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Urinary excretion of connective tissue metabolites under the influence of a new non-steroidal anti-inflammatory agent in adjuvant induced arthritis

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Abstract

The therapeutic effect of boswellic acids and salai guggal in adjuvant induced arthritic rats in relation to urinary excretion of connective tissue metabolites viz. hydroxyproline, hexosamine and uronic acid was thoroughly investigated. Compared to controls, the arthritic animals showed an increase in the excretion of these metabolites in urine. The elevated levels of urinary hydroxyproline (free, total, non-dialysable and dialysable), hexosamine and uronic acid in the arthritic animals were found to be slightly decreased in the acute phase and significantly decreased in the chronic phase of the disease following the administration of boswellic acids or salai guggal. The results of the investigation indicated that both these anti-inflammatory drugs could offer a partial protective action against changes induced by adjuvant induced arthritis.

Introduction

Adjuvant induced arthritis in the rat is a widely used pathological model for the study of chronic inflammatory disease [1] and is used for the detection and evaluation of the efficacy of anti-inflammatory drugs [2]. Certain manifestations of adjuvant induced arthritis resemble in part those of rheumatoid arthritis and adjuvant arthritis is ameliorated by a number of anti-arthritic agents. The inflammatory process of adjuvant induced arthritis is a result of an immunological response to antigens in the bacterial capsule amplified by the oil adjuvant. The bacterial antigen is recognised as a foreign substance by the host's immunological system and induces a sequence of stereotyped cyclic changes of clinical manifestations.

Recently, interest has been focussed on the metabolism of collagen in this inflammatory disease. An increased catabolism of newly synthesized collagen accompanied by a retardation in the conversion of soluble to insoluble collagen is found in arthritic animals [3]. Studies from this laboratory [4, 5] have shown that the synthesis as well as crosslinking of collagen is decreased in the chronic inflammatory process of adjuvant arthritis. The elevated urinary excretion of hydroxyproline, hexosamine and hydroxylysylglycosides in adjuvant induced arthritis has also been reported [6, 7].

Recently, many new anti-inflammatory drugs have been introduced to control the undesirable effects of inflammation. Among them, the non-steroidal anti-inflammatory drugs are beneficial in the symptomatic treatment of inflammatory diseases. Although the non-steroidal anti-inflammatory drugs have great therapeutic efficacy

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in controlling inflammatory symptoms, they usually have undesirable side effects [8]. Therefore, an attempt has been made to search for herbal based anti-inflammatory products reputed to have beneficial effects in rheumatoid arthritis and other inflammatory diseases. It has been reported [9] that the gum resin exudate of *Boswellia serrata*, commonly known as salai guggal, a new non-steroidal anti-inflammatory agent, displayed prominent anti-arthritic activity with marked inhibition of secondary lesions and loss in body weight as compared to phenylbutazone. Various pharmacological studies demonstrated that salai guggal did not show any gastric ulcerogenic activity in rat stomach even in doses as high as 2 g/kg body weight, a point of distinct advantage, whereas the ulcerogenic index with phenylbutazone was found to be significantly greater even at low dose of 0.1 g/kg body weight. The therapeutic efficacy claimed for this new investigational drug is, therefore of considerable clinical interest worthy of laboratory investigation. The purpose of the present investigation was to demonstrate a pharmacological basis for the suggested therapeutic effect of salai guggal on the urinary excretion of connective tissue metabolites in the experimental model of rheumatoid arthritis.

Earlier studies on chemical analysis of salai guggal have shown it to be comprised of a mixture of triterpene pentacyclic acid derivatives of boswellic acids and other essential oils [10–12]. Since boswellic acids are major constituents of salai guggal, it was of interest to compare the therapeutic efficacy of boswellic acids with that of salai guggal.

Materials and methods

Animals

Male albino rats of Wistar strain were kept and bred in our own animal house and used at an age of 6–8 week with initial average body weight of 80 g each. Animals received commercial diet (Hindustan Lever, Bombay) and water *ad libitum*.

Production of adjuvant arthritis

The arthritic group of rats received 0.1 ml of Freund's adjuvant per animal injected intradermally into the dorsum of the tail root. The adjuvant contained 10 mg heat-killed *Mycobacterium tuberculosis* (obtained from Tuberculosis Research Centre, Madras) per ml sterile paraffin oil. A group of animals with the same initial body weight received equal amount of sterile paraffin oil alone and served as control.

Evaluation of arthritis

The severity of adjuvant induced arthritis was assessed by quantitation of the hind paw swelling and the arthritic index. The volume of the hind paw was determined plethysmographically at weekly intervals throughout the experimental period. The arthritic index was estimated, according to the evaluation system [6] ranging from 0 to 16 points. Depending on the severity of the lesions and the number of joints involved (in any single paw), 0 to 4 points were given per limb. Score 4 corresponds with total functional loss of the paw. Thus by this index the maximum score obtained for one animal is 16.

Drug treatment

Animals with fully developed arthritis were selected and from day 14 the animals were divided into three groups viz. Arthritic (A), Arthritic + Boswellic acids (AB) and Arthritic + Salai guggal (AS). Animals in AB and AS groups received orally boswellic acids and salai guggal (each 10 mg/100 g body weight/day), respectively. Animals in the control group (C) and arthritic group (A) received only drug vehicle (C.M. cellulose 0.5%).

Body weights were recorded at weekly intervals during the experimental period of 49 days. At the end of every week urine samples were collected in toluene over a period of 24 hrs following gastric loading with 3 ml saline/100 g body weight whilst keeping the animals in metabolic cages.

Analysis of urine

The total hydroxyproline content of urine was measured in the hydrolysed sample and the free hydroxyproline was measured in the unhydrolysed sample by the method of Podenphant et al. [13]. The non-dialysable fraction of urine was separated by dialysing aliquots of urine against 0.15 *M* NaCl and distilled water at 4 °C for 24 hrs as adopted by Haddad et al. [14] and the hy-

droxyproline content of the non-dialysable fraction was determined. The dialysable hydroxyproline content was obtained by subtracting non-dialysable hydroxyproline from the total hydroxyproline. Results are expressed as mole of hydroxyproline per gram creatinine in order to account for eventual incomplete collection of urine samples as well as to balance the effect of any body weight dissimilarities [15].

Urinary hexosamine was determined by the procedure of Elson and Morgan [16] as adopted by Remington [17]. Urinary glycosaminoglycan was isolated and estimated as uronic acid content by the method of Ritchie et al. [18]. The creatinine content in the urine was estimated by the Jaffee reaction [19].

Statistical analysis

The results were statistically evaluated using Students' '*t*' test. *P* values less than 0.05 were considered to be statistically significant.

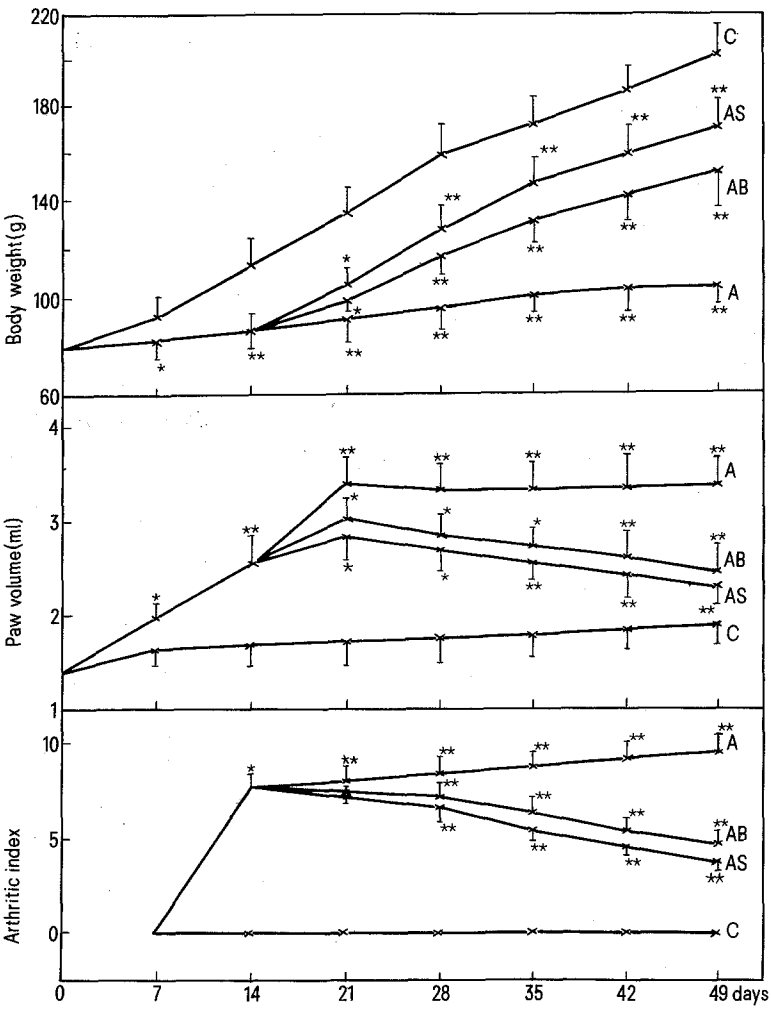

Figure 1
Effect of boswellic acids and salai guggal on Body weight, Paw volume and Arthritic index in adjuvant induced arthritis. Each point represents the mean of 4 rats with the S.D. indicated by the vertical bars. * $P<0.05$, ** $P<0.01$, C=Control, A=Arthritis, AB=Arthritis+Boswellic acids and AS=Arthritis+Salai guggal. A compared to C, AB and AS compared to A.

Results

The mean body weight of control animals progressively increased from an initial value of 80 g to 204 g on day 49 (Fig. 1). The growth rates of the animals in groups A, AB and AS were found to be slower than those of control animals of the same weight but reached a weight of 105 g, 154 g and 171 g respectively on day 49. Administration of boswellic acids or salai guggal to arthritic animals caused a gain in body weight steadily and significantly ($p < 0.01$) as compared to the arthritic animals. The paw volume of arthritic animals increased rapidly during the second and third

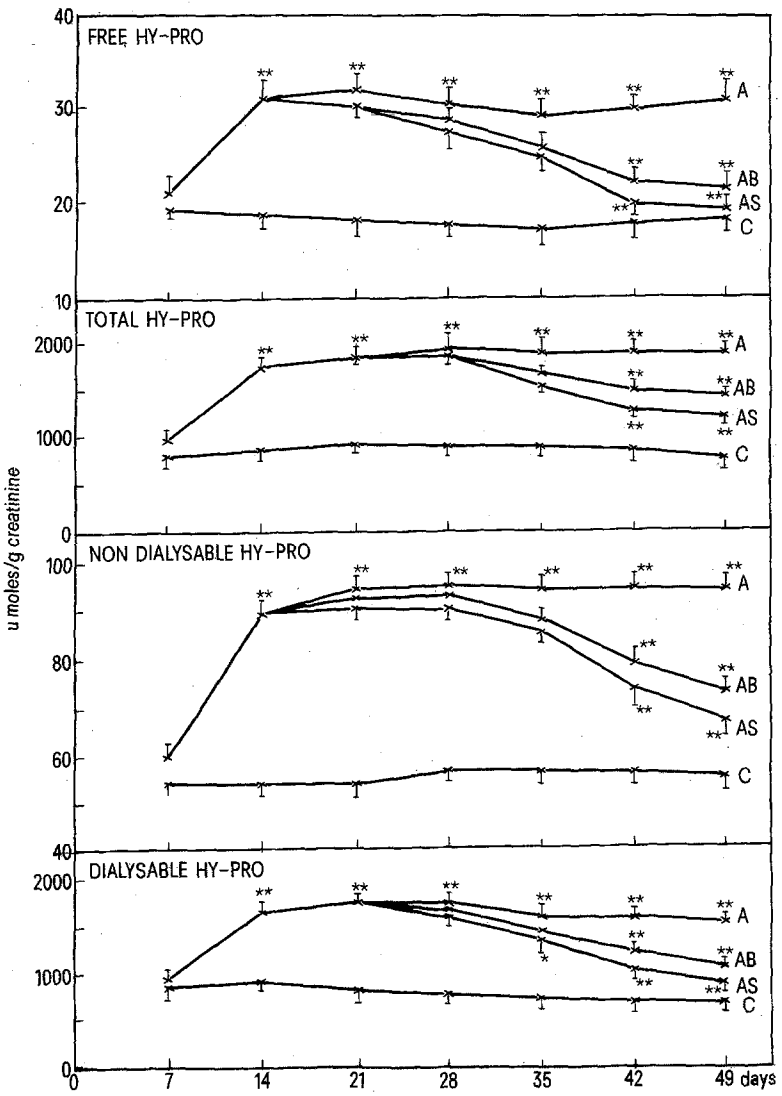

Figure 2
Effect of boswellic acids and salai guggal on urinary excretion of free, total, nondialysable and dialysable hydroxyproline (expressed as μmoles/g creatinine) in adjuvant induced arthritis. Values are mean±S.D. * $P<0.05$, ** $P<0.01$. Six samples from each group containing urine of 4 rats were analysed. C = Control, A = Arthritis, AB = Arthritis + Boswellic acids and AS = Arthritis + Salai guggal. A compared to C, AB and AS compared to A.

Table 1
Effect of boswellic acids and salai guggal on urinary excretion of hexosamine in adjuvant induced arthritis.

Days	Group			
	C	A	AB	AS
21	72.62 ±4.26	136.42 ±6.32**	135.26 ±4.96 (NS)	132.46 ±5.26 (NS)
28	76.43 ±3.82	146.38 ±8.46**	142.68 ±5.62 (NS)	138.54 ±5.92 (NS)
35	80.64 ±3.86	150.38 ±7.96**	140.42 ±6.24 (NS)	132.28 ±5.24**
42	77.62 ±3.42	144.96 ±5.26**	124.76 ±4.96**	114.64 ±4.26**
49	78.96 ±3.64	152.35 ±6.62**	120.36 ±5.23**	110.74 ±4.36**

Values are expressed as mg/100 mg creatinine. Six samples from each group containing urine of 4 rats were analysed.
Values are mean ± S.D.; ** $P<0.01$; NS = Not significant; C = Control; A = Arthritis; AB = Arthritis + Boswellic acids; AS = Arthritis + Salai guggal. A compared to C; AB and AS compared to A.

Table 2
Effect of boswellic acids and salai guggal on urinary excretion of uronic acid in adjuvant induced arthritis

Days	Group			
	C	A	AB	AS
21	9.24 ±1.92	14.98 ±2.26**	12.68 ±2.18 (NS)	11.96 ±2.16 (NS)
28	8.46 ±1.82	17.84 ±2.38**	13.56 ±2.42*	12.98 ±2.26*
35	9.62 ±1.87	18.82 ±2.54**	13.28 ±2.26**	12.24 ±1.87**
42	8.94 ±1.68	18.96 ±2.48**	12.26 ±1.98**	10.98 ±1.56**
49	9.76 ±1.94	19.29 ±2.68**	12.18 ±2.08**	10.48 ±1.92**

Values are expressed as mg/100 mg creatinine. Six samples from each group containing urine of 4 rats were analysed.
Values are mean ± S.D.; * $P<0.05$; ** $P<0.01$; NS = Not significant; C = Control; A = Arthritis; AB = Arthritis + Boswellic acids; AS = Arthritis + Salai guggal. A compared to C, AB and AS compared to A.

week onwards (Fig. 1) and thereafter there was little change. The animals treated with boswellic acids or salai guggal were found to be decreased in paw volume significantly compared to arthritic animals. Arthritic rats showed a progressive increase in arthritic index (Fig. 1) and reached 7.6 after 2 weeks, 8.7 after 3 weeks and then slowly increased up to 9.48 ($p<0.01$). Administration of these anti-inflammatory drugs caused a significant decrease in arthritic index compared to arthritic rats.

The urinary excretion of free, total, non-dialysable and dialysable hydroxyproline in arthritic rats was found to be significantly increased ($p<0.01$) throughout the experimental period (Fig. 2). The drug-treated arthritic animals were found to be affected during the chronic phase of the arthritic syndrome. The enhanced urinary excretion of free, total, non-dialysable and dialysable hydroxyproline in arthritic animals significantly decreased after treatment with boswellic acids or salai guggal between day 42 and 49 (Fig. 2).

The urinary excretion of hexosamine and uronic acid in the arthritic group significantly increased ($p<0.01$) from the second week onwards up to the end of the experimental period as compared to the control group. Administration of anti-inflammatory drugs to arthritic animals caused a significant reduction in the excretion of hexosamine and uronic acid in urine (Tables 1 and 2).

Discussion

Hydroxyproline, a non-essential amino-acid is found to occur almost exclusively in collagen, where it accounts for 13–14% of the total amino-acids [20]. The synthesis of hydroxyproline occurs by the hydroxylation of large polypeptides as one of the terminal steps in the formation of collagen, and apparently there is no other mechanism for synthesizing hydroxyproline in vertebrates. These relations make hydroxyproline a convenient, naturally occurring label for studying the collagen metabolism, and the presence of hydroxyproline in tissues, plasma, or urine can be used as a measure of collagen or degradative products of collagen. The measurement of urinary excretion of hydroxyproline thus reflects the index of collagen metabolism, because the final breakdown products of collagen which include both free and peptide bound hydroxyproline fragments are not re-utilized in biosynthetic pathways of collagen [21–23]. Although changes in the excretion of hydroxyproline peptides in urine are not specific for any given disease, measurement of urinary excretion of hydroxyproline peptides has been

found to be useful in a number of clinical situations. Many studies on the urinary hydroxyproline excretion have proved [22, 23] to affect primarily the connective tissues in a number of bone diseases, several endocrine disorders and various similar diseases. An increased excretion of urinary hydroxyproline would thus appear to indicate an alteration in the patterns of collagen metabolism due to either changes in collagen synthesis or in the rate of conversion of one form of collagen to another or in the rate at which any form of molecules is degraded [24].

Haddad et al. [14] have studied patients with connective tissue disorders and have shown that the proportion of dialysable and non-dialysable hydroxyproline peptides serves as an index of metabolism of bone collagen. It has been shown [25] that non-dialysable hydroxyproline peptides in urine reflect newly synthesized fragments of collagen, whereas the dialysable hydroxyproline peptides are derived from the catabolism of bone [14]. An increased proportion of non-dialysable hydroxyproline peptides in the urine of inflammed animals indicates either increased rate of synthesis of new collagen or decreased utilization of newly formed collagen for matrix formation. The increased proportion of dialysable hydroxyproline indicates that the relative rate of bone catabolism in the disease may be higher than normal. Studies on metabolism of dermal collagen indicated [4, 5] that an increase in both neutral salt soluble collagen and acid soluble collagen contents in adjuvant induced arthritis is due to the reduced maturation and cross-linking of collagen in arthritic disease.

In the present investigation, the increased amounts of non-dialysable hydroxyproline peptides in the urine or arthritic animals (Fig. 2) indicate that this fraction of hydroxyproline peptides was derived either from recently synthesized and rapidly degraded collagen or from fragments of newly synthesized collagen but not incorporated into the tropocollagen. Thus appraisal of non-dialysable and dialysable hydroxyproline peptides in urine may contribute to an evaluation of relative contribution of bone formation and resorption to total bone turnover.

The increased excretion of hexosamine and uronic acid in the urine (Tables 1 and 2) may indicate the excessive catabolism of glycoproteins and glycosaminoglycans in adjuvant induced arthritis. The increased catabolism of glycoproteins and glycosaminoglycans may be explained on the basis of altered levels of glychohydrolases in the process of adjuvant induced arthritis. In our previous studies [26] we observed elevated levels of glycohydrolases viz. β-glucuronidase, β-N-acetyl glucosaminidase, cathepsin B_1, B_2 and D in adjuvant arthritic animals. We have also observed that arthritic symptoms were found to be reduced in the arthritic animals treated with boswellic acids or salai guggal by suppressing the activities of glycohydrolases. It is, therefore, evident from the present investigation that these new anti-inflammatory drugs displayed prominent anti-arthritic activity against inflammatory process of arthritis as assessed by urinary excretion of hexosamine and uronic acid (Tables 1 and 2).

In the light of present findings which are in line with the published evidence [3, 27] the increase in urinary excretion of free total, non-dialysable and dialysable hydroxyproline peptides (Fig. 2) and hexosamine and uronic acid (Tables 1 and 2) may thus be connected with an increased rate of collagen, glycoprotein and glycosaminoglycan catabolism in arthritic syndrome. It is evident from the data presented that the enhanced catabolism of collagen, glycoproteins and glycosaminoglycans in arthritic animals is partially reversed by the administration of either boswellic acids or salai guggal.

Earlier studies [3] indicated that the breakdown of *de novo* synthesized collagen is increased and accompanied by an impaired conversion of soluble to insoluble collagen in the inflammatory process of adjuvant induced arthritis. It is quite likely that these new anti-inflammatory agents may also protect the arthritic lesions by promoting the collagen maturation thereby lowering the excretion of hydroxyproline in the urine.

The mechanism of action of these new anti-inflammatory agents in arthritic animals is not very clear. But the beneficial action of these new drugs appears to act by a mechanism similar to that of the non-steroidal group of anti-arthritic drugs. Recently we have shown [28] that both boswellic acids and salai guggal subdued the arthritic symptoms with a beneficial action in stabilizing lysosomes in adjuvant induced arthritis.

The data presented in this investigation with respect to the excretion of hydroxyproline peptides, hexosamine and uronic acid in urine have shown

that these new anti-inflammatory drugs viz. boswellic acids and salai guggal afford partial protective action against changes induced by adjuvant arthritis. Of the two anti-inflammatory agents tested, salai guggal has shown more protective action than boswellic acids.

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