

Anti inflammatory effect of thymoquinone in comparison with methotrexate on pristane induced arthritis in rats

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Abstract

Objective: To determine the anti-inflammatory effects of thymoquinone on body weight, clinical score of inflammation, total leukocyte count and differential leukocyte count in arthritic rats and compare it with that of methotrexate.

Methods: The study was conducted at the Post-Graduate Medical Institute, Lahore, from March to September 2013, and comprised female Sprague-Dawley rats randomised into four equal groups; group A (healthy control), group B (positive control), group C (thymoquinone treated) and group D (methotrexate treated). Arthritis developed in Group B, C and D within two weeks after a single intra-dermal injection of pristane. Body-weight measured on electronic balance in grams and clinical score of inflammation scored on macroscopic scoring system were monitored on every alternate day while total leukocyte count and differential leukocyte count were taken at day 0, 16 and 30. After day 15, groups A and B were given 0.5ml of distilled water by intra-peritoneal injection daily for 15 consecutive days; group C was given thymoquinone 2mg/kg by intra-peritoneal injection daily for 15 consecutive days, and group D received methotrexate 0.5mg/kg by intra-peritoneal injection, daily for 15 consecutive days. SPSS 20 was used for statistical analysis.

Results: The 32 rats in the study were randomised into four groups of 8(25%) each. In group A the body-weight continued to increase and reached a mean of $144.13 \pm 10.8\%$ of the baseline at day 30. In group B the weight reduced to $93.13 \pm 4.19\%$ at day 16 and to $88.3 \pm 6.97\%$ at day 30. In groups C and D the weight reduced to $87.25 \pm 7.69\%$ and $88.5 \pm 7.07\%$ respectively at day 16; then the animals in the two groups regained their weight which increased to $108.63 \pm 10.89\%$ and $103.38 \pm 6.25\%$ respectively at day 30. The score was zero in group A throughout the study period. The score of group B, which was zero at day 0, reached a mean of 16 ± 0 at day 16. In groups C and D, the mean score increased till day 16 and reached 16 ± 1 and 16 ± 0 respectively, and then reduced to 5 ± 2 and 4 ± 1 at day 30 respectively.

Conclusion: Evaluation of data supported the anti-inflammatory activities of thymoquinone, so it may be investigated as an effective anti-inflammatory drug in rheumatoid arthritis.

Keywords: Rheumatoid arthritis, Pristane, Thymoquinone, Methotrexate. (JPMA 65: 519; 2015)

Introduction

Rheumatoid arthritis (RA) is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for deformity and disability. The consequent morbidity and mortality has a substantial socioeconomic impact. The prevalence of RA is consistent worldwide, affecting about 0.5-1% of the population. Women are affected more than men at ratio of 3:1.¹

RA progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which

causes the synovium to thicken. In the third stage, the inflamed cell releases enzymes that may digest the bone and cartilage, often causing the joints to lose shape and movement.

The most commonly prescribed medication for RA treatment is non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs, corticosteroids and immunosuppressant drugs. The goal of these drugs has been to relieve pain, to decrease joint inflammation and to prevent joint destruction. Among disease-modifying anti-rheumatic drugs, methotrexate (MTX) remained the cornerstone for RA. These drugs are known to produce various side effects, including gastrointestinal (GI) disorders, immunodeficiency, hormonal disturbances and increased tendency to cause infections. Accordingly, reduced side effects should be considered while designing improved therapeutics for RA.

Nigella Sativa has been used traditionally in herbal

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medicine for the treatment of various diseases. Thymoquinone (TQ) is major active agent of *Nigella Sativa*. It has antioxidant effects and has been shown to protect against heart, liver and kidney damage,² has anti-cancer,³ anti-bacterial,⁴ anti-convulsant,⁵ anti-inflammatory,⁶ anti-tussive,⁷ anti-asthmatic⁸ and anti-diabetic effects.⁹

Considering the anti-inflammatory property, the current study was conducted to evaluate the anti-inflammatory activity of TQ in arthritic rats and to compare it with that of MTX.

Subjects and Methods

The comparative study was conducted at the Post-Graduate Medical Institute (PGMI), Lahore, from March to September 2013. Adult healthy female Sprague Dawley rats weighing 120-220 grams were kept in the PGMI Animal House in iron cages under hygienic conditions. Room temperature was maintained at $25 \pm 2^\circ\text{C}$ and they were fed rat chow and water ad libitum. They received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.¹⁰ They were kept for acclimatisation for one week.

The sample size was estimated by using power and precision 3.0 software and the rats were randomly divided into four equal groups; A, B, C and D. The rats were numbered 1, 2, 3 and 4; all rats having number 1 were included in group A, while the numbers 2, 3 and 4 rats formed groups B, C and D respectively.

All the four groups received rat chow and drinking water for 30 days. Group A was given 0.5ml of distilled water at the base of tail on day 0. After day 15, group A was given 0.5ml of distilled water by intra-peritoneal injection daily for 15 consecutive days. Groups B, C and D were given a single intra-dermal injection of 0.5ml pristane on day 0 at the base of tail.¹¹ After day 15, group B was given 0.5ml of distilled water by intra-peritoneal injection daily for 15 consecutive days; group C was given TQ 2mg/kg dissolved in distilled water by intra-peritoneal injection daily for 15 consecutive days¹² and group D received MTX 0.5mg/kg intra-peritoneal injection daily for 15 consecutive days.¹³

Parameters measured were body-weight, clinical score of inflammation, total leukocyte count (TLC) and differential leukocyte count (DLC). The weight of the animals was not similar at day 0 after random allocation so it was converted into percentage and was considered as 100%. Then the trend of change in weight was studied in percentages. Arthritis development was monitored by a macroscopic scoring system for the four limbs ranging from 0-4, where 0 = no swelling, no redness, no joint

involvement; 1 = swelling and redness of one joint; 2 = two joints involved; 3 = more than two joints involved; and 4 = severe arthritis in the entire paw.¹⁴

(Minimum score = 0 and maximum = 4 for one limb; the score of 4 limbs was collectively taken so it became minimum score = 0 and maximum = 16.)

Clinical scoring of arthritis and body-weight for all the rats was performed at day 0 and then on every alternate day. Scores at day 0, 16, 24 and 30 were analysed statistically.

At day 0, 15 and 30, 1ml blood was collected by cardiac puncture under light anaesthesia (inhalation of chloroform). Blood was put in the test tubes containing Ethylenediaminetetraacetic acid (EDTA) and checked for TLC and DLC.

Data was entered and analysed using SPSS 20. Mean and standard deviations were used as descriptive measures in quantitative variables like body-weight, inflammatory score, TLC, and DFC for the four groups at different reading times. Data was tested for normality by using Shapiro's Wilkes test, and homogeneity of variance was tested by using Leven's statistics. Data following normal distribution and having homogeneity of variances were compared among groups by using one-way analysis of variance (ANOVA), and Tukey's test was used for post hoc analysis. Data deviating from normality and homogeneity was compared among groups by using Kruskal Wallis ANOVA, and Mann Whitney U test was used for post hoc analysis. Normally-distributed data was compared between times by using paired t-test. $P \leq 0.05$ was considered statistically significant.

Results

The 32 rats in the study were randomised into four groups of 8(25%) each. In group A the body-weight continued to increase and reached a mean of $144.13 \pm 10.8\%$ of the baseline at day 30. In group B the weight reduced to $93.13 \pm 4.19\%$ at day 16 and to $88.3 \pm 6.97\%$ at day 30. In groups C and D the weight reduced to $87.25 \pm 7.69\%$ and $88.5 \pm 7.07\%$ respectively at day 16; then the animals in the two groups regained their weight which increased to $108.63 \pm 10.89\%$ and $103.38 \pm 6.25\%$ respectively at day 30 (Figure-1).

The clinical score of inflammation was zero in group A throughout the study period. The score of group B, which was zero at day 0, reached a mean of 13 ± 1 at day 8, and increased to 16 ± 0 at day 16. Then it remained constant on 16 ± 1 and 15 ± 1 at day 24 and day 30 respectively. In groups C and D, the mean score increased till day 16 and reached 16 ± 1 and 16 ± 0 respectively. At day 24 the mean

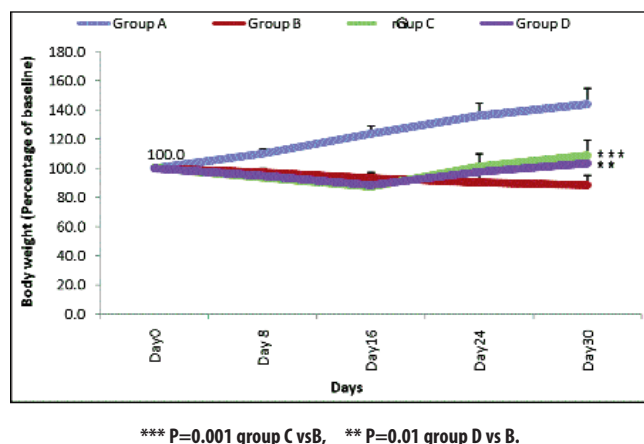


Figure-1: Changes in body-weight of animals (mean $\pm$ standard deviation) of rats with control (A), pristane-induced arthritis (B), pristane-induced arthritis treated with thymoquinone (C) and pristane-induced arthritis treated with methotrexate (D) from day 0 to day 30 measured on every alternate day (n=8).

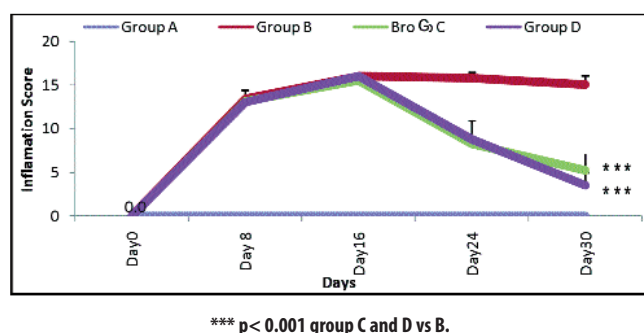


Figure-2: Clinical score of inflammation (mean $\pm$ SD) of rats with control (A), pristane-induced arthritis (B), pristane-induced arthritis treated with thymoquinone (C) and pristane-induced arthritis treated with methotrexate (D) from day 0 to day 30 taken on every alternate day (n=8).

score reduced to 8 ± 1 and 9 ± 2 with further reduction to 5 ± 2 and 4 ± 1 at day 30 in the two groups respectively (Figure-2). The score was normal in group A, marked in group B, moderate in group C, and mild in group D (Figure-3).

Mean TLC at day 0 was $5025 \pm 509/\text{cmm}$ in group A, $5263 \pm 941/\text{cmm}$ in group B, $4738 \pm 590/\text{cmm}$ in group C and $4913 \pm 671/\text{cmm}$ in group D. In group A, TLC remained unchanged over time. In group B at day 15 and day 30 TLC was $12563 \pm 795/\text{cmm}$ and $11738 \pm 1108/\text{cmm}$ ($p < 0.001$). In groups C and D, TLC at day 15 was $13150 \pm 924/\text{cmm}$ and $13450 \pm 875/\text{cmm}$ ($p < 0.001$). At day 30, TLC in groups C and D was $6938 \pm 886/\text{cmm}$ and $5938 \pm 760/\text{cmm}$ ($p < 0.001$) (Table-1 and 2).

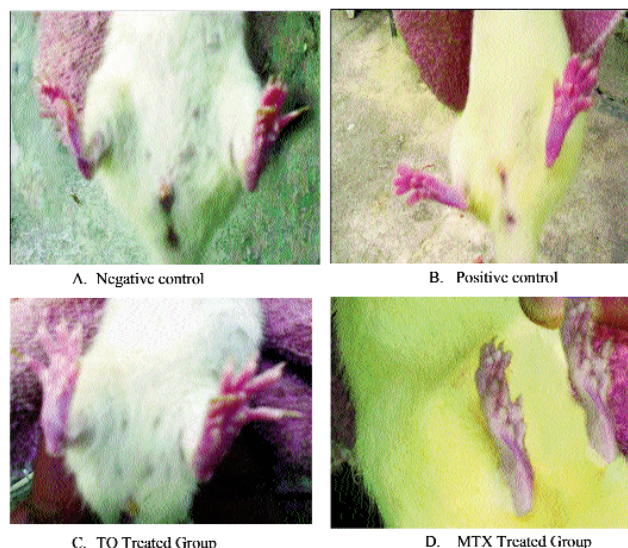


Figure-3: (A) Normal paws of a healthy control rat (B) Arthritic paws showing redness and swelling after 15 days of pristane administration (C) Reduction in inflammation and swelling after 15 days of treatment with thymoquinone (D) Marked reduction in soft tissue swelling and inflammation after 15 days of treatment with methotrexate.

When pair-wise comparison was made between the groups at day 15, the mean TLC was significantly higher in groups B, C and D compared to group A ($p < 0.001$ each). There was no significant difference between groups B, C and D at day 15. At day 30, group B had significantly higher mean TLC compared to group A, C and D ($p < 0.001$ each). There was no significant difference between groups C and D ($p = 0.119$) and A and D ($p = 0.298$), but difference between groups A and C was significant ($p = 0.002$) (Table-3). Neutrophil percentage at day 0 was the highest 32.3 ± 2.2 in group C and lowest 30.1 ± 2.8 in group A. In group A, it remained unchanged over time. In group B, the neutrophil percentage at day 15 and day 30 was 35.1 ± 2.5 and 34.4 ± 2.7 respectively which was significantly higher than day 0 ($p < 0.001$ and $p = 0.008$). The difference between day 15 and day 30 was insignificant ($p = 0.080$). In groups C and D the neutrophil percentage at day 15 was 36.4 ± 2.4 and 34.4 ± 2.1 which was significantly higher than day 0 ($p < 0.001$ each). At day 30, in groups C and D it was 33.8 ± 2.2 and 30.6 ± 2.3 which was significantly lower than day 15 ($p < 0.001$ each).

Comparison made at day 15 between the groups and the percentage of neutrophil were significantly higher in groups B, C and D compared to group A ($p < 0.001$ each). The difference among groups B, C and D was insignificant ($p = 0.663$; $p = 0.900$; and $p = 0.277$). At day 30, groups B and C had significantly high neutrophil compared to group A ($p = 0.001$; $p = 0.003$) while difference between groups D and A was insignificant ($p > 0.05$). In group A, lymphocyte count (LC)

Table-1: Effect of Thymoquinone on total and differential leukocyte count (TLC and DLC) in blood of prisaine-induced arthritic rats (n=8) (mean±standard deviation).

Day Group	Day 0				Day 15				Day 30			
	A	B	C	D	A	B	C	D	A	B	C	D
TLC mm ³	5025±509	5263±941	4738±590	4913±671	5175±542	12563±795#	13150±924#	13450±875#	5163±632	11738±1108#	6938±886#*	5938±760*
Neutrophil %age	30.1±2.8	32.0±2.3	32.3±2.2	30.3±1.9	29.0±1.7	35.1±2.5#	36.4±2.4#	34.4±2.1#	29.4±1.6	34.4±2.7#	33.8±2.2#	30.6±2.3*γ
Lymphocyte %age	67.9±3.3	66.4±3.2	66.5±2.8	69.5±2.0	69.1±1.6	61.5±2.7#	61.1±3.9#	63.0±3.5#	69.0±2.0	62.4±2.6#	65.0±3.0#	68.1±2.6*
Neutrophil: Lymphocyte	0.45±0.06	0.48±0.06	0.49±0.05	0.44±0.04	0.42±0.03	0.57±0.07#	0.60±0.07#	0.55±0.06#	0.43±0.03	0.55±0.06#	0.52±0.06#	0.45±0.05*

#p ≤ 0.05 compared with group A. *p ≤ 0.05 compared with group B. γp ≤ 0.05 compared with group C. €p ≤ 0.05 compared with group D.

Table-2: Comparison of weight, clinical score of inflammation, Total leukocyte count (TLC) and neutrophils among groups at various reading times (ANOVA).

Parameter			Sum of Squares	Df	Mean Square	F	Sig.
Weight	Day 16	Between Groups	7191.625	3	2397.208	62.193	<0.001
		Within Groups	1079.250	28	38.545		
		Total	8270.875	31			
	Day 30	Between Groups	13383.000	3	4461.000	55.159	<0.001
		Within Groups	2264.500	28	80.875		
		Total	15647.500	31			
Clinical score of inflammation	Day 16	Between Groups	1.3	2	0.7	2.3	0.122
		Within Groups	6.0	21	0.3		
		Total	7.3	23			
	Day 30	Between Groups	614.3	2	307.2	141.8	0.000
		Within Groups	45.5	21	2.2		
		Total	659.8	23			
Total leukocyte count	Day 16	Between Groups	375748437.5	3	125249479.2	196.870	<0.001
		Within Groups	17813750.0	28	636205.4		
		Total	393562187.5	31			
	Day 30	Between Groups	209323750.0	3	69774583.3	93.322	<0.001
		Within Groups	20935000.0	28	747678.6		
		Total	230258750.0	31			
Neutrophils	Day 16	Between Groups	253.8	3	84.6	17.86	< 0.001
		Within Groups	132.6	28	4.7		
		Total	386.5	31			
	Day 30	Between Groups	139.8	3	46.6	9.52	< 0.001
		Within Groups	137.1	28	4.9		
		Total	277.0	31			

ANOVA: Analysis of variance.

remained unchanged over time. The lymphocyte percentage was at day 0 the highest 69.5±2.0 in group D and the lowest 66.4±3.2 in group B. In group A the lymphocyte percentage remained unchanged over time. In group B LC at day 15 and day 30 was 61.5±2.7% and 62.4±2.6% which was significantly lower than day 0 (p<0.001 each). In groups C and D the lymphocyte percentage at day 15 was 61.1±3.9 and 63.0±3.5 which was significantly lower than day 0 (p<0.001). At day 30 the lymphocyte percentage in groups C and D was 65.0±3.0 and 68.1±2.6 which was significantly higher than day 15 (p<0.001 each).

Pair-wise comparison made between the groups at day 15 showed mean LC was significantly lower in groups B, C

and D compared to A (p<0.001; p<0.001; p=0.002 respectively). There was no significant difference among groups B, C and D at day 15 (p>0.05 each). At day 30, groups B and C had significantly lower LC compared to group A (p<0.001 and p=0.022 respectively). Group D had non-significant difference with group A (p>0.05).

In group A, the neutrophil-to-lymphocyte ratio remained unchanged over time. At day 0 it was the highest 0.49±0.05 in group C and the lowest 0.44±0.04 in group D. In group A, the ratio remained unchanged over time. In group B at day 15 and day 30 it was 0.57±0.07 and 0.55±0.06 which was significantly higher than day 0 (p<0.001 and p=0.003 respectively). The difference

Table-3: Pair-wise comparison of weight (percent of day 0) among groups at various reading times (Tukey's test).

Parameter	Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	P-value
Weight	Day 16	Group A	Group B	30.75(***)	3.10	<0.001
			Group C	36.63(***)	3.10	<0.001
			Group D	35.38(***)	3.10	<0.001
		Group B	Group C	5.88	3.10	0.254
			Group D	4.63	3.10	0.457
			Group C	-1.25	3.10	0.977
	Day 30	Group C	Group D	3.75	3.70	0.743
			Group A	55.75(***)	4.50	<0.001
			Group C	35.50(***)	4.50	<0.001
		Group B	Group D	40.75(***)	4.50	<0.001
			Group C	-20.25(***)	4.50	0.001
			Group D	-15.00(**)	4.50	0.012
Clinical score of inflammation	Day 24	Group C	Group D	5.25	4.50	0.652
		Group B	Group C	7.50(***)	0.74	<0.001
			Group D	7.00(***)	0.74	<0.001
	Day 30	Group C	Group D	-0.50	0.74	0.782
		Group B	Group C	9.75(***)	0.74	<0.001
			Group D	11.50(***)	0.74	<0.001
Total leukocyte count	Day 16	Group C	Group D	1.75	0.74	0.067
		Group A	Group B	-7387.5(***)	398.812	<0.001
			Group C	-7975.0(***)	398.812	<0.001
			Group D	-8275.0(***)	398.812	<0.001
		Group B	Group C	-587.5	398.812	0.467
			Group D	-887.5	398.812	0.141
	Day 30	Group C	Group D	-300.0	398.812	0.875
		Group A	Group B	-6575.0(***)	432.342	<0.001
			Group C	-1775.0(**)	432.342	0.002
			Group D	-775.0	432.342	0.298
		Group B	Group C	4800.0(***)	432.342	<0.001
			Group D	5800.0(***)	432.342	<0.001
Neutrophil	Day 16	Group C	Group D	1000.0	432.342	0.119
		Group A	Group B	-6.1***	1.1	< 0.001
			Group C	-7.4***	1.1	< 0.001
			Group D	-5.4***	1.1	< 0.001
		Group B	Group C	-1.3	1.1	0.663
			Group D	0.8	1.1	0.900
	Day 30	Group C	Group D	2.0	1.1	0.277
		Group A	Group B	-5.0***	1.1	0.001
			Group C	-4.4*	1.1	0.003
			Group D	-1.3	1.1	0.675
		Group B	Group C	0.6	1.1	0.942
			Group D	3.8**	1.1	0.011
		Group C	Group D	3.1*	1.1	0.041

between day 15 and day 30 was insignificant ($p=0.059$).

In groups C and D, the neutrophil-to-lymphocyte ratio at day 15 was 0.60 ± 0.07 and 0.55 ± 0.06 which were significantly higher than day 0 ($p<0.001$ each). At day 30, the ratio in group C and D was 0.52 ± 0.06 and $0.45.1 \pm 0.5$ which were significantly lower than day 15 ($p<0.001$ each).

Pair-wise comparison made between the groups at day 15 showed that the neutrophil-to-lymphocyte ratios were

significantly higher in groups B, C and D compared to A ($p<0.001$; $p<0.001$; and $p=0.001$ respectively). There was no significant difference among groups B, C and D at day 15 ($p>0.05$). At day 30, groups B and C had significantly higher ratio compared to group A ($p<0.001$ and $p=0.006$ respectively). Group D had no difference with group A.

Discussion

RA is the most common form of polyarticular

inflammatory arthritis characterised by persistent synovial inflammation, bony erosions and progressive articular destruction leading to varying degrees of physical disability.¹⁵

Pristane (synthetic mineral oil) is often used to induce arthritis in rats with interesting immunological and genetic features. Pristane-induced arthritis (PIA) is of great importance for the understanding of the pathogenesis and genetics of human autoimmune diseases, such as RA.¹³

In the present study, arthritis was induced by pristane and monitored by body-weight, clinical score of inflammation, TLC and DLC. PIA in rats is characterised by an early acute phase of severe inflammation.¹⁶ In the present study, induction of arthritis was associated with decrease in body-weight and rise in clinical score of inflammation and TLC. DLC demonstrated increase in neutrophil percentage and decrease in lymphocyte percentage, indicating acute phase of disease. Both the standard drug MTX and test compound TQ significantly suppressed inflammation as seen from the significant increase in body-weight, decrease in clinical score of inflammation and TLC, and normalisation of DLC.

When comparison was made between TQ and MTX, it was observed that increase in body-weight and decrease in clinical score of inflammation were statistically same in both groups, but they did not return to normal at the end of the study. TLC and DLC improved with both drugs, but the effect of MTX was more marked and cell counts returned to normal values in this group.

Many studies have been carried out on TQ in order to explore its anti-inflammatory effects. One study investigated the preventive effects of TQ on collagen-induced arthritis in rats. A significant decrease in the incidence and severity of arthritis by clinical and radiographic assessments was found in recipients of the therapy compared with the controls. In our study also, clinical score of inflammation improved.¹¹

Another study evaluated the effects of TQ on polyarthritis index and pain response score in Freund's adjuvant arthritis in rats. Biochemical parameters like Leukotriene B₄ (LT-B₄), interleukin 6 (IL-6) were also considered. TQ-treated rats showed significant reduction in polyarthritis index, pain response, IL-6 and LT-B₄ compared to the non-treated rats.¹⁷

In another study, the anti-inflammatory effect of TQ on adjuvant-induced arthritis in rat model was studied. Signs of inflammation on the claw and radiological signs were searched for and tumour necrosis factor (TNF)-alpha and

IL-1beta were measured. The results of the control and other groups were compared and TQ significantly suppressed arthritis both clinically and radiologically.¹⁸ Improvement in clinical score of inflammation is in accordance with our study.

Studies suggest multiple possible anti-inflammatory mechanisms of TQ. It is found to inhibit the production of nitric oxide, a pro-inflammatory mediator, and thus anti-inflammatory action might be mediated partly through this mechanism.¹⁹ TQ also inhibits the synthesis of prostaglandins (PGEs) and LTs by blocking the cyclooxygenase (COX) and lipoxygenase (LO) pathways of arachidonate metabolism, and is thus responsible for anti-inflammatory activity.²⁰ TQ highly reduces the levels of IL-1 β , IL-6, TNF- α and interferon gamma (IFN- γ) which are considered to be important pro-inflammatory mediators.²¹

TQ administration, at a dose of 2mg/kg/day for 15 days improved clinical features of PIA in rats. This effect was comparable to that of MTX.

Conclusion

TQ had anti-inflammatory effects in pristane-induced arthritis and can be investigated as potential anti-arthritic agent.

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