COMPARATIVE EVALUATION OF THYMOQUINONE AND METHOTREXATE IN LUNG INFLAMMATION IN MURINE MODEL OF RHEUMATIOD ARTHRITIS

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ABSTRACT

Objective: To determine the effect of thymoquinone and methotrexate on lung in pristine induced inflammation in rats.

Methodology: It was a comparative study which was carried out at Post Graduate Medical Institute Lahore. Rats were divided into four equal groups each consisting of 8 rats (n=8); group A served as healthy control, group B was taken as positive control, group C was treated with thymoquinone and group D with methotrexate. Arthritis developed within two weeks after a single pristane injection which is assessed through total leukocyte count. Total leukocyte count was taken at day 0, 15 and 30 while lung tissue for histopathology was taken at the time of dissection (day 30).

Results: The result of this study indicated that development of arthritis was accompanied by significant raise in total leukocyte count and the presence of inflammatory cells in lung histopathological sections as compared to healthy control groups. Both thymoquinone and methotrexate significantly reduced total leukocyte count and number of inflammatory cells as compared to positive control group of arthritic rats.

Conclusion: Evaluation of results supported the beneficial effects of both thymoquinone and Methotrexate in lung inflammation produced by murine model of rheumatoid arthritis. Thus thymoquinone can be a potential therapy for rheumatoid lung and needs further evaluation in human models.

Key Words: Rheumatoid arthritis, Pristane, Thymoquinone, Methotrexate, Total leukocyte count.

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INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory disorder that typically affects the small joints of the hands and feet. It results in chronic pain with very high disability rates. Furthermore, the life expectancy of the patients suffering from RA is 10 years less than the normal individuals. The ratio of mortality for RA ranges from 1.28 to 3.0[1]. Being a systemic disease the involvement of the joints, heart and lungs is the most common. Respiratory involvement of rheumatoid arthritis may be serious and sometimes even causes death[2].

One of the important contributors to increase the mortality in patients of RA is the respiratory causes. The treatment of rheumatoid articular diseases has significantly improved with the discovery of the new drugs in recent years but these have not significantly benefited the RA-associated lung disease.

RA is a cause for a number of pulmonary manifestations[3]. The interstitial lung disease (ILD) is the most common one which ultimately leads to pulmonary fibrosis (PF). ILD is the most serious form of lung involvement in RA[1]. Researches has shown that ILD is present in 25% of RA patients. Not only the prevalence of ILD is increasing but also various studies have proved its involvement in about 6% of all RA deaths. RA patients hardly complained of dyspnea associated with ILD until the end of the last century. Pain was usually reducing their mobility. But nowadays as more effective treatment for the articular manifestations of RA has become the standard, the involvement of the respiratory system in the form of ILD has become increasingly recognized as a major factor in determining morbidity and mortality in RA. During the last ten years most of the studies were performed to know the prognosis of the RA-ILD. Such studies revealed the mean survival is about 3 years from diagnosis[4].

Methotrexate (MTX) is considered as a gold standard for RA. Disappointingly, it leads to pulmonary fibrosis.

Therefore while using this drug one should monitor the respiratory status closely. MTX can produce other adverse effects like alopecia, hepatotoxicity, mucosal ulcers and gastrointestinal disturbances[2].

Thymoquinone (TQ), an active principal of Nigella Sativa has been used traditionally in herbal medicine for the treatment of various diseases. It is locally known as Kalonji in Southern Asia, Habat-ul-sauda in Arabic while Black Cumin in english[5]. It has antioxidant effects and has been shown to protect against heart, liver and kidney damage[6], anti bacterial activity[7], anti tussive effect[8] and anti diabetic effect[9]. It also has an anti-inflammatory and immune modulatory effects[10].

TQ is used both for the prevention and treatment of lung inflammation associated with arthritis[10,11]. A lot of researches have been done on its preventive role but data regarding the efficacy of TQ in the treatment of arthritis associated inflamed lung is very limited. Based upon the above facts, the current study was aimed to determine and compare the efficacy of TQ and MTX in arthritis associated inflammatory lung diseases in rats.

METHODOLOGY

Adult healthy female Sprague Dawley rats weighing 120-220 grams were kept in animal house at Post Graduate Medical Institute Lahore in iron cages under hygienic conditions. The room temperature was maintained at 25 ± 2°C and was fed rat chow and water ad libitium. They were kept for acclimatization for one week.

Induction of arthritis was performed by single intra-dermal injection of 0.5ml Pristane (synthetic mineral oil), at the base of tail. The rats developed arthritis within two weeks[12].

All the rats were randomly divided in to four groups by balloting method; as A, B, C and D. Each group consists of eight adult healthy female Sprague Dawley rats. The 32 sample size was estimated by using power and precision 3.0 software. It was estimated at 5% level of significance and 90% power of test with expected arthritis score of 5.98 ± 0.3, 3.26 ± 0.29, 1.95 ± 0.28 in untreated, treated with TQ and treated with MTX groups respectively[13].

All the four groups received rat chow and drinking water for 30 days. On day 0, Group A was given 0.5ml of distilled water at the base of tail. After day 15, group A was given 0.5 ml of distilled water by intra peritoneal injection daily for 15 consecutive days. On day 0 group B, C and D were given a single injection of 0.5ml pristane intradermally at the base of tail. After day 15, group B was given 0.5 ml of distilled water by intra peritoneal injection daily for 15 consecutive days; group C was given thymoquinone 2 mg/kg dissolved in distilled water by intra-peritoneal injection daily for 15 consecutive days and group D received methotrexate 0.5 mg/kg intra-peritoneal injection, daily for 15 consecutive days[13,14].

Induction of arthritis was evaluated by total leukocyte count (TLC) and its effect on lungs was evaluated by the presence of inflammatory cells in histopathological sections of lungs. At day 0, 15 and 30 (at the time of dissection), 1ml blood was collected by cardiac puncture under chloroform anesthesia and was checked for TLC while at day 30 the sections of lungs were taken for histopathology.

Samples of lung tissue were fixed in 10% neutral buffered formalin solution and embedded in paraffin. 6um thick slices were cut in serial sections, they were stained with hematoxylin-eosin and examined for inflammatory cells under light microscope. The degree of perivascular and peribronchial inflammation was evaluated on a subjective scale of 0-3. A value of 0 was considered Normal (when no inflammatory cells were found); a value of 1 was considered as Mild (occasional presence of inflammatory cells), a value of 2 was taken as Moderate (when most bronchi or vessels were surrounded by a thin layer of inflammatory cells {one to five cells thick}); and a value of 3 was taken as Severe (when most bronchi or vessels were surrounded by a thick layer {more than five cells thick} of inflammatory cells[15]. The protocols of the experiment of this study were approved by the ethical committee of Post Graduate Medical Institute Lahore.

Statistical Analysis: After collection data was entered and analyzed by using SPSS 20.0 software. The mean and standard deviation was determined for total leukocyte count. One way ANOVA was used for data following normal distribution and for the comparison of homogeneity of variances among groups, and for post hoc analysis Tukey's test was used. Data deviating from normality and homogeneity were compared among groups by using Kruskal Wallis ANOVA, and for post hoc analysis Mann Whitney U test was used. P-value ≤ 0.05 was considered statistically significant.

RESULTS

The leukocyte count was same for all the four groups at day 0. In group A the leukocyte count remained unchanged over time. In group B the leukocyte count at day 15 and day 30 was significantly higher than day 0 with p-values <0.001. In both groups C and D increase till day 15 was significant with p-values <0.001 and then the decrease between day 15 and day 30 was also significant with p-values <0.001.

The inflammatory cells in lungs were not present in group A. There were 12.5% cases with mild, 37.5% moderate and 50.0% cases with severe inflammation in group B. Majority (62.5%) of cases of group C had mild while 37.5% had moderate inflammation. In group

D 71.0% had mild and just 29% had moderate inflammation.

When comparison of histological parameters was performed among groups it was found that the difference was significant for inflammatory cells with p-values <0.001. When pair wise comparison was made for inflammatory cells, it was noted that the group B, C and D had severe inflammation as compared to group A with p-values <0.001, <0.001 and 0.001 respectively. Similarly the group C and D had mild inflammation as compared to group B which was severe with p-values 0.006 and 0.003 respectively. Both C and D had mild inflammation with insignificant difference having p-value 0.062.

Table 1: Total leukocyte count for animals in four groups at three reading times (n=8)

Day	Day 0				Day 15				Day 30			
Group	A	B	C	D	A	B	C	D	A	B	C	D
TLC mm3	5025± 509	5263± 941	4738± 590	4913± 671	5175± 542	12563± 795	13150± 924	13450± 875	5163± 632	11738± 1108	6938 ±886	5938± 760

Table 2: Comparison of total leukocyte count among groups at three reading times

			Sum of Squares	df	Mean Square	F	p-value
Day 0	TLC	Between Groups	1160937.5	3	386979.2	0.797	0.506
		Within Groups	13601250.0	28	485758.9		
		Total	14762187.5	31			
Day 15	TLC	Between Groups	375748437.5	3	125249479.2	196.870	<0.001
		Within Groups	17813750.0	28	636205.4		
		Total	393562187.5	31			
Day 30	TLC	Between Groups	209323750.0	3	69774583.3	93.322	<0.001
		Within Groups	20935000.0	28	747678.6		
		Total	230258750.0	31			

Table 3: Comparison of inflammatory cells in both lungs of four groups

	Groups	N	Severity of inflammation at day 15	Severity of inflammation at day 30
Inflammatory cells	Group A	8	Normal	Normal
	Group B	8	Severe	Severe
	Group C	8	Severe	Mild
	Group D	8	Severe	Mild

Table 4: Pair wise comparison of inflammatory cells among groups for both lungs

Histological parameter	(I) group	(J) group	Mann Whitney U	Z. approximation	p-value
Inflammatory cells	Group A	Group B	0.0	-3.60	<0.001
		Group C	0.0	-3.66	<0.001
		Group D	4.0	-3.26	0.001
	Group B	Group C	7.0	-2.73	0.006
		Group D	4.5	-2.93	0.003
	Group C	Group D	15.5	-1.86	0.062

DISCUSSION

In this study the total leukocyte count was significantly increased till day 15 in group B (positive control), C (TQ treated) and D (MTX treated) as compared to group A (healthy control). After day 15 the TLC in both group C and D was markedly decreased as compared to group B. Inflammatory cells in lungs were not present in group A; 12.5% cases with mild, 37.5% with moderate and 50.0% cases with severe inflammation in group B; 62.5% cases of group C had mild while 37.5% had moderate inflammation; in group D 71.0% had mild and just 29% had moderate inflammation.

Boskabady and co-workers investigated the effect of thymoquinone on lung pathology, interferon-γ (IFN-γ) and blood interleukin-4 (IL-4) of sensitized guinea pigs. Twenty seven guinea pigs were divided randomly into three groups (n=8) and were sensitized to ovalbumin (OA). They were given drinking water alone, and drinking water containing low and high concentrations of thymoquinone, respectively. Control group (n=8) was given saline instead of OA and drinking water. The inflammatory changes in the lungs, including infiltration of eosinophils and lymphocytes and blood IL-4 and IFN-γ of sensitized guinea pigs were evaluated. Marked pathological changes were found in the lungs of the sensitized group. In contrast to the control group the blood IL-4 and IFN-γ were high in sensitized animals. Thymoquinone led to a significant decrease in the inflammatory changes of the lungs in sensitized animals. The results of the study showed the preventive effect of thymoquinone on lung inflammation of sensitized guinea pigs[16].

A study was performed in Egypt to evaluate the anti-inflammatory and anti-fibrotic effects of TQ. Male Wister rats were injected bleomycin intraperitoneally. Bleomycin highly increased the total leucocytic count, lung weight and the levels of Lactate dehydrogenase, total protein and mucin in bronchoalveolar lavage. Lung histopathology was also done. These markers were restored in TQ treated groups. Furthermore, the anti-inflammatory effect of TQ was also shown by histopathological examination[17].

Kanter evaluated the protective effects of TQ in lung inflammation produced by toluene (volatile aromatic compound) in rats. The total number of rats were 10 which were randomly divided into three experimental groups: one was control, second was toluene treated and the third was toluene treated with Nigella sativa (NS); each group contained 10 animals. 1ml serum physiologic was given to control rats, toluene by inhalation of 3000 ppm was given to the second group, and the rats in TQ treated group was given TQ started just after their exposure to toluene. For histopathological investigation Lung tissue samples were obtained. Result showed that in toluene treated rat the inflammatory pulmonary responses assessed by peribronchial inflammatory cell infiltration, alveolar septal infiltration, alveolar exudate, alveolar edema, interstitial fibrosis and necrosis formation were significantly reduced by treatment with NS[18].

In another study, the preventive effect of TQ was determined on tracheal responsiveness, lung inflammation and white blood cell (WBC) count in lung lavage of sensitized guinea pigs. Lung lavage was also evaluated for total WBC and its differential count. The tracheal responsiveness to methacholine, OA and WBC of positive control group were significantly higher than those of healthy controls. Tracheal responsiveness in TQ treated groups to both methacholine and OA and total WBC count was markedly decreased in comparison to positive control group. There was also a decrease in neutrophils, lymphocytes and monocytes in the positive control group. These results confirmed a preventive effect of thymoquinone on inflammatory cells of lung lavage and tracheal responsiveness of sensitized guinea pigs[19].

In all the above mentioned studies the decline in total leukocyte count and reduction in inflammatory cells seen on lung histopathology is in accordance to our study.

CONCLUSION

Treatment with both TQ and MTX significantly reduced the total leukocyte count and histopathological changes almost equally. This study indicates that TQ can be used as an effective therapy for inflammatory lung diseases and may be useful for the treatment of RA.

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CONTRIBUTORS

RF conceived the idea and planned the study and wrote manuscript. UI did acquisition of data, statistical analysis and interpretation of data. Both the authors contributed significantly to the submitted manuscript.