

## Research Article



## STUDYING THE EFFECT OF THE COMBINATION OF LOSARTAN WITH DEXAMETHASONE ON SERUM CRP LEVELS AND ANKLE JOINT CHANGES IN ADJUVANT ARTHRITIS RATS

<sup>1</sup>Rana Makhose, <sup>1</sup>Sawsan Maddi, <sup>2</sup>Shareef Barakat, <sup>3</sup>Ahmad Agil

<sup>1</sup>Department of Pharmacology, Faculty of Pharmacy, Damascus University, Syria.

<sup>2</sup>Department of Histology, Faculty of Dentistry, Damascus University, Syria.

<sup>3</sup>Department of Pharmacology and Institute of Neurosciences, Faculty of Medicine, University Granada, Spain.

\*Corresponding author's E-mail: [ranamakhous@gmail.com](mailto:ranamakhous@gmail.com)

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### ABSTRACT

Drugs used in the treatment of rheumatoid arthritis, have many serious side effects. This study investigated the potential therapeutic effect of the combination of angiotensin receptor blocker (losartan) with low dose of glucocorticoid (dexamethasone) in ameliorating joint dysfunction arising from adjuvant arthritis (AA) in comparison with the effects of that produced by losartan and dexamethasone, and latter one at double dose, in purpose of achieving a new strategy in the treatment of rheumatoid arthritis with lower side effects than that produced by glucocorticoids in the higher doses. Adjuvant arthritis was induced in male Wistar rats by complete Freund's adjuvant injected in the right hind paw. Ankle joint width was assayed by calliper, serum C- reactive protein (CRP) levels were detected by immunoassay method, and the ankle joint tissue sections were examined histopathologically. The efficacy of combination of losartan with low dose of dexamethasone in reducing significantly the prominent ankle joint width, serum CRP levels, synovial hyperplasia, synovial inflammatory cells infiltration and cartilage erosion was equi-effective to that produced by high-dose of dexamethasone and even more effective than each of losartan or dexamethasone at low dose. This combination may represent a novel approach in the management of rheumatoid arthritis.

**Keywords:** Angiotensin receptor blockers, glucocorticoids, rheumatoid arthritis, adjuvant arthritis.

### INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by infiltration of inflammatory cells in the synovium, and hyperplasia of the synovial lining cellular components, resulting in the formation of pannus tissue, which invades and destroys adjacent cartilage and bone<sup>1</sup>. Recent studies have shown that the prevalence of the preclinical atherosclerosis is increased in RA patients<sup>2</sup>. RA treatment usually consists of disease modifying antirheumatic drugs, cytokine modulators, and glucocorticoids (GCs)<sup>3</sup>. GCs has had a special place in the treatment of rheumatoid arthritis and it initially was widely used for RA, however within several years the classical adverse effects of GCs including an increased prevalence of hypertension, insulin resistance, hyperlipidemia and osteoporosis were frequently noted particularly at the high doses, which may limit its use in spite of its efficacy<sup>3,4</sup>. Recent studies have shown that GCs also has disease modifying properties<sup>5,6</sup>.

Interestingly, previous researches have demonstrated a role of angiotensin II in joint inflammation and synoviocytes expansion in rheumatoid arthritis<sup>7,8</sup> and it has been approved that ARBs ameliorate arthritis in the murine collagen-induced arthritis (CIA) model<sup>9</sup>. Furthermore, many researchers have demonstrated too that angiotensin II may play a significant role in the development of insulin resistance and the use of angiotensin receptor blockers (ARBs) may attenuate it<sup>10,11,12,13</sup>, and other scientists established the role of angiotensin II in osteoporosis and the potential effects of ARBs in the treatment of osteoporosis<sup>14</sup>.

In the present study we hypothesized that combined treatment with losartan and dexamethasone may have positive advantage in ameliorating arthritis in vivo. Our study aimed to evaluate and compare the potential effects of combined treatment with losartan and low dose of dexamethasone beyond that produced by each corresponding dexamethasone and losartan alone, included the steroid at double dose, on the treatment of RA in an animal model of RA (adjuvant arthritis), in purpose of reducing the severity of the side effects of GCs, through reducing its dose, and through the potential effects of ARBs in counteracting these effects, in addition to the advantage may be obtained by the use of ARBs in preventing the development of RA associated atherosclerosis. To characterize these changes and to test our hypothesis, a rat model of adjuvant-induced arthritis was used using complete Freund's adjuvant (CFA). CFA-induced arthritis is the most widely used chronic test model and shares some features with human RA, including swelling of the extremities, cartilage degradation, loss of joint function and lymphocyte infiltration to the joints<sup>15,16</sup>.

### MATERIALS AND METHODS

Losartan potassium and dexamethasone sodium phosphate were obtained as a gift from (Obarri Company for pharmaceutical industries, Damascus, Syria). Complete Freund's adjuvant (CFA) was obtained from (Sigma- Aldrich, St Louis, USA).

The experimental protocol was approved by the Institutional Animal Care and Use Committee of



Damascus University, School of Pharmacy, Damascus, Syria.

Male Wistar rats (obtained from Leen institute for experimental animals and animal Facility, Faculty of Pharmacy, Damascus, Syria), 15 weeks old, weighing  $242.52 \pm 4.41$ g were housed in a cage, at a constant room temperature, relative humidity of 50–70% and an airflow rate of 15 exchanges/h. In addition, a time controlled system provided 07.00–21.00 h light and 21.00–07.00 h dark cycles. All rats were given standard rat chow diet and water ad libitum. At 16 weeks of age, the animals were divided into six groups (all groups  $n = 15$ ): I animals injected with 0.5 ml saline in the right footpad (hind paw) as a control group (C); the five subsequent arthritic groups were injected with 0.5 ml of CFA in the right footpad (hind paw): II drug untreated animals as adjuvant arthritis group (AA); or treated from day 12 of CFA injection for a 16 days as follows, III losartan (AAL) (s.c., 15mg/kg/48h); IV dexamethasone (AAD<sub>h</sub>) (i.m., 0.05 mg/kg/12h); V both, losartan (s.c., 15mg/kg/48h) and dexamethasone (i.m., 0.025 mg/kg/12 h) (AALD); VI dexamethasone (AAD<sub>i</sub>) (i.m., 0.025 mg/kg/12 h)

Arthritis was induced by the CFA inoculation in the rats. Briefly, rats were injected with 0.5 ml of CFA (1 ml of CFA contains 1 mg of heat killed mycobacterium tuberculosis) in the right footpad (hind paw) under ether anesthesia. The rats serving as controls were injected with 0.5 ml of saline<sup>17</sup>.

At the end of the treatment period, the animals (20-weeks-old) were anesthetized with ether after overnight fasting and killed. Blood was collected via heart puncture into tubes and centrifuged, the plasma was frozen at  $-80^{\circ}\text{C}$  for subsequent determinations.

**Joint swelling measurement:** Joint swelling was assessed by measurement of right hind paw ankle joint width by using Vernier calipers (Mitutoyo, Japan) prior to arthritis induction and every 2 days after arthritis induction.

**Histological ankles changes:** Immediately after killing, the joints of the ankles from the right hind paw were removed, fixed in 10% formalin, then in HNO<sub>3</sub> for decalcification, and then were embedded in paraffin. Sections at 5 microns were cut with a microtome (Leica RM 2155, Germany). The slides were stained with haematoxylin–eosin and submitted for histological evaluation. Synovial hyperplasia, synovial inflammatory cells infiltration, and cartilage erosion were determined<sup>18,19</sup>. The histopathological parameters in the ankles were scored as following: synovial hyperplasia: grade 0; (1-3 layers of synoviocytes), grade 1; (4-5 layers), and grade 2; ( $\geq 6$  layers), Inflammatory cell infiltration to the synovium: grade 0; (no inflammatory cells infiltration to the synovium), grade 1; (few), grade 2; (modest), and grade 3; (severe), cartilage erosion: grade 0; (normal), grade 1; (minor erosion), grade 2; (modest) and grade 3; (severe).

**Serum C-Reactive Protein:** Serum C-Reactive Protein levels were measured by immunoassay method using Rat CRP kit (ELISA), (Biovender company, Modice, Czech Republic).

**Statistical analysis:** Data are presented as means  $\pm$  SD. The significance of the differences between groups for ankle joint width changes and serum CRP levels were estimated by ANOVA test and Turkey's Multiple Comparison test. Histological results were estimated by Kruskal-Wallis test and Multiple Comparison Dunn test. The significance of the differences between control and AA group were estimated by unpaired T-test (two-tailed) for ankle joint width changes and serum CRP levels, and by Mann-Whitney test (two-tailed) for histological study.  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

**Ankle joint width:** Table 1 shows the changes in ankle joint width of the experimental groups (ankle joint width on day 28 of CFA injection – ankle joint width on day 0 of CFA injection). The injection of CFA alone (AA) induced a significant increase in ankle joint width, when compared with control group ( $p < 0.001$ ). Losartan or dexamethasone (0.025 mg/kg) treatment reduced significantly the induced ankle joint width when compared with AA group by 34% and 33% respectively ( $p < 0.001$ ). The efficacy of combination of losartan with dexamethasone (0.025 mg/kg/24 h) in reducing significantly ankle joint width (55%,  $P < 0.001$ ), was equal to the efficacy of high dose dexamethasone (0.05 mg/kg/24 h) by 58%, and was better than each of them alone.

**Serum CRP levels:** Table 1 shows the serum CRP levels of the experimental groups. The injection of CFA induced a significant increase in serum CRP levels, when compared with control group ( $p < 0.001$ ). Losartan or dexamethasone (0.025 mg/kg) treatment reduced significantly the induced CRP levels when compared with AA group by 25% and 27% respectively ( $p < 0.001$ ). The efficacy of combination of losartan with dexamethasone (0.025 mg/kg/24 h) in reducing the induced CRP levels was significantly equal to the efficacy of dexamethasone (0.05 mg/kg/24 h) (54 vs 55%,  $P > 0.05$ ) respectively.

**Histological study:** Table (2) shows the results of histological study, figure (1) shows sections from ankle joints of the experimental groups.

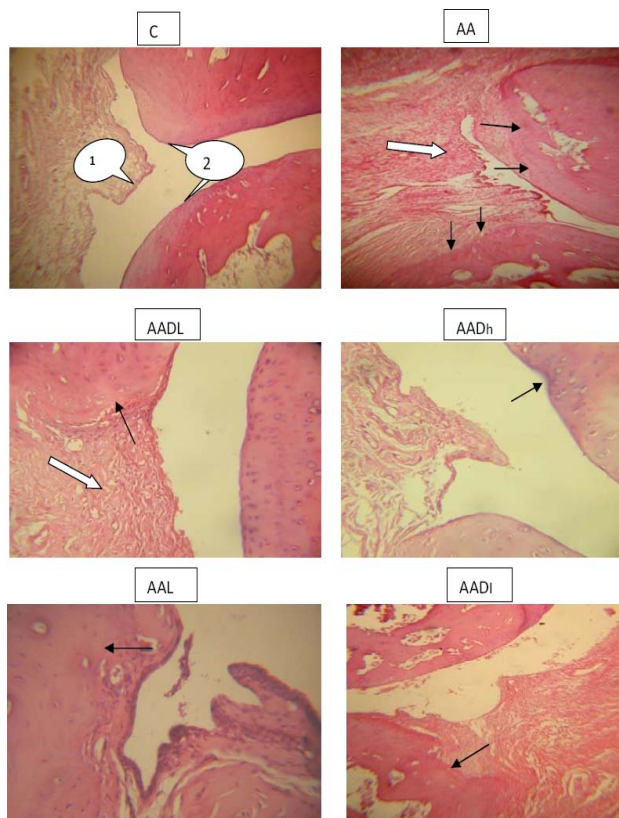
The injection of CFA induced significantly hyperplasia of the synovium, increased inflammatory cells infiltration to the synovium and severe cartilage erosion ( $P < 0.001$ ). While losartan alone did not significantly improve cartilage erosion and synovial hyperplasia, it reduced significantly the synovial inflammatory cells infiltration ( $P < 0.05$ ), in this experimental animal model, the combination of losartan with low dose of dexamethasone reduced all these parameters by more than 52% ( $p < 0.001$ ), and the efficacy of this combination was equal to the efficacy obtained by high dose dexamethasone ( $P > 0.05$ ).



To our knowledge, it's the first study that investigates the potential therapeutic effects of combination of losartan with low dose of dexamethasone on; ankle width changes, serum CRP levels, synovial inflammatory cells infiltration, synovial hyperplasia and cartilage erosion in adjuvant arthritis Wistar rats. The present study demonstrates that this combination improves significantly all these parameters. The higher significant increase in ankle joint width, serum CRP levels, synovial hyperplasia, synovial inflammatory cells infiltration, and cartilage erosion of the adjuvant arthritis group than that of control group are in agreement with previous reports <sup>20,21,22,23</sup>.

The determination of ankle joint width changes is an apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and the therapeutic efficacy of the drugs. Arthritic rats showed soft tissue swelling that was noticeable around ankle joints and was considered to be due to oedema of periarticular tissues such as ligament and joint capsule. Elevated levels of serum CRP have been found in the blood during virtually all diseases associated with active inflammation or tissue destruction, particularly in patients with rheumatoid arthritis<sup>24,25</sup>, and there is a significant correlation between serum CRP levels and rheumatoid arthritis disease activity<sup>26</sup>. In the present study the efficacy of combination of losartan with low dose of dexamethasone in reducing significantly the prominent ankle joint width, and CRP levels was equi-effective to that produced by high-dose of dexamethasone and even more effective than each of losartan or dexamethasone at low dose.

Furthermore, this combination reduced significantly synovial hyperplasia (53 %,  $P < 0.01$ ), synovial inflammatory cells infiltration and cartilage erosion (59 and 45%,  $P < 0.001$ ; respectively), and this combination was equi-effective to that obtained by dexamethasone at high dose ( $P > 0.05$ ).



**Figure 1:** Representative histopathology of ankle joints of experimental groups (C) Control rats, the note indicates the normal synovium (1), and normal cartilage (2). (AA) Adjuvant arthritic rats non-treated rats, white arrow indicates the severe inflammatory cells infiltration to the synovium, black arrows indicate the severe expansion of the inflamed synovium on the cartilage and severe cartilage injury. (AADL) Arthritic rats treated with losartan (15 mg/Kg/48h) in combination with low dose dexamethasone (0.025 mg/Kg/24h), the white arrow indicates the mild synovial inflammatory cells infiltration and the black arrow indicates the mild expansion of the synovium on the cartilage. (AADh) Arthritic rats treated with dexamethasone (0.05 mg/Kg/24h), the arrow indicates a mild injury in the cartilage surface in spite of the absence of synovial expansion on it. (AAL) Arthritic rats treated with losartan (15 mg/Kg/48h) the black arrow indicates the moderate expansion of the synovium on the cartilage; the white arrow indicates the hyperplasia of the synovium. (AADi) Arthritic rats treated with dexamethasone (0.025 mg/Kg/24h), arrow indicates the severe expansion of the synovium on the cartilage and the severe cartilage injury.

**Table 1:** Serum CRP levels and ankle joint width changes of experimental groups

	Group	Serum CRP (µg/ ml)	Ankle joint width changes (mm)
C	Control	207.98±12.88	0.06±0.09
AA	Arthritic	760.43±17.08 <sup>###</sup>	4.77±0.3 <sup>###</sup>
AAD <sub>h</sub>	AA+ Dexamethasone (0.05 mg/kg)	339.7±9.56 <sup>###, ***</sup>	2.00±0.29 <sup>###, ***</sup>
AAL	AA+ Losartan (15mg/kg)	570.3±21.64 <sup>###, ***, ...</sup>	3.13±0.19 <sup>###, ***, ...</sup>
AADL	AA+ Dexamethasone (0.025 mg/kg) + Losartan (15mg/kg)	353.02±6.73 <sup>###, ***</sup>	2.15±0.25 <sup>###, ***</sup>
AAD <sub>i</sub>	AA+ Dexamethasone (0.025 mg/kg)	558.03±15.99 <sup>###, ***, ...</sup>	3.18±0.26 <sup>###, ***, ...</sup>

Serum CRP levels and ankle joint width of experimental groups. All results were expressed as the mean ±SD. <sup>###</sup> Significant difference with respect to control group,  $P < 0.001$ . <sup>\*\*\*</sup> Significant difference with respect to arthritic group,  $P < 0.001$ . <sup>\*\*\*</sup> Significant difference with respect to combination (dexamethasone and losartan) group,  $P < 0.001$ .

**Table 2:** Ankle histology results of experimental groups

	Group	Synovial hyperplasia (0-2)	Inflammatory cells (0-3)	Cartilage erosion (0-3)	Total score (0-8)
C	Control	0.0±0.00	0.14±0.36	0.00±0.00	0.14±0.36
AA	Arthritic	1.67±0.49 <sup>###</sup>	2.60±0.51 <sup>###</sup>	2.6±0.63 <sup>###</sup>	6.87±1.3 <sup>###</sup>
AAD <sub>h</sub>	AA+ Dexamethasone (0.05 mg/kg)	0.73±0.36 <sup>**</sup>	1.0±0.35 <sup>#,***</sup>	1.27±0.47 <sup>#,***</sup>	3.00±1.36 <sup>#,***</sup>
AAL	AA+ Losartan (15mg/kg)	1.53±0.54 <sup>###,*</sup>	1.73±0.59 <sup>###,*</sup>	2.2±0.56 <sup>###,*</sup>	5.47±0.83 <sup>###,*</sup>
AADL	AA+ Dexamethasone (0.025 mg/kg) + Losartan (15mg/kg)	0.79±0.58 <sup>**</sup>	1.07±0.47 <sup>#,***</sup>	1.43±0.52 <sup>#,***</sup>	3.29±0.91 <sup>#,***</sup>
AAD <sub>i</sub>	AA+ Dexamethasone (0.025 mg/kg)	1.53±0.74 <sup>###,*</sup>	1.67±0.62 <sup>###,*</sup>	2.13±0.44 <sup>###,*</sup>	5.33±0.9 <sup>###,*</sup>

Histological was measured at end of the experiments. Results were expressed as mean ± SD. <sup>#</sup> Significant difference with respect to control group,  $P < 0.05$ . <sup>##</sup> Significant difference with respect to control group,  $P < 0.01$ . <sup>###</sup> Significant difference with respect to control group,  $P < 0.001$ . <sup>\*</sup> Significant difference with respect to arthritic group,  $P < 0.05$ . <sup>\*\*</sup> Significant difference with respect to arthritic group,  $P < 0.01$ . <sup>\*\*\*</sup> Significant difference with respect to arthritic group,  $P < 0.001$ . <sup>\*</sup> Significant difference with respect to combination (dexamethasone and losartan) group  $P < 0.05$ . <sup>\*\*\*</sup> Significant difference with respect to combination (dexamethasone and losartan) group,  $P < 0.001$ .

It is noteworthy that histological improvement in the present study was accompanied by improvement of ankle width changes and serum CRP levels. These results lead to suggestion of a synergistic effect between losartan and low doses of dexamethasone, but the mechanism by which the combined treatment of losartan and dexamethasone attenuates these parameters in this model need further studies.

In the inflamed RA joint the synovium is highly infiltrated by inflammatory cells and the intimal lining becomes hyperplastic owing to the increased number of synoviocytes which invade cartilage and bone structures, this process is mediated by number of cytokines, chemokines, cell adhesion molecules, matrix metalloproteinases, and free radicals released from inflammatory cells<sup>1</sup>. The powerful anti-inflammatory effects of GCs rest on their capacity to suppress the action of many proinflammatory mediators (cytokines, chemokines), and to increase the expression of many anti-inflammatory proteins (lipocortin1), through genomic actions (binding to GC receptors and interactions with glucocorticoid response elements in DNA), and these genomic effects are dose dependent<sup>27</sup>. This can explain the difference in the efficacy between low and high doses of dexamethasone that was clear in our study.

Renin angiotensin system (RAS) is a major regulator of blood pressure, but there is a great deal of evidence that angiotensin II plays a major role too in inflammation and autoimmunity through the inducing of the production of reactive oxygen species, adhesion molecules, inflammatory cytokines and chemokines<sup>28,29</sup>. In our study losartan reduced significantly the serum CRP levels and the synovial inflammatory cells infiltration, and this suggest the potential role of angiotensin II in inflammatory process in this model.

Previous studies have shown that there is a significant increase in AT1 receptor protein content in synovium from chronically inflamed rat knee joints induced by CFA, and that the administration of 15 mg/Kg/48 h of losartan at 16-day time point, similar to our study, substantially reduced knee joint swelling (51%,  $P < 0.001$ ) in rats, and this high dose of losartan was well tolerated by the animals and had negligible effects on arterial blood pressure<sup>7</sup>.

The adverse effects of GCs including an increased prevalence of hypertension, insulin resistance, and osteoporosis were frequently noted particularly at the high doses. The present combination which was evaluated in our study and was equi-effective to the high dose of dexamethasone, was associated with reducing the dose of dexamethasone and this may lead to reduce the severity of GCs side effects, in addition to the potential effects of losartan in counteracting these side effects depending on the previous finding about the potential therapeutic effects of ARBs in the attenuation of the development of insulin resistance and osteoporosis<sup>10,11,14</sup>.

Since the increased mortality in RA patients is mostly associated with cardiovascular disease, this new combination that we have suggested in this work will carry additional benefits through the therapeutic effects of ARBs in cardiovascular disease.

## CONCLUSION

Our results indicate that combination of losartan with low dose of dexamethasone may be a novel, safe, inexpensive, and effective therapeutic strategy in the treatment of rheumatoid arthritis.



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