Original Article

Fimasartan attenuates edema and systemic changes in egg albumin-induced paw inflammation in rats

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ABSTRACT

Angiotensin receptor blockers are well known for their therapeutic efficacy and fimasartan has been used safely and efficiently since 2010 in the treatment of hypertension. The study aimed to examine the anti-inflammatory effect of fimasartan in egg albumin-induced inflammation in rats. Rats were treated with diclofenac (25 mg/kg, intraperitoneally) or fimasartan at two different doses (3 or 10 mg/kg, intraperitoneally). The increase in the thickness of the paw was considered to be edema, which was measured using a vernier caliper. Serum was collected to analyze the systemic production of the inflammatory mediators TNF-α, and IL-6. The results showed that fimasartan in both doses significantly reduced edema in inflamed rat paws and produced a significant reduction in serum levels of TNF-α and IL-6, which increased significantly following egg albumin injection. Fimasartan effects were comparable to the effect produced by the standard anti-inflammatory drug diclofenac. Thus, the study confirms that egg albumin injection induces a systemic elevation in the levels of TNF-α and IL-6 and fimasartan was successful in reducing the rat paw edema size and inhibiting the production of proinflammatory mediators. In conclusion, fimasartan is a valuable anti-inflammatory agent in inflammatory conditions.

Keywords: Egg albumin, Paw edema, Fimasartan, Diclofenac, IL-6, TNF-α

Introduction

Inflammation is a complex process that plays a key role in homeostasis and regenerative processes [1]. It is involved in the acute phase reactions against harmful stimuli, like in conditions of tissue damage [2]. In such conditions, inflammation is a controlled process in which numerous cells and soluble factors interact with each other in an attempt to limit or prevent the spread of injury [3, 4]. However, when the inflammatory process becomes uncontrolled, it would contribute to the development of many complex disorders like autoimmune diseases and chronic inflammatory diseases [1, 5]. Many factors may become involved in the inflammatory process, among which is angiotensin II (Ang II). Ang II is the main peptide of the renin-angiotensin-aldosterone system (RAAS), and it can induce or potentiate inflammation via activation of the angiotensin II type 1 receptor (AT1R) [6]. AT1R is not abundant in normal conditions, but their expression becomes upregulated in stressful conditions like in patients suffering from heart failure and vascular injury. When bound to the AT1R, Ang II initiates a complex cascade of inflammatory reaction via induction of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases, reactive oxygen species (ROS), and nuclear factor-kappa-B (NF-κB) pathway, which mediates transcription and gene expression, and enhances chemokines and adhesion molecules, hence exerting a pro-inflammatory effect on leukocytes and vascular smooth muscle cells [7]. Thus, there is an increasing interest in RAAS modulating agents concerning their effects on inflammation, as these medications are among the most widely prescribed antihypertensives in old people who may suffer from disorders like atherosclerosis and arthritis, with a likely inflammatory etiology [8]. Importantly, a wide range of anti-inflammatory agents already exists, including NSAIDs and steroid classes of

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Najim SM, Abd MR, Fadhil AA, Hassan AF. Fimasartan attenuates edema and systemic changes in egg albumin-induced paw inflammation in rats. J Adv Pharm Educ Res. 2023;13(1):127-33. https://doi.org/10.51847/EdWlwyapjy

drugs. However, long-term treatment with these agents is not free of side effects, which predispose such patient population to an increased risk of developing or progression of other ailments [9, 10]. Therefore, safer alternatives are needed.

The acute inflammatory reaction involves two stages: vascular and cellular and could be reproduced in experimental animals [11]. Among the various available methods utilized for the screening of anti-inflammatory agents, one of the most commonly used is the ability of the tested compounds to inhibit the edema induced in the rats' paws upon injection of the phlogistic agent [12]. Paw edema induced by egg albumin is a highly reproducible and widely studied model of acute inflammation [13-15]. Edema, erythema, and hyperalgesia have been found to develop instantly following the injection of egg albumin into the rats' hind paw, due to the pro-inflammatory action of various mediators and ROS generated at the site of the tissue injury or by infiltrating cells [16].

Little information exists concerning the systemic effects of the subplantar injection of egg albumin. Of note, Carrageenan was considered a non-antigenic phlogistic agent that is free of any noticeable systemic effects [17]. However, increased serum levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were demonstrated in several previous investigations that utilized phlogistic-induced edema, including carrageenan injection [9, 16, 18]. Thus, it is rational to test the systemic reaction of the seemingly transient, localized acute inflammation following egg albumin injection.

Fimasartan is the ninth-developed angiotensin receptor blocker (ARB), which has a selective blocking activity at the AT1R with an insurmountable binding. It was developed and approved in South Korea for the management of hypertension and heart failure. Experimental data exists concerning fimasartan's ability to exert beneficial effects beyond its hemodynamic effects, including antioxidant and anti-inflammatory effects [19]. When examined in a mice model of carotid artery injury, fimasartan was able to ameliorate the hyperplastic changes in neointima by regulation of inflammatory and immune responses [20]. Similar outcomes were obtained in a double-blinded preclinical study, in which fimasartan administration reduced atherosclerosis plaque progression in rabbits' aorta via suppression of inflammation [21]. The high potency, excellent efficacy, minimum adverse effects profile, availability, and convenient cost, make ARBs like fimasartan an attractive candidate to be tested as a possible remedy for various inflammatory states. Many members of ARBs, namely: losartan, candesartan, irbesartan, and valsartan were found to possess anti-inflammatory activity in experimentally-induced paw edema in rats [22, 23]. Paradoxically, researchers have found that the local administration of losartan resulted in a significant enhancement of the edema in carrageenan-induced paw edema and the effect was dose-dependent [8].

The current study was designed to assess the possible anti-inflammatory activity of two doses of fimasartan in an egg albumin-induced paw edema model of acute inflammation in rats, in comparison to a standard anti-inflammatory drug.

Materials and Methods

Materials

Fimasartan potassium trihydrate was obtained from Novachemistry (Loughborough, UK). Diclofenac sodium was supplied from Hangzhou Hyper Chemicals Limited (Zhejiang, China). Hen's egg was purchased from a local market in Baghdad, Iraq. A rat tumor necrosis factor α (TNF-α) sandwich enzyme-linked immunosorbent assay (ELISA) kit was obtained from Shanghai YL Biont Co., Ltd. (Shanghai, China). Rat IL-6 sandwich ELISA kit was also used and obtained from Bioassay Technology Laboratory (BT Lab), Shanghai Korain Biotech Co Ltd. (Zhejiang, China). Dimethyl sulphoxide (DMSO) obtained from Thomas Baker (Chemicals) Pvt. Ltd. (Mumbai, India) and diethyl Ether (Romil Ltd., Cambridge, UK) were also used in the study. Solutions of 0.2% fimasartan potassium trihydrate and 0.5% diclofenac sodium were prepared on the day of the experiment by dissolving the drugs in a 4% DMSO solution [24]. The drugs' solutions were mixed properly by vortex mixer (LABINCO BV, the Netherlands) before use.

Experimental animals

Thirty adult Wistar rats (female), weighing 180-220 g, were used for the study. They were acquired from the Animal House at the College of the Pharmacy/University of Baghdad under conditions of the controlled temperature, humidity, and a twelve hours light/dark cycle. All rats have been acclimatized for one week before the experimental session to keep the stress levels low in order to obtain a good inflammatory response [25]. During the acclimatization period, they received a standard pellet diet and water *ad libitum* and were observed for any sign of sickness. The experimental procedures were conducted after approval from the University of Baghdad-College of Pharmacy Research Ethics committee according to the principles of Committee Office International des Epizooties' (OIE) on animal research.

Egg white-induced paw edema

The study was approved by the Scientific and Ethical Committees of the College of Pharmacy-University of Baghdad. After overnight fasting, twenty-four rats were grouped into four groups (n=6) and treated intraperitoneally as previously described [12]: Group I rats (positive control) received vehicle only (5 ml/kg) by intraperitoneal route [26], group II rats received diclofenac sodium intraperitoneally in a dose of 25 mg/kg [11, 27, 28], group III rats were given fimasartan intraperitoneally in a dose of 3 mg/kg [29], and group IV received fimasartan intraperitoneally in a dose of 10 mg/kg [30, 31]. Another group (negative control, group V) treated with an equivalent volume of vehicle as in the positive control group (5 ml/kg) intraperitoneally was also used for the TNF-α and IL-6 assay [9]. Thirty minutes after the treatment, inflammation was induced by injecting 0.1 ml of freshly taken hen's egg white (egg

albumin) subcutaneously into the subplantar region of the right hind paw of each rat in all groups (except the negative control group) [12]. The paw thickness was then measured with the aid of a digital vernier caliper (Kunshan Xy-Top Electronic Co., Ltd., Jiangsu, China) immediately before (0 hours) the injection of egg albumin and then at (0.5, 1, 2, 3, 4, and 5) hours thereafter [2, 25]. The intensity of edema has been assessed in terms of the mean increase in paw thickness (mm), which is determined by calculating the difference in paw thickness at "0 hours" and paw thickness at the respective hours [32]. The ability of the tested compound to suppress inflammation was then expressed as a percentage of inhibition of paw edema, which was calculated according to the following formula [27]:

$$Percentage\ of\ inhibition\ (\%) = 100 \times [1 - \frac{dt}{dc})] \quad (1)$$

Where (dt) represents the mean difference in paw thickness of treated animals after egg albumin injection and (dc) is the mean difference in paw thickness of control animals after egg albumin injection.

Sample collection

After measuring the mean increase in paw thickness at the fifth hour after egg albumin injection, blood samples were obtained by heart puncture under diethyl ether anesthesia. The obtained blood was then transferred into serum separator tubes, and allowed to stand for 30 minutes to clot. Then, to isolate sera, the clotted samples were centrifuged at 3000 rpm for 15 minutes using EBA 20® centrifuge (Andreas Hettich GmbH & Co. KG, Germany). The obtained sera were immediately collected and stored at −20°C until the day of analysis [33, 34].

Biochemical analysis

To determine whether egg albumin injection induced systemic inflammatory effects, the concentrations of IL-6 and TNF-α were quantified in the serum by sandwich ELISA method according to the kits manufacturers' instructions. For IL-6 determination, the microwell plate provided with the kit had been pre-coated with antibodies specific for rat IL-6 and the detecting antibodies are polyclonal antibodies labeled with biotin. The standard and serum samples were added into the wells and incubated at 37°C for 60 minutes. During this step, IL-6 is present in the samples bound to the wells by the pre-coated capture antibodies. Then, the ELISA plate was washed with phosphate buffer saline (PBS) and the biotinylated rat IL-6 antibody liquid was added to each well and incubated at 37°C for 60 minutes and was then washed to remove any unbound substance. Next, the avidin-peroxidase conjugate liquid was added to the wells, and again incubated at 37°C for 30 minutes and washed. Subsequently, the chromogenic substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated at 37°C in darkness until a blue color gradient appears. Finally, the enzymatic reaction was stopped by adding a stop solution which changes the color from blue to yellow. After that, a HumaReader HS® microplate reader (Human Diagnostics Worldwide GmbH, Wiesbaden, Germany) was used to read the ELISA microplate at 450 nanometers (nm) to measure the optical density (OD). The absorbance is proportional to the amount of IL-6 present in the sample [35]. The level of rat serum IL-6 was expressed as nanogram per liter (ng/L).

For TNF-α analysis, the method is the same as that of IL-6 determination except the microwell plate provided with the TNF-α kit was precoated with antibodies specific for rat TNF-α [35, 36]. The level of rat serum TNF-α was also expressed as nanogram per liter (ng/L).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Analysis was carried out using Statistical Package for Social Sciences (SPSS, version 25) software. Unpaired t-test and One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were used for the statistical analysis. Values were considered statistically significant at a *p*-value of less than 0.05 ($P<0.05$).

Results and Discussion

The present study showed edema formation (manifested as the characteristic increase in paw thickness) along with elevation in the serum levels of proinflammatory cytokines after the injection of egg albumin. As shown in **(Table 1 and Figure 1a)**, edema began to develop rapidly within thirty minutes and reaches its maximum at the first-hour post-injection in all egg albumin-injected rats (except diclofenac treated rats, which has maximum edema within thirty minutes after the induction of inflammation). These outcomes are in agreement with previous research, where egg albumin injection was reported to cause a significant, time-dependent increase in rat's paw thickness [2, 14, 15]. Edema is one of the fundamental parameters of acute inflammation and resulted from the release of many pro-inflammatory mediators from cells involved in the inflammatory response. The earliest derived mediators are histamine and serotonin, which mediate the initial phase (0-2 hours) of egg albumin-induced edema. The intermediate phase is governed by bradykinins, whereas the late (third) phase is regulated by the prostaglandins (PGs) synthesized by COX [13, 37]. The process is followed by the migration of leukocytes to the inflamed tissues which intensify the inflammatory response by releasing various mediators and toxic radicals [16, 38].

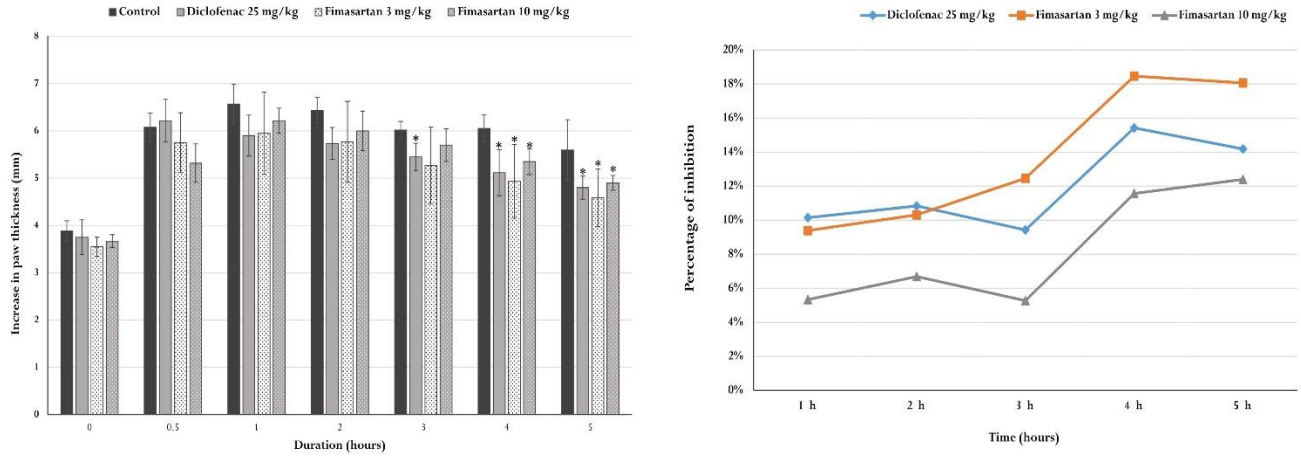
Both fimasartan doses produced a progressive reduction in paw edema starting at the second hour ($p>0.05$ for both groups) and reached a statistically significant reduction ($p<0.05$ for both groups) at the fourth and fifth hours in comparison to the positive control group. There were no significant differences ($p>0.05$) among the two tested doses of fimasartan. Treatment with the reference drug, diclofenac, decreased egg albumin-induced paw edema at the first and second hours nonsignificantly ($p>0.05$); but reached a statistically significant reduction ($p<0.05$) at 3, 4, and 5 hours after egg albumin injection, as compared to the

positive control group **(Table 1 and Figure 1a)**, indicating an earlier onset of action than fimasartan. Importantly, both doses of fimasartan showed nonsignificant changes in paw thickness compared with the standard NSAID, diclofenac. Additionally, the results showed that the maximum inhibition of the resultant edema occurred at the fourth hour by 3 mg/kg fimasartan (18.5%) followed by diclofenac (15.4%) and 10 mg/kg fimasartan (11.6%), as shown in **(Table 1 and Figure 1b)**. The effects of fimasartan on the edematous response may be due to its ability to impede PGs synthesis via inhibition of cyclooxygenase-1 (COX-1) or -2 (COX-2) because the egg albumin-induced edematous reaction in rats reflects PGs actions [9]. In agreement, fimasartan was found to suppress cyclooxygenase-2 (COX 2) expression in an *in vitro* study of hemolysate-induced inflammation in astrocytes [39], suggesting that fimasartan act on the late phase of egg albumin-induced paw edema.

Table 1. The Effect of control, diclofenac (as a standard), and fimasartan (3 and 10 mg/kg) on egg albumin-induced paw edema in rats.

Groups	Mean increase in paw thickness (mm)							% of inhibition					
	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h	0 h	1 h	2 h	3 h	4 h	5 h
Positive Control	3.883 ±0.214	6.076 ±0.301	6.567 ±0.417	6.430 ±0.2813	6.017 ±0.183	6.050 ±0.2865	5.5933 ±0.6437	--	--	--	--	--	--
Diclofenac 25 mg/kg	3.750 ±0.367	6.217 ±0.449	5.900 ±0.434	5.733 ±0.339	5.450 ±0.288*	5.117 ±0.488*	4.800 ±0.245*	--	10.15	10.8	9.4	15.4	14.2
Fimasartan 3 mg/kg	3.550 ±0.207	5.750 ±0.635	5.950 ±0.869	5.767 ±0.857	5.267 ±0.814	4.933 ±0.781*	4.583 ±0.611*	--	9.4	10.3	12.5	18.5	18.1
Fimasartan 10 mg/kg	3.667 ±0.137	5.317 ±0.407	6.217 ±0.264	6.000 ±0.420	5.700 ±0.346	5.350 ±0.274*	4.900 ±0.157*	--	5.3	6.7	5.3	11.6	12.4

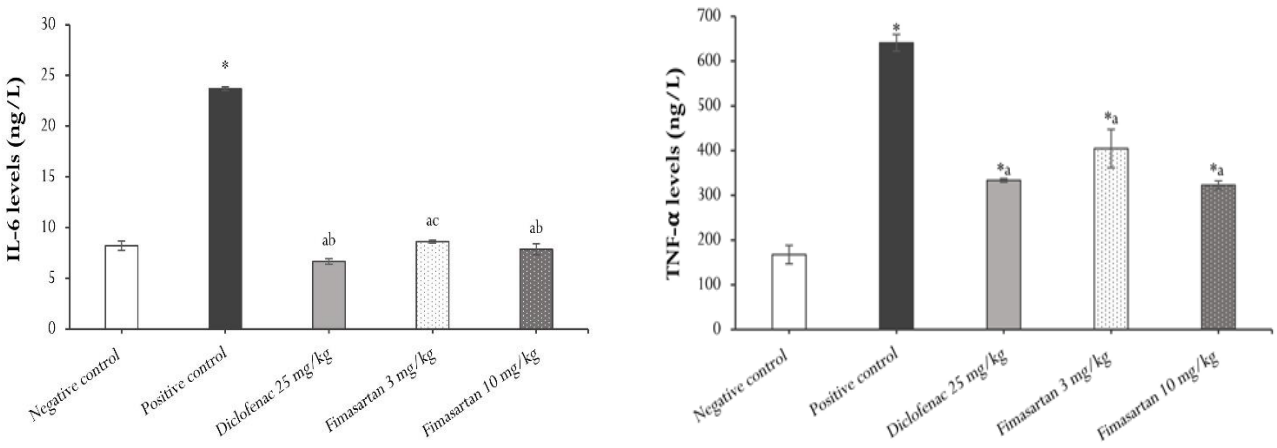
Data were expressed as mean ± SD; n=6; * P< 0.05 compared to the positive control group.



a) b)

Figure 1. a) The Effect of control, diclofenac (25 mg/kg), and fimasartan (3 and 10 mg/kg) on egg albumin-induced paw edema in rats. Data were expressed as mean ± SD; n=6; * P< 0.05 compared to the positive control group. b) The dose-dependent effect of fimasartan on egg albumin-induced acute inflammation in rats.

As shown in **(Figures 2a and 2b)**, serum levels of TNF-α and IL-6 were significantly elevated after egg albumin-induced inflammation compared to the negative control rats (p<0.05). The resultant systemic reaction to the locally-induced disturbance by egg albumin could be attributed to the responses of the injured tissue itself; which involve the production and release of pro-inflammatory cytokines, nitric oxide, and glucocorticoids as well as the activation of the vascular system and inflammatory cells [40]. These responses in turn are associated with the induction of COX-2 expression, and the production of more cytokines and other inflammatory mediators, including TNF-α and IL-6, which diffuse to the extracellular fluid and circulate in the blood [41].



a) b)

Figure 2. The Effect of control, diclofenac (25 mg/kg), and fimasartan (3 and 10 mg/kg) on the serum levels of a) IL-6; b) TNF-α following egg albumin-induced inflammation in rats. Data were expressed as mean ± SD, n=6.
(*) $P<0.05$ versus the negative control group; (a) $P<0.05$ versus the positive control group; Data with nonidentical small letter superscripts (b and c) were considered significantly different ($P<0.05$).

The study showed that pretreatment with diclofenac significantly reduced the serum levels of TNF-α and IL-6 in egg albumin-administered rats ($p<0.05$), **(Figures 2a and 2b)**. Diclofenac is known to act by inhibition of COX enzymes that are necessary for the production of PGs important in mediating cell–cell communication during inflammatory reactions [42]. However, diclofenac and related NSAIDs are not free of adverse consequences and their prolonged usage was found to result in many adverse effects including gastrointestinal, cardiac, and renal complications [9]. On the other hand, the regulatory role of RAAS modulators on inflammatory pathways has been extensively studied [43]. Aziz *et al.* (2020) reported that Aliskiren produced an attenuating effect on the granuloma, paw edema, and elevated serum TNF-α in a rat model of inflammation by directly inhibiting the rate-limiting enzyme of the RAAS, renin [44]. In the current study, preadministration of fimasartan at either dose (3 or 10 mg/kg) reduced the serum levels of IL-6 and TNF-α after egg albumin injection. Of note, the 10 mg/kg fimasartan dose produced similar effects to diclofenac in reducing serum IL-6 levels, with a nonsignificant difference between both treatment groups ($p>0.05$). In contrast, the reduction of serum IL-6 levels in the 3 mg/kg fimasartan was significantly lower ($p<0.05$) than in the diclofenac-treated group **(Figure 2a)**. On the other hand, the results observed with serum levels of TNF-α after both doses of fimasartan (3 or 10 mg/kg) were comparable to diclofenac (25 mg/kg), with nonsignificant differences between the three treatment groups ($p>0.05$) **(Figure 2b)**. Presumably, no previous work has reported the effects of egg albumin injection on systemic levels of IL-6 and TNF-α.

The findings in the current study were in line with previous investigations, which demonstrated the inhibitory effects of various ARBs, including fimasartan, in different inflammatory conditions. Yang and colleagues (2018) have found that fimasartan reduced the activation of NF-κB, a transcription factor that upregulates the expression of genes of many proinflammatory cytokines, including IL-6 and TNF-α [29]. Moreover, both Ang II and TNF-α have been recognized as inducers of COX-2, and blockade of Ang II signaling by fimasartan resulted in an inhibitory action on COX-2 expression, as described by Yang X-L *et al.* (2016) [39].

Conclusion

In conclusion, the present work showed that the locally produced edema in rat's paw via egg albumin injection was associated with systemic effects, evidenced by the elevated serum levels of the inflammatory cytokines IL-6 and TNF-α. Importantly, pretreatment with fimasartan has a significant inhibitory effect on the production of IL-6 and TNF-α and decreased the associated paw edema in rats. The observed results could be attributed to the inhibitory action of fimasartan on COX-2 and the subsequently released inflammatory mediators in the third phase of egg albumin-induced acute inflammation. However, detailed mechanistic studies are required to confirm the current results and to find out the extent of the anti-inflammatory actions in both normal and hypertensive animal models.

Acknowledgments: The authors are extremely grateful to the College of Pharmacy-University of Baghdad for supporting the study.

Conflict of interest: The authors declare that they have no conflicts of interest.

Financial support: None.

Ethics statement: All the experimental procedures were performed after the approval of the University of Baghdad-College of Pharmacy Research Ethics committee according to the principles of the Committee Office International des Epizooties' (OIE) on animal research.

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