA rats and when these rats were treated with leflunomide alone or with meloxicam then chances of depression became higher due to severe depletion of brain indoleamines especially serotonin.

## Conclusion

We want to highlight here that not only DMARDs along with NSAIDs are enough to treat all pathologies related to RA and secondly, not all the combination among them works synergistically as in case of leflunomide and meloxicam. Both individually reduced joint inflammation but produce severe adverse effects when given together with the same dose. While, significant depression was observed in all treated case when compared with normal, AIA control or AIA saline, which is dominant in AIA lef and AIA lef+melox. So, an antidepressant should be added in the regimen of RA treatment especially when lef or melox are there.

These results significantly show that both of these drugs have antagonistic effects to each other. Meloxicam is a mild pain reliever and is usually co-administered with leflunomide to boost up its action and to give mild relief in pain. The results of present study show that there is no use of this combined therapy, albeit meloxicam depresses or antagonizes the effect of leflunomide.

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