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EVALUATION OF ANTI-INFLAMMATORY EFFECTS OF LOSARTAN IN EXPERIMENTALLY INDUCED RHEUMATOID ARTHRITIS

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ABSTRACT:

Aim: To evaluate anti-inflammatory effects of losartan in experimentally induced rheumatoid arthritis (RA).

Materials and Methods: Adjuvant induced arthritis model is used in this study. Albino-Wistar rats of either sex were used. Arthritis was induced by single intra-plantar injection of Freund's complete adjuvant (FCA) suspended in oil in right hind paw. Rats were divided in to three groups (n=8) namely disease control, standard and test group. Drug treatment was carried out for 21 days. Effect of test drug on acute inflammatory phase was evaluated on day 5 by assessing right hind paw edema. After 21 days animals were sacrificed and evaluated for left hind paw edema, weight changes, histo-pathological synovitis grading in left hind limbs and secondary lesion score.

Results: Results showed that losartan significantly reduced right hind paw edema on day 5 ($p<0.05$) and showed significant weight gain ($p<0.05$). Losartan though reduced the histo-pathological synovitis grade and secondary lesion score was not statistically significant ($p>0.05$).

Conclusion: Our study suggests that losartan could be a potential candidate for treatment of RA. It is therefore recommended that studies using different doses and routes of administration of losartan needed to be conducted in future.

INTRODUCTION: Rheumatoid arthritis affects 1% of population worldwide [1,2]. It is a chronic inflammatory disorder of the joint characterized by inflammation of the synovial membrane, pain, and restricted joint movement. It is poly-articular joint disease characterized by massive synovial proliferation and subintimal infiltration of inflammatory cells, which along with angiogenesis leads to the formation of a very aggressive tissue called 'pannus'. Expansion of the pannus induces bone erosion and cartilage thinning, leading to the loss of joint function [3].

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and fluid homeostasis. Two distinct subclasses of the angiotensin II (Ang II) receptors, AT_1 and AT_2, have been described. This octapeptide hormone also has a number of other effects and, in particular, autocrine and paracrine pro-inflammatory properties [4].

Angiotensin II has significant pro-inflammatory actions in the vascular wall, inducing the production of reactive oxygen species (ROS), inflammatory cytokines and adhesion molecules [5].

AngiotensinII signalling through the AT1 receptor leads to activation ofthe transcription factor nuclear factor κB (NF-κB) with subsequent production of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6, ROS and adhesion molecules [6, 7].

Some studies demonstrated inhibition of collagen induced murine arthritis using AT1 receptor blockers like olmesartan, candesartan and telmisartan[8].Manabe et al. demonstrated the pro-inflammatory cytokines lowering effects of valsartan in hypertensive patients [9].Anti-inflammatory effects on vascular inflammation injury were also demonstrated for Valsartan and olmesartan by Lan Wu *et al*., [10] and Carlos *et al*., [11] respectively. While Peeters et al. reported neutral effect on cytokine production with captopril and valsartan, *in vivo* [12].

In view of the different studies, our study was undertaken to find out the anti-inflammatory role of losartan in experimental RA.

MATERIAL AND METHODS:

Animals: Albino-Wistar rats of either sex, 8-10 weeks old and weighing 150-200 grams were procured from Haffkine Biopharma Corporation, Parel. Animals were fed with standard pellet diet and water *ad libitum*. They were housed in animal laboratory at temperature (25℃+2℃), relative humidity (45%-55%) and 12 hours light dark cycle was maintained. The experimental protocol was reviewed and approved by institutional animal ethics committee (IAEC), B.Y.L. Nair Ch. Hospital and T.N.M.C., Mumbai. All experiments carried out according to guidelines suggested by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Drugs and Chemicals: losartan was used in pure form (Cipla Pharmaceuticals, Mumbai), indomethacin 50 mg capsule(Drug store of B.Y.L. Nair Ch. Hospital, Mumbai) and Freund's Complete Adjuvant (Sigma Chemicals Ltd., Mumbai) were used.

Losartan (4.5 mg/kg) and indomethacin (4.5 mg/kg) was suspended in 0.3% Carboxy-methyl cellulose (CMC) in distilled water for oral administration to each rat.

Study design: The study comprised of three groups of eight animals each (n=8) and treatment given is described below:

Disease control group:1 ml suspension of 0.3% CMC in distilled water was fed daily for 21 days.

Standard group: 1 ml suspension of indomethacin (4.5 mg/kg) in 0.3% CMC in distilled water was fed daily for 21 days.

Test group: 1 ml suspension of losartan (4.5 mg/kg) in 0.3% CMC in distilled water was fed daily for 21 days.

On day 0 of the experiment, the baseline measurements recorded were right and left hind paw edema and animal body weights. Experimental arthritis was induced on day 1. The animals were fed with the drugs orally daily for 21 days. On day 5, right hind paw edema was measured in all the groups. On day 21, left hind paw edema was measured, secondary lesion score and body weights were recorded, rats were sacrificed and left hind limb joints were sent for histopathology.

Induction of adjuvant induced Arthritis [13]: Arthritis was induced in all animals on day 1 by intradermal injection of 0.1 ml of FCA suspended in oil by no.26 needle in to the foot pad of right hind paw.

The subplantar injection of 0.1 ml of this suspension in the right hind paw results in primary, non-immune localized inflammatory response in the right hind paw. This local swelling (primary lesion) begin in the injected paw within 24 hrs, reach peak on 3^{rd} -5^{th} day and subsides by day 8-9. This local swelling on day 5 corresponds to acute inflammatory reaction, indicating the influence of therapeutic agents on this phase.

This is followed by the secondary immune systemic response after a delay of 11-12 days after injection of FCA. Secondary lesions are characterized by inflammation of non-injected sites (left hinpaw, forepaws, ears, nose and tail), and by decrease of weight. The secondary lesions are present till day 21. Day 21 was used for the influence of drugs on this phase of RA.

Histopathology: On day 21 the animals were sacrificed by ether anaesthesia. Left hind-paws with ankle joints obtained from rats were harvested post-mortem, fixed in 10% buffered formalin, decalcifiedin 5% nitric acid for up to 48 hours, routinely processed and embedded in paraffin wax. Sagittal sections (5 µm) were taken from the paraffin blocks and stained with haematoxylin–eosin (H & E) stain. The joints were studied by blinded examiner from the Pathology Department (B.Y.L. Nair Ch. Hospital, Mumbai).

Histopathologicalsynovitis grading [14]: The synovial inflammation was scored in 3 categories as follows:

I. Hyperplasia/enlargement of synovial lining cell layer.

II. Activation of resident cells/synovial stroma.

III. Inflammatory infiltration.

Each category was scored from 0 to 3 as follows: 0 = nil, 1= mild, 2 = moderate and 3 = severe.

Synovitis graded as **grade 0** (nil), **grade 1** (mild), **grade 2** (moderate) and **grade 3** (severe) with summaries ranging from 0 to 9 as: 0 to 1 corresponds to grade 0, 2 to 3corresponds to grade 1, 4 to 6 corresponds to grade 2 and 7 to 9 corresponds to grade 3.

Assessment of rat paw edema: Two methods were used to assess rat paw edema.

Mercury Plethysmography: A mark was put on the right and left hind limb of rat at the malleolus to facilitate uniform dipping at subsequent readings. The rat was held with left hand and the hind limb was dipped in the mercury column up to the mark. In this study, paw volume (in ml) was expressed as displacement of mercury in plethysmograph tube in cm.

Micrometer screw gauge: The micrometer screw gauge uses an auxiliary scale (measuring hundredths of mm) which is marked on a thimble with rotating vernier scale. In order to measure the diameter of the rat paw, hind paw was placed between the jaws, and the thimble was rotated using the ratchet until the object was secured. The maximum diameter of the paw was measured in mm.

Secondary lesion score [15]**:** Secondary lesions scored as; **Ears, Nose and Tail:** absence of nodules (0), Presence of nodules (1). For each; **Fore paws:** absence of inflammation (0), Inflammation of at least one joint (1). **Hind paws:** absence of inflammation (0), Slight inflammation (1), moderate inflammation (2), marked inflammation (3).

Secondary lesions were graded as: **grade 0** (nil), **grade 1** (mild), **grade 2** (moderate) and **grade 3** (severe) with summaries ranging from 0 to 7. 0 to 1 corresponds to grade 0, 2 to 3 corresponds to grade 1, 4 to 5 corresponds to grade 2 and 6 to 7 corresponds to grade 3.

Statistical analysis: Results were expressed as mean + SEM. One Way ANOVA followed by Tukey's post hoc test was used to analyze rat paw edema and difference in body weight between day 0 and day 21. Kruskal Wallis test was used to analyze histopathological grades and grades of secondary lesions. *P* value less than 0.05 was considered as statistically significant.

RESULTS:

We assessed the rat paw edema by mercury plethysmography as well as by micrometer screw gauge. On day 5 Losartan 4.5 mg/kg significantly reduced the rat paw edema on right side as compared to disease control group ($p<0.001$) by both methods (**Figure 1 & 2**). On day 21 losartan 4.5 mg/kg though showed the anti-inflammatory effect as compared to disease control group, it was not statistically significant ($p>0.05$) (**Figure 3 & 4**).

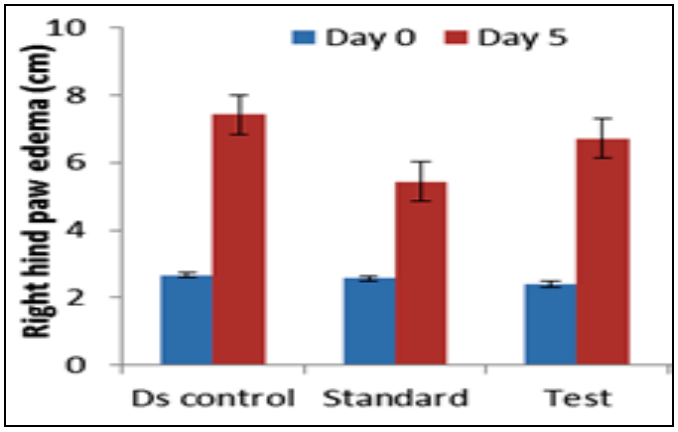

FIGURE 1: RIGHT HIND PAW EDEMA MEASURED ON DAY 0 AND DAY 5 BY MERCURY PLETHYSMO-GRAPHY

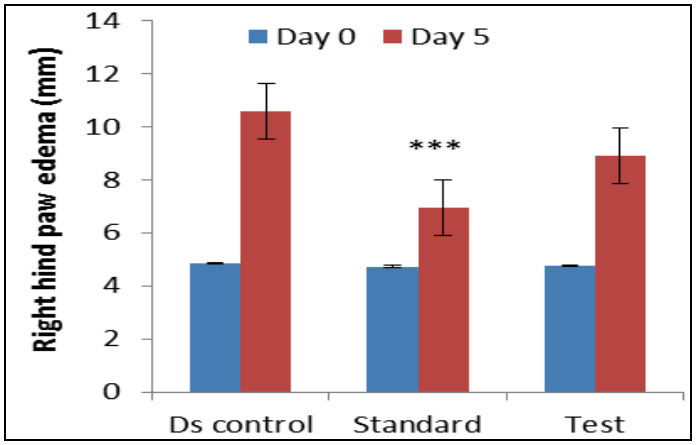

FIGURE 2: RIGHT HIND PAW EDEMA MEASURED ON DAY 0 AND DAY 5 BY MICROMETER SCREW GAUGE

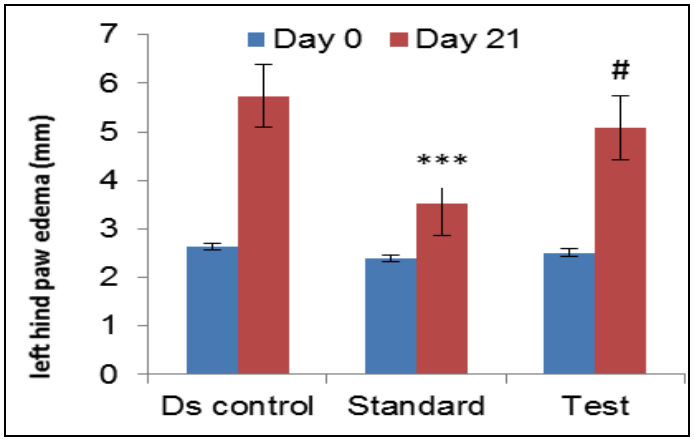

FIGURE 3: LEFT HIND PAW EDEMA MEASURED ON DAY 0 AND DAY 21 BY MERCURY PLETHYSMO-GRAPHY

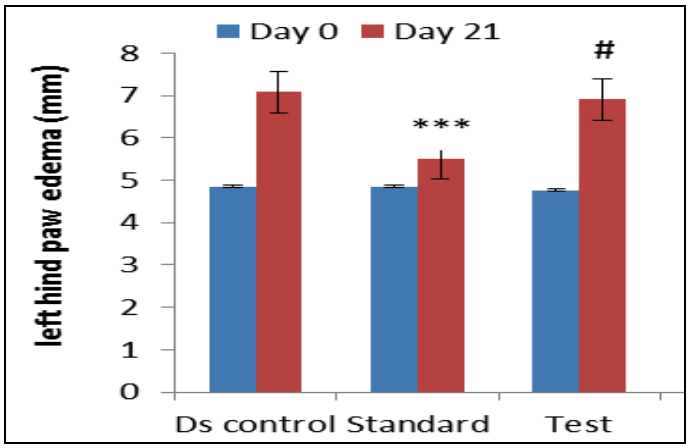

FIGURE 4: LEFT HIND PAW EDEMA MEASURED ON DAY 0 AND DAY 21 BY MERCURY PLETHYSMO-GRAPHY

Each bar is expressed as mean ± SEM. (n=8); ***p<0.001, # p>0.05 as compared with control group; (One way ANOVA followed by Tukey's post hoc test)

Histo-pathological grade of synovitis and secondary lesion score were also reduced by losartan 4.5 mg/kg as compared to disease control group but statistically was not significant (p>0.05) (**Table 1**).

The synovitis was graded in to four grades. The absence of infiltration of inflammatory cells was considered as grade 0, mild infiltration of inflammatory cells was grade 1, moderate infiltration of inflammatory cells was grade 2 and marked infiltration of inflammatory cells was grade 3 (**figure 5**).

On comparing the change in the body weight from day 0 to day 21, the weight gain in indomethacin and losartan group was significantly greater than disease control group (p<0.05) (**Table 1**).

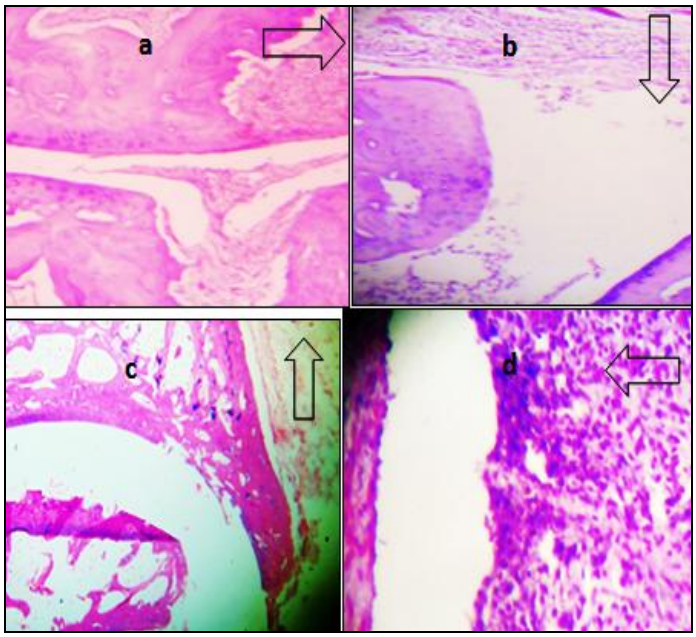

FIGURE 5: HISTOPATHOLOGICAL GRADES OF SYNOVIAL INFLAMMATION: (a) inflammatory grade 0 with no inflammatory infiltration, **(b)** inflammatory grade 1 with minimal infiltration of inflammatory cells, **(c)** inflammatory grade 2 with moderate infiltration of inflammatory cells, **(d)** inflammatory grade 3 with marked infiltration of inflammatory cells.

DISCUSSION: Several studies have been conducted for determining the role of AT1 receptor blockers as anti-inflammatory agents in RA. Some studies showed anti-inflammatory activity [8] while some showed pro-inflammatory activity [16] or no anti-inflammatory activity [12, 17]. The present study was undertaken to clarify those conflicting anti-inflammatory results about the role of AT1 receptor blockers in RA. Losartan is a widely prescribed antihypertensive drug with favorable safety profile. In our study, dose of the study drug (losartan 4.5 mg/kg) used in rats was extrapolated from the human dose[18] (losartan 50 mg) which was in the dose range normally used as anti-hypertensive therapy in humans [19].

TABLE 1: EFFECT OF LOSARTAN ON HISTOPATHOLOGICAL GRADE, GRADE OF SECONDARY LESIONS AND CHANGE IN BODY WEIGHT IN ADJUVANT INDUCED ARTHRITIS IN RATS.

Groups	HSP grade	Grade of Secondary lesions	Change in body weight (day 21-day 1)
Ds control	2 ± 0.76	2.75 ± 0.74	4.62 ± 3.41
Standard	0.12 ± 035***	0.5 ± 0.63***	13.75 ± 3.66***
Test	1.125 ± 083[#]	1.75 ± 0.48[#]	10.06 ± 2.29***

All values expressed as mean ± SEM (n=8). ***P< 0.001 as compared to disease control group.[#] p>0.05 (Kruskal Wallis test for HSP and secondary lesion grading. One Way ANOVA followed by Tukey's post hoc test for change in body weight). **HSP:** histopathology.

Freund's adjuvant induced arthritis in rats is the most commonly used model for experimental induction of RA [20]. This model involves immunological process for induction of arthritis which closely resembles the process of rheumatoid arthritis in human beings [21].

We assessed rat paw edema by mercury plethysmography as well as by micrometer screw gauge. Significant reduction of right hind-paw edema by losartan on day 5 ($p<0.001$) by both methods (**Figure 1& 2**) suggest that losartan 4.5 mg/kg is effective anti-inflammatory agent in acute phase of inflammation in experimental RA. This was supported by Laudanno *et al*., [22] who showed that the three ATI receptor antagonists such as candesartan, losartan and valsartan yielded anti-inflammatory effect in carrageenan foot-pad edema. In contrast to this, Raghavendra *et al*., [16] showed thatAT1 receptor antagonist losartan enhances angiotensin-II facilitated carrageenan-induced paw edema.

On day 21, losartan reduced the left hind-paw edema (**Figure 3 & 4**), histo-pathological grade of synovitis and secondary lesion score (**Table 1**) as compared to disease control group; but statistically significant results were not obtained ($p>0.05$).This could be attributed to the fact thatlosartan is a selective AT1 receptor blocker and Ang II activates NF-κB via both AT1 and AT2 receptors [23, 24]. Esteban *et al*., [25] showed that only combined treatment with AT1 and AT2 antagonists completely blocked renal inflammatory infiltration and NF-κB activation in Ang II infused mice. Therefore, therapy combining AT1 and AT2 antagonists may be more effective than therapy using AT1 antagonist alone in reducing the inflammation of arthritis.

In our study, we administered a relatively low dose of Losartan. Shao *et al*., [26] demonstrated that the imbalance in T cell subsets was reversed by olmesartan, only in a dose-dependent manner. Furthermore, Kayo *et al*., [8] proposed that for more effective suppression of arthritis, the means of administration and the doses of ARBs need to be modified.

Rats with arthritic syndrome invariably lose weight [13, 27]. In our study, weight gain in Indomethacin and losartan group was significantly greater than disease control group ($p<0.05$) (**Table 1**).

In conclusion our study suggests that losartan could be a potential candidate for treatment of RA. It is therefore recommended that studies using different doses and routes of administration of Losartan should be conducted in future.

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Conflicts of interest: None

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