Pitavastatin is a potent anti-inflammatory agent in the rat paw model of acute inflammation

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Abstract: Statins are used extensively as anti-hyperlipidemic agents. In addition to curtailing cholesterol synthesis they have been found to have multiple actions unrelated to cholesterol lowering" the pleiotropic effects," which includes inhibition of inflammation. We aimed at investigating the effect of pitavastatin a 3rd generation statin, in suppressing acute inflammation in rat paw edema model. Male Sprague-Dawley rats were randomly assigned to one of five groups (n=8): Control, indomethacin and pitavastatin (0.2mg/kg, 0.4mg/kg, 0.8mg/kg) treated. 1hour following treatment, inflammation was induced by sub-planter injection of egg albumin into the hind paw. Anti-inflammatory effect was evaluated by measurement of edema formation every half hour for three hours, assessment of polymorphonuclear leukocyte (PMNL) infiltration and measurement of tissue damage in skin biopsies. Ascending doses of pitavastatin were found to attenuate these parameters. The lowest dose of pitavastatin (0.2mg/kg) was found to significantly reduce edema volume, PMNL infiltration and tissue damage. The efficacy of the smallest dose was found comparable to indomethacin.

Keywords: Acute inflammation, pitavastatin, rat foot pad edema, pleiotropic effects.

INTRODUCTION

HMG-CoA reductase inhibitors or statins are extensively used worldwide as anti-hyperlipidemic drugs for primary (Sever *et al*., 2003) and secondary (Vrecer *et al*., 2003) prevention of cardio-vascular disorders. They inhibit the initial reaction in synthesis of cholesterol from mevalonate particularly in the liver. However, other products derived from mevalonic acid, originating further down-stream would invariably be curtailed. These include isoprenoids farnesyl pyrophosphate (FPP) and geranyl geranyl pyrophosphate (GGPP), which play important part in cell membrane attachment and intra-cellular localization of small GTP binding proteins and hetero-trimeric G proteins. These proteins play vital roles in cellular transduction pathways involved in cellular functions like growth and proliferation, apoptosis, cell motility etc (James, 2002). Such activities produced independently of cholesterol lowering have been labeled "pleiotropic effects" of statins.

The efficacy of statins as anti-atherosclerotic agents is based not just on a simple lowering of serum cholesterol. Evidence of angiographic regression of plaques appears earlier than a significant reduction of cholesterol and the beneficial effect in reducing risk of coronary heart disease is disproportionately greater than would be expected form the magnitude of cholesterol reduction (WOSCOP group, 1998). Contemporary views attribute inflammation as a strong component of atherosclerosis. Statins have been shown to reduce C-reactive protein, interleukin-1, interleukin-6, and tumor necrosis factor-α, levels and reduce migration of inflammatory cells into inflamed tissues (Ascer *et al*, 2004; Rezaie-Majd *et al*., 2002). Investigators have also demonstrated an anti-oxidant effect due to reduced isoprenylation of NADPH, which in the isoprenylated form generates superoxide ion (Christ *et al*., 2002). Consequently the use of statins was expanded for applications in conditions such as rheumatoid arthritis (McCarey *et al*., 2004), pneumonia (Floyd *et al*., 2007), and sepsis (Daniel *et al.* 2006).

Pitavastatin the newest member of the statin family has some unique characteristics. It has good lipid solubility (N-octanol: water partition coefficient =1.49) hence is able to easily penetrate cell membranes. It is well absorbed following oral administration. Metabolism by CYP enzymes is minimal and therefore it has maximal bioavailability (80%) compared to other statins (Ose, 2010). High level of pitavastatin in the systemic circulation makes it a favorable candidate to explore effects on peripheral tissues.

In this study, we aimed to evaluate the effects of pitavastatin in acute inflammation and to test its efficacy against a strong anti-inflammatory drug like indomethacin. We used the rat paw model of inflammation, which is a well-established model for screening of anti-inflammatory agents. It also aimed to assess the dose of pitavastatin capable of demonstrating anti-inflammatory activity.

MATERIALS AND METHOD

The study was performed in BMSI, Jinnah Post-graduate Medical Center, Karachi. For the use of experimental animals, An approvable was taken from Animal Ethics

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Animals

The anti-inflammatory activity of pitavastatin was evaluated as described previously (Ojewole, 2004). Male Sprague-Dawley rats weighing 250-300 grams, aged 3 months were used. The animals were housed in cages (4 animals per cage) with *ad libitum* access to food and water. On the day prior to the experiment the rats were fasted overnight with free access to water only.

Drugs

Pitavastatin tablets (4mg) Livalo Japan. Pitavastatin suspension (0.4mg/ml) was prepared by grinding a tablet and preparing a suspension in distilled water making the volume up-to 10ml.

Indomethacin powder (Sigma Chemicals): Indomethacin (1mg/ml) was prepared by dissolving 10mgs of the powder making the volume up-to 10ml with distilled water. Undiluted fresh egg albumin (0.1ml) was used for inducing inflammation.

Calculation of drug doses

Drug doses for rats were calculated by method of conversion from human doses to animal doses (Sharma and Mc Neil, 2009) based on dosage conversion factor, which is 6.2 for conversion of human to rats. Human dose range for pitavastatin in 60kg adult is 2-4mg/day
Human dose 2mg/60 kg=0.03 mg/kg
Equivalent dose in rats=0.03 × 6.2=0.186mg/kg or approx. 0.2mgs/kg (Group P)
Human dose 4 mgs/kg=0.4mg/kg in rats (Group 2P)
Human dose 8mgs/ kg=0.8 mg/kg in rats (Group 4P)
Each animal was weighed for dose calculation

Animal groups

Rats were randomly allotted to one of the five groups.
Control Group (n=8) received 0.1ml of distilled water by oral tube.
P Group (n=8) received pitavastatin (0.2mg/kg) suspension orally.
2P Group (n=8) received pitavastatin (0.4mg/kg) orally.
4P Group (n=8) received pitavastatin (0.8mg/kg) orally.
I Group (n=8) received indomethacin (10mg/kg) orally.

Procedure for egg albumin-induced inflammation of rat hind paw

A mark was made by a permanent fine tipped marker just above the malleolus of right hind paw. The volume of the paw at baseline (0hr) was measured using IITC Life Science Plethysmometer for mice and rats. The animals were pretreated according to the group allocated, with the drugs given through an oral feeding tube, 1 hour before induction of inflammation. All rats were injected with sub-planter injection of fresh egg albumin (0.1ml) into the right footpad. Paw volume was measured every 30 minutes up to three hours. Preliminary experiments showed that inflammatory response starts to develop in about 20 min and reaches maximum in 2.5 to 3 hours, thereafter a decrease in intensity was observed.

Assessment of edematous exudates

Mean edema volume (ml)
was calculated for each group at 0.5hr, 1hr, 1.5 hr, 2 hr, 2.5 hr and 3 hr.
EV_t (ml) =. Vt-Vo
(EV_t is the edema volume at time t, Vo is the pre-injection paw volume, and V_t is the paw volume at time t after egg-albumin injection).

% inhibition of inflammation =
Mean EV_t Control group – Mean EV_t Treated group/ Mean EV_t Control group × 100
The volumes were measured at the same point in time (t) after egg albumin injection.

Histopathological study

After completion of the experiment the rats were sacrificed by exsanguination. Right paw was removed at the level of the lateral malleolus and fixed in 10% neutral buffered formaldehyde. 7 days after fixation, sub-planter skin samples were divided into 5 portions for preparation of tissue sections. The samples were dehydrated in graded alcohol (100%, 96%, 70%), xylol and embedded in paraffin blocks. 2-µm thick paraffin sections were stained with hematoxylin and eosin method. From each specimen, whole visual fields magnified 40X by using a light microscope.

Semi-quantitative evaluation of tissue damage

The severity of tissue damage was evaluated in five randomly selected fields in each animal under magnification 40X. The severity was graded according to the tissue damage score (TDS) on a scale 0-4 as described by Nezić *et al*. (2009).
0 = normal findings
1+ = mild damage (mid dilation of blood vessels with no changes in wall continuity. A few foci of inflammatory cell infiltrates)
2+ = moderate damage (discrete edema and hyperemia, various number of inflammatory infiltrates)
3+ = severe and focal damages (increased blood volume and vasodilation associated with extensive hyperemia, edema and accumulation of inflammatory cells)
4+ = severe and diffuse damages (strong vasodilation with erythrocyte accumulation (stasis) associated with massive hyperemia and edema; intensive accumulation of inflammatory cells).

Polymorphonuclear leukocyte count

The number of PMNL was counted in five random high-power fields in each sample using a light microscope. Areas of hemorrhages were excluded from the count.

STATISTICAL ANALYSIS

The data was analyzed using SPSS version 19. Results are shown as mean ±SD. The data was subjected to One Way ANOVA. Post hoc analysis using the Scheffe's multiple comparison was performed to compare mean values between treatment groups and control. Differences between the groups were considered significant at p-value <0.05.

RESULTS

Effect of pitavastatin on egg albumin-induced paw edema

As shown in table 1 and fig. 1, pitavastatin given orally in dose range 0.2mg/kg (low dose) -0.4mg/kg (middle dose) -0.8mg/kg (high dose), 1 hr before egg albumin challenge inhibited edema formation in a dose-dependent manner.

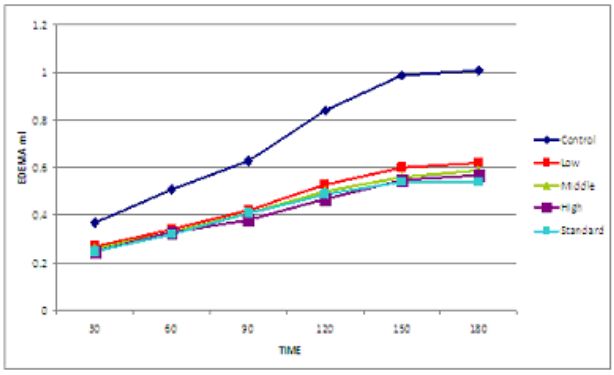


Fig. 1: Comparison of edema volume at different time intervals across groups

The mean edema volume in control group increased progressively from 0.37ml±0.058 at 30min to 1.01ml±0.11 at 3hours.

Treatment with pitavastatin (0.2mg/kg) 1hr prior to albumin challenge produced edema of 0.27ml±0.05 at 30mins, which was not a significant reduction over the control value (p value>0.05). However significantly less edema of 0.34ml±0.06 was observed at 1 hour (p=0.009). Similarly at 3hours the edema volume 0.62ml±0.08 was a significantly lower in comparison to control (p=0.000). Doubling the dose of pitavastatin caused a reduction in edema to 0.26ml±0.07 and 0.25ml±0.067 at 30mins and 0.59ml±0.07, 0.57ml±0.09 at 3 hours with the middle and high doses of pitavastatin respectively. This was a significant reduction in comparison to control. However there was no significant difference in edema volumes between the three dose levels of pitavastatin.

Prior treatment with indomethacin markedly inhibited paw swelling from 0.25ml±0.04 at 30 mins and 0.54±0.07 at 3 hrs in comparison to control group (p value <0.05) but this volume was not significantly different compared to any of the three-pitavastatin doses.

When calculated as % reduction of edema volume, administration of low, middle and high doses of pitavastatin increasingly inhibited edema from 27.02% to 29.73% to 34.43% at 30mins respectively (table 1). The same doses caused a greater inhibition of edema formation i.e. 38.61% to 41.58% to 43.56% at the 3rd hr. This was highly comparable to the effect of indomethacin showing 32.43% reduction at 30mins and 46.53% reduction at the 3rd hour. The maximal effect of pitavastatin was observed at 2.5 hours, showing 39.39%, 43.43%, 44.44% edema reduction with the low, middle and high doses respectively. Maximum effect of indomethacin at 3 hrs was 46.53% reduction in footpad swelling.

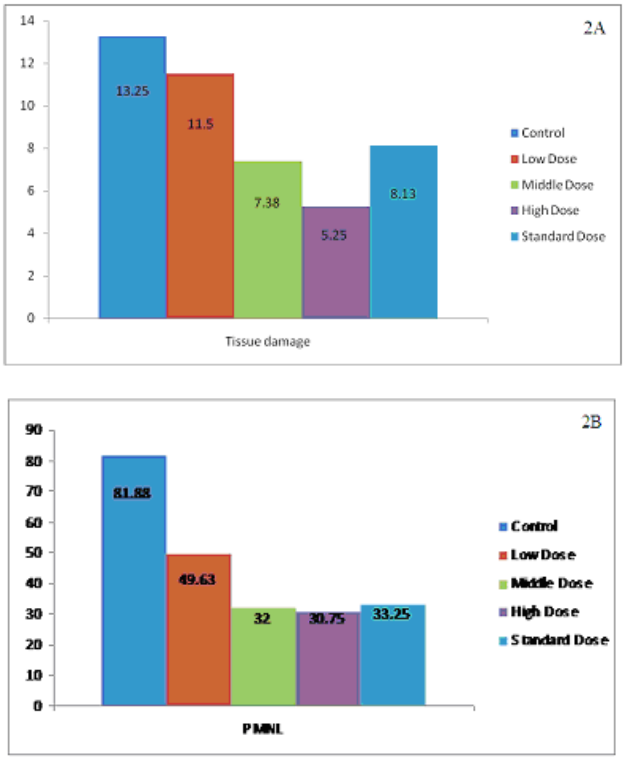


Fig. 2: Comparison of (A) Tissue Damage Score (B) Polymorpho nuclear Leukocyte Count among Control group, and following Pitavastatin (0.2mg/kg, 0.4mg/kg and 0.8mg/kg) and Indomethacin (10mg/kg) administration.

Evaluation of tissue damage and PMNL infiltration

As seen in table 2 and fig. 2, in the control group the mean TDS was 13.25±2.76 and mean PMNL count was 81.88±18.83. Low dose TDS of 11.50±2.56 was not significantly different Vs control. TDS with the middle dose and high doses was 7.38±1.99 and 5.25±1.28 respectively, which was significantly lower than both control and low dose. Similarly PMNL count reduced to 32.00±3.46 and 30.75±6.11 with middle and high doses respectively, which was a significant reduction compared to low dose (49.63 ±7.28) and control (p<0.05).

Table 1: Effect of Pitavastatin (0.2mg/kg, 0.4mg/kg, 0.8mg/kg) and Indomethacin (10mg/kg) on egg-albumin induced rat paw edema volume and inhibition of inflammation.

Time after induction of inflammation (t)	Control Group Edema volume (ml) n=8	Pitavastatin Treated Group Edema volume (ml)			Indomethacin Group Edema volume (ml)
		0.2 mg/kg n=8	0.4 mg/kg n=8	0.8 mg/kg n=8	10 mg/kg n=8
0.5hrs	0.37±0.058	0.27±0.05 (27.02%)	0.26±0.07* (29.73%)	0.25±0.067* (34.43%)	0.25±0.04* (32.43%)
1hr	0.51±0.08	0.34±0.06* (31.33%)	0.33±0.11* (35.29%)	0.33±0.07* (35.29%)	0.32±0.04* (37.25%)
1.5 hrs	0.63±0.15	0.42±0.08* (33.33%)	0.41±0.11* (34.92%)	0.38±0.09* (39.68%)	0.41±0.05* (34.92%)
2 hrs	0.84±0.14	0.53±0.07* (36.90%)	0.50±0.07* (40.47)	0.47±0.09* (44.04%)	0.49±0.07* (41.67%)
2.5 hrs	0.99±0.13	0.60±0.09* (39.39%)	0.56±0.08* (43.43%)	0.55±0.09* (44.44%)	0.54±0.07* (45.45%)
3 hrs	1.01±0.11	0.62±0.08* (38.61%)	0.59±0.07* (41.58%)	0.57±0.09* (43.56%)	0.54±0.07* (46.53%)

Values are mean ± SEM (n=8). * Statistically significant as compared to control. Values in parenthesis indicate inhibition %

Table 2: Effect of Pitavastatin (0.2mg/kg, 0.4mg/kg, 0.8mg/kg) and Indomethacin (10mg/kg) on tissue damage and polymorphonuclear leucocyte count.

	Tissue Damage Score in 5 random fields (n=8)						PMNL Count	
	0	1	2+	3+	4+	Mean TDS ±SD	Total in 5X8 fields	Mean ±SD
Control	0	0	14	20	6	13.25±2.76	655	81.88±18.83
Pitavastatin (0.2 mg/kg)	0	4	23	10	2	11.50±2.56	397	49.63±7.28*
Pitavastatin (0.4 mg/kg)	1	21	16	2	0	7.38±1.99*□	256	32.0±3.46*□
Pitavastatin (0.8mg/kg)	3	32	5	0	0	5.25±1.28*□	246	30.75±6.11*□
Indomethacin (10mg/kg)	0	18	19	3	0	8.13±1.88*	266	33.25±7.54*

*Statistically significant as compared to control $p<0.05$ □Statistically significant as compared to low dose $p<0.05$

TDS and PMNL count in the indomethacin treated animals was 8.13±1.88 and 33.25±7.54, which was a significant reduction in comparison to control ($p<0.05$). However there was no significant difference between indomethacin and the three tested doses of pitavastatin.

Histopathological analysis

Control Group: Light microscopic observations in the untreated control specimen (fig 3A) revealed no remarkable changes in the epidermis. The mid-dermis revealed marked edema and infiltration of polymorphonuclear leukocytes moderately admixed with lymphocytes and histiocytes. Focal changes in small blood vessels in the form of hyperemia, congestion, and vasculitis also appeared. The junction between the deeper dermis and subcutis showed polymorph infiltration. Unremarkable skeletal muscle fibers and skin appendages were also seen.

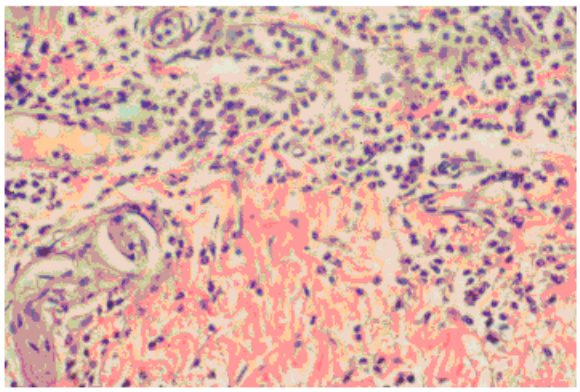
Pitavastatin was seen to diminish inflammatory effect of egg albumin in a dose-dependent manner. Tissues treated with low dose pitavastatin (fig. 3B) revealed microscopic findings similar to control. Marked edema was seen in the dermis, however infiltration of neutrophils was less and there were occasional lymphocytes and histiocytes. Rats treated with 0.4 and 0.8mg/kg pitavastatin (fig. 3C, D), exhibited highly diminished inflammatory response. Edema was present in dermis, neutrophil mobilization was minimal with focal patchy hyperemia and congestion in the border of deeper dermis and subcutis.

In the tissue specimen of indomethacin treated animals (fig. 3E), evidences for tissue destruction were less prominent. There was edema and focal infiltration of neutrophils and occasional lymphocytes predominantly at the dermis and subcutis junction. Blood vessels showed early changes of vasculitis and mild hyperemia. Rest of the tissue was unchanged.

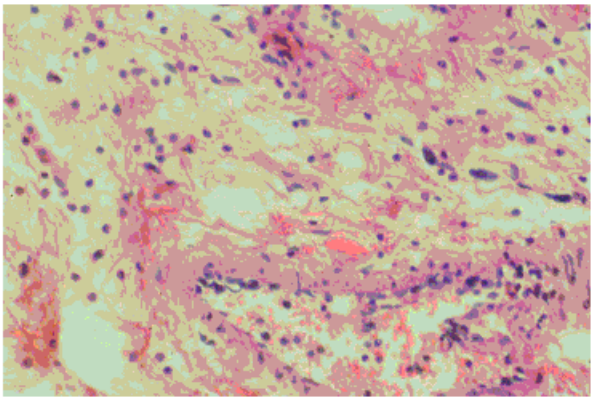
DISCUSSION

In this study the efficacy of ascending doses of pitavastatin against edema formation, leukocyte diapedesis and tissue damage, was evaluated using egg albumin-induced inflammatory model of the rat hind paw.

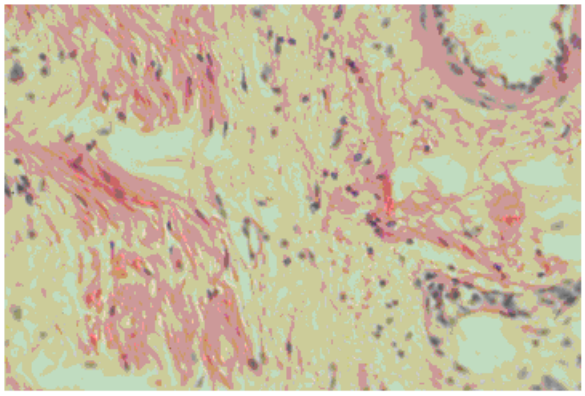
In our experiments, untreated rats showed a progressive increase in edematous exudation, which was maximum between 2-2.5 hours and then leveled off. An acute tissue insult provokes an inflammatory response manifested as



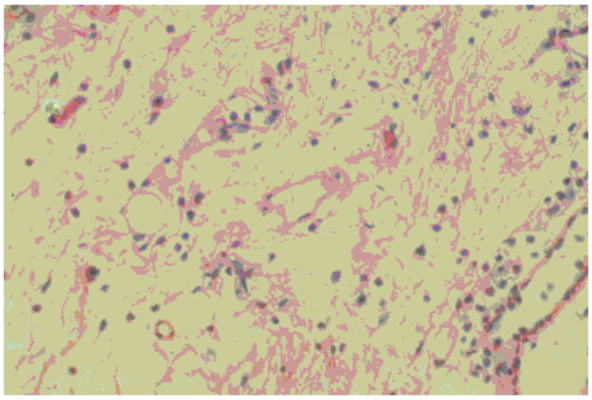
3A: Control Group shows edema and moderate inflammatory infiltrate with prominent polymorphonuclear leukocytes.



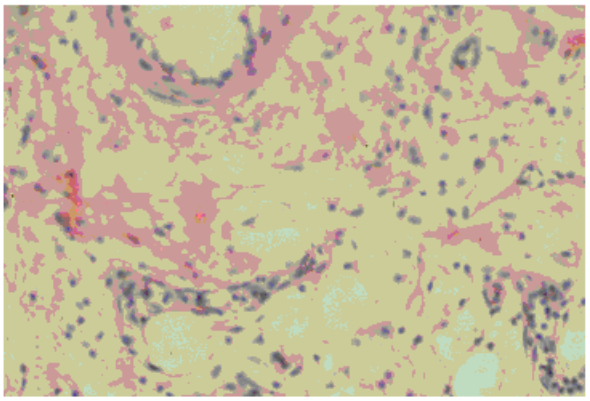
3B: Pitavastatin 0.2 mg/kg shows persistent edema, hyperemia and fewer PMNLs



3C: Pitavastatin 0.4 mg/kg shows edema, slight hyperemia and scant inflammation



3D: Pitavastatin 0.8 mg/kg shows very mild edema, scant inflammation and no hyperemia



3E: Indomethacin (10mg/kg) shows mild edema and hyperemia and limited inflammatory infiltrates

Fig. 3: Representative histological sections (x40 magnification) of acute inflammation in paw skin samples of rats.

redness and swelling. This is due to vasodilation, increased blood flow and vascular permeability in the inflamed tissue. The initial transient vasodilation lasting about 30 minutes is mediated by histamine, serotonin and nitric oxide (NO). The later phase of vasodilation starting around 2 hours is mediated by cytokines like tumor necrosis factor-α and interleukin-1 (Winyard, 2003). Proteolytic enzymes and reactive oxygen species released from the leukocytes also causes injury to capillary endothelium and contributes to fluid exudation into extra-vascular tissues. Inhibition of vascular permeability and resultant edema is a measure of the anti-inflammatory effect of tested compounds. Pretreatment with ascending doses of pitavastatin produced strong anti-inflammatory effect evidenced by a significant decrease in edema volume and an increase in inhibition of inflammation with all three doses of pitavastatin. Pitavastatin 0.8mg/kg produced the maximal inhibition (44.44%) at 2.5 hours

but even the lowest dose of pitavastatin (0.2mg/kg) reduced the edema volume to a level comparable to indomethacin. Previous studies have also demonstrated the effectiveness of statins against acute inflammation. Nezić *et al*., (2009) showed that simvastatin (10mg/kg) had a potent anti-inflammatory effect similar to indomethacin. In our study the dose of pitavastatin producing anti-inflammatory effect comparable to indomethacin was 0.2mg/kg. This shows the higher potency of pitavastatin probably due to its higher bioavailability and greater lipid solubility than simvastatin. Gargani *et al*., (2008) established that lovastatin and atorvastatin given orally reduced edema and maximum exudate production and leukocyte recruitment in acute carrageenan-induced rat paw edema and chronic mouse air-pouch models.

Leukocyte-endothelial interaction is an early event in the development of inflammation. Neutophils are the earliest cells to be recruited into the inflamed tissue. Proteolytic enzymes released from these cells contribute to tissue injury. Our study demonstrates pitavastatin attenuates inflammatory changes in paw skin samples. In the untreated group, there was marked edema, a remarkable increase in neutrophilic infiltration and occasional lymphocytes. Blood vessels showed hyperemia and vasculitis. Pretreatment with pitavastatin reduced the inflammatory response as revealed by a progressive reduction in edema, minimal neutrophil infiltration and focal patchy hyperemia and no congestion. These changes were less prominent with low dose of pitavastatin and more striking with higher doses. The indomethacin treated group likewise showed mild edema and hyperemia and limited inflammatory infiltrates.

The tissue damage score measured in five random fields decreased significantly in groups administered indomethacin, 0.4mg/kg and 0.8mg/kg pitavastatin. The effect of the middle and high dose was also significantly lower Vs the low dose. The outcome with indomethacin was analogous to all doses of pitavastatin. Similarly polymorphonuclear leukocyte count exhibited significant dose dependent reduction with pitavastatin, which corresponded to the indomethacin outcome. Recently Adami M *et al.,* (2012), showed that topically applied simvastatin ointment decreased leukocytic migration and edema formation in inflammation induced in ear skin of mice. Statins inhibit endothelial adhesion and trans-endothelial migration of leukocytes by attenuating endothelial adhesion molecules ICAM-1, E-selectin, VCAM-1 levels (Patti *et al*., 2006). Lefer *et al*. (1999) also demonstrated that a single dose of simvastatin blocked the influx of PMN leukocytes into rat cardiomyocytes subjected to ischemia and reperfusion injury. Barsante *et al.* (2005) found decreased concentration of cytokines IL-1β, IL-6, TNF-α and chemokines CCL5 and CCL2 in arthritic rats treated with atorvastatin. Simvastatin was found to reduce edema formation, oxidative stress and decrease exudate level of TNF-α, IL-6 and malondialdehyde as efficaciously as aspirin in air-pouch granuloma inflammation model.

It could be argued that this anti-inflammatory effect was due to reduction in serum cholesterol. However, statins do not reduce cholesterol level in rodents (Sparrow *et al.*, 2001), and even in sensitive species cholesterol level decline after several days of therapy. In our study anti-inflammatory effects manifested within 4 hours, much before changes in lipid level was possible.

Further studies are needed to identify the exact mechanism by which pitavastatin reduces acute inflammation. This could lead to novel therapeutic strategies with pitavastatin as an adjuvant to anti-inflammatory drug therapy in acute inflammatory conditions.

CONCLUSION

Treatment with pitavastatin, prior to induction of acute inflammation is effective in modifying fundamental pathological processes such as edema, neutrophil influx and tissue destruction in the inflamed skin and subcutaneous tissues. The dose necessary for such effects is equal to the minimum dose necessary for control of hypercholesterolemia in humans and is comparable to indomethacin a highly efficacious non-steroidal anti-inflammatory drug. The anti-inflammatory effect of pitavastatin is an important component of their potential benefit in acute inflammatory states. However large randomized clinical trials are necessary before putting pitavastatin for use in such conditions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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