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A comparative study of anti-inflammatory activity of lovastatin, simvastatin, atorvastatin and rosuvastatin on acute and chronic inflammation in animal models

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ABSTRACT

Objective: To study the anti-inflammatory activity of Lovastatin, Simvastatin, Atorvastatin, and Rosuvastatin on acute and chronic models of inflammation, to compare with the effect of Diclofenac sodium and amongst themselves in rats. **Methods:** Carrageenin induce rat paw edema method in which 5 animals of each group (6 groups) received orally 4% gum acacia, Diclofenac and 4 statins respectively 1 h before Carrageenin injection in paw. The paw edema volume measured with plethysmograph after 3 h and percentage inhibition of edema in various groups calculated. Rexin pellet granuloma method in which 4 rexin pellets were implanted into dorsum of skin of each rat of 6 groups (n=5) including control, Diclofenac and 4 statin groups respectively. Rats were orally fed with drugs daily for 7 days and on 8th day rexin pellets were removed after sacrificing the rat and dried in incubator 60oC overnight. Pellets were then weighed and percentage inhibition of granulation tissue was calculated and sent for histopathological examination. **Results:** All the 4 statins showed significant anti-inflammatory activity in the present study in both acute as well as chronic models of inflammation. The anti-inflammatory activity of the 4 statins was significant on comparison with Diclofenac. Lovastatin and Simvastatin demonstrated 10-20% more anti-inflammatory activity than Atorvastatin and Rosuvastatin. **Conclusions:** The present study revealed the anti-inflammatory effect of statins and thus suggests that the statins have a potential anti-atherosclerotic activity along with its lipid lowering property.

1. Introduction

Inflammation is a complex reaction in vascularized connective tissue, which is elicited by the same exogenous and endogenous stimuli causing cell injury. Although inflammation helps in clearing infection and wound healing, both inflammation and repair have tremendous potential to cause harm. Inflammation may contribute to a variety of disease that are not thought to be primarily due to abnormal host response. For instance, chronic inflammation may play a role in Atherosclerosis, Type -2 diabetes, Alzheimer disease and Cancer[1].

Atherosclerosis, which was earlier thought to be always associated with hypercholesterolemia, has now been proved as inflammatory disease[2]. 3-hydroxy 3-methyl glutaryl co-enzyme A(HMG Co-A) reductase inhibitors (*i.e.* Simvastatin, Lovastatin, Atorvastatin, Rosuvastatin, *etc*) commonly referred to as "statins" have been widel y used in the treatment of hyperlipidemia and coronary artery disease, by their actions of blocking the conversion of HMG-CoA to Mevalonate, the rate limiting step in the cholesterol biosynthesis[3]. Statins have recently been proved to possess various pleiotropic effects like anti-inflammatory[4], Immune-modulatory[5-7], Plaque stability[8,9], Endothelial dysfunction improvement[10-12]. Studies carried out on acute inflammatory model with Simvastatin[13] and Rosuvastatin[14] has shown significant anti-inflammatory effects of these statins. Atorvastatin and Lovastatin were shown to repress interferon- γ (IFN- γ) induced expression of major histocompatibility complex class II (MHC II) molecules on various cell types[15].

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Some studies have also shown that lipophilic statins like Lovastatin[16,17] and Atorvastatin[17] induce different pro inflammatory responses both in-vitro and in-vivo. Rosuvastatin has not shown any anti-inflammatory effect in one study[18].Decrease adhesiveness of monocytes to endothelium[19] , reduction of endothelial adhesion molecule *i.e.* intracellular adhesion molecule[20], decrease macrophages activation and proliferation[21].

Available literature suggests that most of the statins have anti-inflammatory effects, but a comparison among them regarding their anti-inflammatory activity is not reported. Hence the present study was planned to study and compare the anti-inflammatory effects of statins and to prove or disprove the controversial reports regarding their anti-inflammatory activity.

2. Materials and methods

Ethical clearance was obtained from Institutional Ethics Committee M.R.Medical College, Gulbarga. In the present study, Statins like Lovastatin, Simvastatin, Atorvastatin, and Rosuvastatin are evaluated for their anti-inflammatory activity. The results obtained from these statins were compared with known anti-inflammatory agent Diclofenac sodium.

2.1.Materials

Diclofenac sodium. It is an aryl acetic acid derivative; pure form was obtained from Amoli Organics Pvt. Ltd., Vapi, Gujarat.

Statins are white to off-white crystalline powders. Pure forms were obtained from Biocon Limited, Bangalore.

Carrageenin: Carrageenin is obtained from Mulberry Chemicals Private limited, Mumbai. (Irish Sea moss is a mixture of polysaccharides composed of sulfated GA lactose unit). Plethysmograph is obtained from INCO, Ambala.

2.2.Study centre

The study was carried out in the Post graduate research laboratory, Department of Pharmacology, Mahadevappa Rampure Medical College, Gulbarga, Karnataka.

Albino rats obtained from the National Institute of Nutrition, Hyderabad, and maintained at the Central animal house of M.R.Medical College Gulbarga, were used for the study. Adult albino rats (Wister strain) of either sex weighing about 150-200 g, maintained at room temperature of (25±2) ℃ , in a well-ventilated animal house under natural photo period conditions were used for the study. They were provided with standard diet and water ad-libitum.

2.3. Methods

2.3.1. Rat paw edema method

Albino rats of either sex weighing 150-200g were used. They were grouped in batches of 5 animals[22]. Total 30 rats were selected and they were divided into 6 groups of5in each. The first group served as control and received 4% gum acacia suspension (without drug) by mouth. The remaining groups received drugs prepared in 4% gum acacia as the following:

Group 1 (Control): 4% Gum acacia 2 mL/kg.
Group 2 (Standard drug): Diclofenac sodium (4.5 mg/kg) in 4% Gum acacia.
Group 3 (Test drug): Lovastatin (7.2 mg/kg) in 4% Gum acacia.
Group 4 (Test drug): Simvastatin (7.2 mg/kg) in 4% Gum acacia.
Group 5 (Test drug): Atorvastatin calcium (7.2 mg/kg) in 4% Gum acacia.
Group 6 (Test drug): Rosuvastatin calcium (3.6 mg/kg) in 4% Gum acacia.

All the drugs were administered orally followed by constant volume of distilled water after each administration to ensure the entry of drug. One h after feeding, each rat is anaesthetized with ether and under anesthesia 0.1 mL of 1% Carrageenin is injected into sub-plantar region of the hind paw of the rat and the volume of paw is measured. Volume of edema is recorded at the end of 3 h after Carrageenin administration. Same procedure was adopted for rats of all the groups.

The percent inhibition of edema in drug treated rats (standard and test drugs) is calculated by using the formula:

$$\text{Percent inhibition} = \frac{Vc - Vt}{Vt} \times 100$$

Where,
Vc= Volume of paw edema in control animals
Vt= Volume of paw edema in drug treated animals.
The dose of the drug under study was calculated by using the dose conversion table (Table 1)[23].

Table 1
Dose conversion table.

Drugs	Human (70kg)	Conversion factor	Dose for rat (mg)	
	Dose (mg)	Rat	For 200 g	Per kg body weight (100 g)
Diclofenac sodium	50	0.018	0.9	4.5
Lovastatin	80	0.018	1.44	7.2
Simvastatin	80	0.018	1.44	7.2
Atorvastatin calcium	80	0.018	1.44	7.2
Rosuvastatin	40	0.018	0.72	3.6

2.3.2. Rexin pellet granuloma method

Discs of equal size and weight were punched out from rexin sheet[24]. Two such discs were stitched together with their rough surface exposed outside and rexin covered surfaces facing each other. Rexin pellets were sterilized using 70% ethyl alcohol. Adult albino rats, 30 in number of either sex weighing about 150 to 200 g, were selected and divided into 6 groups of 5 animals each. The first group served as a control and was given 4% gum acacia orally. The remaining groups received following drugs in 4% gum acacia suspension. The dose and route of administration is same as that of rat paw edema method.

All the rats were anaesthetized with ether. The dorsal skin was shaved and alcohol was applied to maintain aseptic condition. On either side of midline of dorsal skin, four small incisions of about 1cm length were made. A curved forceps was passed through incisions to make subcutaneous pouch around it. Similarly, four such pouches were made and a sterilized rexin pellets were implanted into each pouch. An

extra rexin pellet was implanted in one animal of each group to be used for histopathological study. The incision was sutured with sterilized cotton thread and tincture benzoin was applied to prevent any contamination.

All the rats were treated with fixed dose of drugs (as mentioned above) once in every 24 h for seven days including the day of implantation of pellets. The animals were provided with free excess of food and water. During seven days, the rats were observed for any behavioral changes. On the 8th day, the implanted pellets along with granulation tissue were removed. All the pellets were cleaned separately, extraneous tissue removed and dried by incubating in hot air oven at 600 for 24 h. Net granuloma formation was calculated by subtracting initial weight of rexin pellet (17 mg) from the weights noted. The mean weight of granulation tissue for each group was calculated. The difference in weight of granulation tissue of controlled group and drug treated group was made out. The percent inhibition was calculated by using the following formula:

$$\text{Percent inhibition} = \frac{Wc - Wt}{Wc} \times 100$$

Where,
Wc=Weight of pellets in control group.
Wt=Weight of pellets in drug treated group.

Extra rexin pellet implanted in one rat of each group was removed along with the granulation tissue on the 8th day after sacrificing the rat. The pellets was preserved in 10% formalin, and sent for histopathological examination. The specimens of control, standard, and test groups that included different rexin pellet subcutaneous implants were carefully excised, fixed in 10% buffer formalin, properly grossed and processed for paraffin section. Sections were cut at thickness of 5 microns by a rotary microtome. Slides were stained with standard hematoxylin and eosin stain. Stained sections were evaluated for inflammatory exudate, various inflammatory cells and granulation tissue. Results were correlated with other experimental parameters.

2.3.3. Leucocyte immigration in rat paws edema

Procedure: Seven albino rats of either sex weighing 150-200 g were categorized into seven batches, as already mentioned in the rat paw edema model with normal (not treated with any drug or Carrageenin)[25-27]. The rats of category 2-7 were given oral feeding as described above. After one h, rat's category 2-7 was anesthetized with ether, and 0.1mL of Carrageenin was injected into sub-plantar region of right hand paw of rat. 6 hs later, skin of the plantar region of rats from category 1-7 were excised aseptically under ether anesthesia and animals were sacrificed. Excised tissue from each category was preserved in 10% formalin, and sent form histopathological examination.

2.4. Histopathological study

Stained sections were carefully examined for edema, acute inflammatory exudate and various inflammatory cells. Results obtained from the histopathological study were correlated with other experimental parameter.

2.5. Statistical analysis

Statistical analysis of experimental data was done by Student's *t* test and ANOVA (one way analysis of variance).

3. Results

3.1. Results of rat paw edema method

The results obtained from the standard and test drugs shown in Figure 1. The percent inhibition of edema in rats treated with Diclofenac sodium, Lovastatin, Simvastatin, Atorvastatin and Rosuvastatin is calculated with a reference to the control group. The percent inhibition of edema at the end of 3 h, with Diclofenac sodium was 63.46%, whereas with Lovastatin 38.46%, Simvastatin 28.80%, Atorvastatin 19.20% and Rosuvastatin 17.30%.

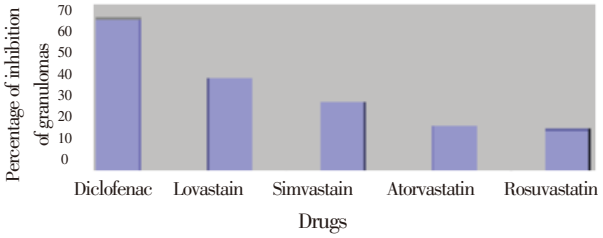

Figure 1. Carrageenin induced rat paw edema (acute study).

On comparison of anti-inflammatory effects of standard and test drugs against control, the anti-inflammatory activity was highly significant with Diclofenac sodium ($P<0.001$) and significant with Lovastatin ($P<0.01$), Simvastatin ($P<0.02$), Atorvastatin ($P<0.05$) and Rosuvastatin ($P<0.05$).

On comparison of anti-inflammatory effects of test drugs with that of standard, the anti-inflammatory activity was significant with Lovastatin ($P<0.02$), Simvastatin ($P<0.01$) and highly significant with Atorvastatin ($P<0.001$) and Rosuvastatin ($P<0.001$).

The application of one way analysis of variance (ANOVA) showed that the anti-inflammatory effects of the four test drugs differed significantly ($F_{3,16}=5.83$, $P<0.05$). The comparison of the anti-inflammatory effects of the 4 statins among themselves is shown in the Table 2.

3.2. Results of Rexin pellet granuloma method

Percent inhibition of granuloma formation was determined by weighing the rexin pellet after 7 days of their implantation in the subcutaneous tissue. Diclofenac sodium has shown 63.42% inhibition of granuloma formation followed by Lovastatin 43.28%, Simvastatin 31.04%, Atorvastatin 19.12% and Rosuvastatin 16.77%.

Table 2
Comparison of anti-inflammatory effects of statin.

Test drugs	Acute study		Chronic study	
	t-value	*P*-value	*t*-value	*P*-value
Lovastatin *vs.* Simvastatin	1.58	>0.05	3.46	<0.01
Lovastatin *vs.* Atorvastatin	2.94	<0.02	7.86	<0.001
Lovastatin *vs.* Rosuvastatin	3.55	>0.01	7.0	<0.001
Simvastatin *vs.* Atorvastatin	1.66	>0.05	3.38	<0.01
Simvastatin *vs.* Rosuvastatin	2.5	<0.05	3.37	<0.01
Atorvastatin *vs.* Rosuvastatin	0.38	>0.05	0.66	>0.05

When compared with control, Diclofenac sodium (P<0.001) Lovastatin (P<0.001) and simvastatin (P<0.001) showed highly significant anti-inflammatory activity whereas Atorvastatin (P<0.01) and Rosuvastatin (P<0.02) showed significant anti-inflammatory activity.

On comparison of anti-inflammatory effects of test drugs with that of standard, the anti-inflammatory activity was highly significant with Lovastatin (P<0.001), Simvastatin (P<0.001), Atorvastatin (P<0.01) and Rosuvastatin (P<0.001). The difference in the anti-inflammatory effects of the four test drugs was found to be highly significant on application of ANOVA ($F_{3,16}$=23.21, P<0.001).

3.3. Histopathological study

Control group immediately surrounding the pellets showed many acute inflammatory cells, many neutrophils, few monocytes, lymphocytes and occasional eosinophil. There was a good amount of granulation tissue in the periphery consisting of many capillaries, fibroblasts and variable collagen.

Diclofenac sodium group showed marked decrease in inflammatory cells like neutrophils, monocytes, lymphocytes and eosinophils immediately surrounding the pellet. Granulation tissue was decreased to around 50%-60% in study under various low and high power fields, when compared to the control. This indicated that Diclofenac sodium exerted good anti-inflammatory activity.

Lovastatin and Simvastatin groups showed moderate decrease in inflammatory cells like neutrophils, monocytes and eosinophil's in the fields nearest to the pellet. There was a decrease in granulation tissue to about 30%-40% in study under various low and high power fields on comparison with the controls. This showed that Lovastatin and Simvastatin revealed anti-inflammatory activity but slightly lesser than the standard drug (Diclofenac sodium) (Figure 2A, 2B).

Atorvastatin and Rosuvastatin groups showed slight decrease in inflammatory cells like neutrophils, monocytes, lymphocytes and eosinophil's. Around 5%-10% decrease in granulation tissue was observed under various low and high power fields, when compared with the control. This correlated with other experimental parameters to suggest that Atorvastatin and Rosuvastatin have little anti-inflammatory activity (Figure 2C, 2D).

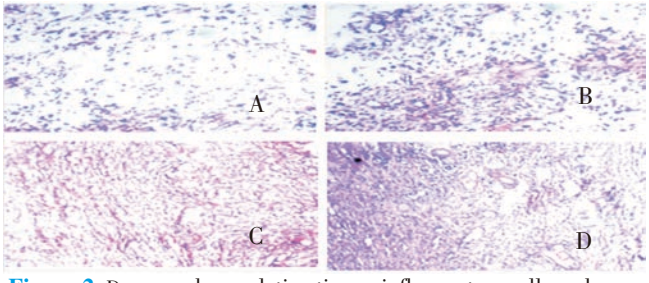

Figure 2. Decreased granulation tissue, inflammatory cells and fibroblasts.
A: Lovastatin; B: Simvastatin; C: Atorvastatin; D: Rosuvastatin.

The dermis of normal skin showed collagenous tissue and subcutaneous tissue showed sub epithelial glands. There was no edema or inflammatory cells.

Control group showed predominantly neutrophils, few macrophages and dense fibro-collagenous tissue present in the deep.

Diclofenac sodium showed minimal edema few inflammatory cells like neutrophils and macrophages. There was a decrease in edema and inflammatory cells to around 50%-60%.

Lovastatin and Simvastatin: Slides from both the groups revealed slightly less edema and inflammatory cells like neutrophils and macrophages as compared to the control group. Edema and inflammatory infiltration was decreased to about 30%-40% in both high and low power fields when compared with the control. This indicated that Lovastatin and Simvastatin have a lesser anti-inflammatory activity than Diclofenac sodium (Figure 3A, 3B).

Atorvastatin and Rosuvastatin: Slides from both the groups showed more of edema and inflammatory cells like neutrophils and macrophages resembling the control group. Around 5-10% decrease in edema and inflammatory cells was observed in low and high power fields when compared to control. This suggests that Atorvastatin and Rosuvastatin exhibit significant anti-inflammatory activity (Figure 3C, 3D).

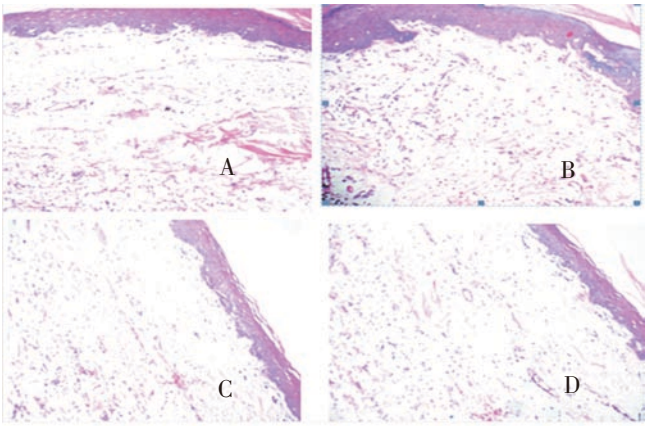

Figure 3: Decreased edema and inflammatory cells like neutrophils and macrophages.
A: Lovastatin; B: Simvastatin; C: Atorvastatin; D: Rosuvastatin.

4. Discussion

The discovery of the pleiotropic effects of statins like anti-inflammatory and Immune-modulatory activity by various clinical and experimental observations, have provided a new scope for these compounds to be tried as therapeutic agents for treatment of chronic immune mediated inflammatory diseases.

The present study was planned to investigate and compare the anti-inflammatory effect of statins like Lovastatin, Simvastatin, Atorvastatin and Rosuvastatin and to prove or disprove the controversial reports regarding their anti-inflammatory activity, as already described in the introduction.

The results obtained from the present study reviewed that Lovastatin, Simvastatin, Atorvastatin and Rosuvastatin exhibited significant anti-inflammatory activity in acute as well as chronic models of inflammation. On comparison of the test drug with that of the standard (Diclofenac sodium), all the four statins used in the study showed significant

anti- inflammatory activity in acute and chronic models of inflammation. Lovastatin, Simvastatin exhibited around 10%-20% more anti-inflammatory activity than Atorvastatin and Rosuvastatin.

The findings obtained from the present study are in agreement with the earlier reports[4,8], but do not agree with the other reports which state that the statins have pro-inflammatory activity[16,17] or no inflammatory activity[18]. *In vitro* studies using mouse monocytes[17], human monocytes[17] and human umbilical vein endothelial cells16 revealed the pro inflammatory activity of statin (Lovastatin, Simvastatin and Atorvastatin *etc*.) The pro-inflammatory activity which was observed in the earlier studies was due to increased infiltration of macrophages , neutrophils in the mouse peritoneal cavity and increased the production of inflammatory cytokines (TNF α , IL-1 β , MCP-1, IL, hv in mouse monocyte cells culture and super induction of E-selectin, ICAM-1, VCAM-1 in cultured human umbilical vein endothelial cells. The variation in the results obtained in our study was mainly due to the difference in species and the experimental methods followed. Similarly experimental condition and variation in species would explain the inefficacy of Rosuvastatin as an anti- inflammatory agent 18.

The observation obtained from the present study does not help to propose the mechanism of anti-inflammatory actions of statins. However, the mechanisms have been proposed by several earlier reports. Lovastatin has been shown to decrease monocyte chemotaxis by interfering with monocyte chemotactic protein (MCP-I)[28] and decreases m-RNA for Cyclooxygenase-II[29]. Lovastatin has also been reported to inhibit expression of inducible NOS and pro-inflammatory cytokines, like TNF α , IL-1 β , and IL-6 in macrophages[30].

Simvastatin has been speculated to decrease m-RNA for cyclooxygenase-II and has also been shown to decrease T-cell proliferation31, IL-8[31], and IL-6[32].

Atorvastatin and Lovastatin were shown to decrease INF - γ induced expression of MHC II molecules on various cell types[15]. Atorvastatin has been proposed to modulate adhesion by interfering mainly with nuclear factor-k β pathway[33] which is activated by Rho GTPase and plays an important role in transcriptional regulation of cytokines, chemokines, adhesion molecules and acute phase proteins such as CRP. Statin decreases the risk of CHD and levels of CRP, an independent marker for inflammation and high CHD risk[34,35]. It also has been shown to decrease P-Selectin expression on platelets[36]. Atorvastatin has been reported to abolish arterial macrophage infiltration and MCP-I[37] like Lovastatin.

Rosuvastatin has shown to preserve e-NOS protein in ischemic perfusion injury which inhibits inflammation[14]. 20 mg of Rosuvastatin daily for 1.9 years was compared with placebo in JUPITER trial which demonstrated the reduction in venous thromboembolism[38].

It has also been reported to attenuate thrombin induced leucocyte rolling adhesion, transmigration, and to down regulate P-selectin[39]. Statin therapy enhances endothelial production of the vasodilator nitric oxide, leading to improved endothelial function.

Atherosclerosis is clearly an inflammatory disease[40]. Results obtained from all the 4 statins used in the present study, support the hypothesis that statins have anti-inflammatory activity, which is relevant for prevention of atherosclerosis by these drugs. Further research may lead to new understanding of the actions of statins and new therapeutic interventions for atherosclerosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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