Effect of Intraperitoneally Injection of Different Doses of Simvastatin on Pain and Inflammatory Response Induced by Formalin in Mice

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Abstract: The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have been unequivocally shown to reduce cardiovascular morbidity and mortality. Their lipid-lowering actions are by reversible and competitive inhibition of the enzyme HMG-CoA reductase, a precursor of cholesterol. It has been suggested that statins appear to have therapeutic benefits in diseases that are unrelated to elevated serum cholesterol levels such as pain and inflammatory. The aim of this study was to determination the effect of intraperitoneally injection of different doses of simvastatin on pain and inflammatory response induced by formalin in mice. Mice were divided into 9 groups randomly, the 1st group received saline normal (ip) (saline group); the 2nd group received Carboxymethylcellulose (CMC) 0.5% (ip) (vehicle group) and the next groups received, respectively different doses of simvastatin (1, 5, 10, 20, 40, 80 and 100 mg/kg/daily) for 4 days (ip) before formalin test. After intrapawly injection of formalin, the time of hind paw biting and licking time measured in 5 min interval for an hour. Results showed that formalin induces biphasic pain response (the 1st phase: 0-5 min and the 2nd phase 15-40 min after injection). Intraperitoneal injection of simvastatin showed significant ($p < 0.05$) decrease of pain responses in the 1st and the 2nd phase in a dose dependently manner. According to the results, simvastatin has analgesic and anti-inflammatory effects on mice. Nevertheless, new studies must be carried out in order to determine the beneficial effects of statins in treatment of pain and inflammatory.

Key words: Simvastatin, intraperitoneal, pain, neurogenic, inflammatory, mice

INTRODUCTION

Pain is a percept consisting of initial nociception followed by a slower but integrated emotional phase. Nociception is the neural recognition of a potentially injurious physical or chemical stimulus. Pain responses occur only after centralized processing and elicitation of an emotional output to the nociceptive input. Long-term painful stimuli may enhance sensitivity in some animals and humans and habituate others. Acute centrally processed pain can be recognized in animals by behavioral changes (Kamerling, 1993; Ley et al., 1990). The inflammatory response is an attempt by the body to restore and maintain homeostasis after injury or infection and is an integral part of body defense.

For the survival of the host, inflammation is a necessary and beneficial process but chronic inflammation can cause harm (Beutler, 2003). Soluble mediators such as Nitric Oxide (NO), Tumor Necrosis Factor-α (TNF-α) and interleukins usually play a role in controlling important functions such as the regulation of blood pressure, platelet aggregation and body temperature (Funk, 2001). Under pathologically inflammatory conditions, however the production of these

molecules promotes events ranging from increased leukocyte infiltration and vascular permeability to organ failure (D'Acquisto et al., 2002). The selective inhibition of these and other inflammatory activities remains an important goal for the effective treatment of inflammation. The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) have been unequivocally shown to reduce cardiovascular morbidity and mortality (Scandinavian Simvastatin Survival Study, 1994; Shepherd et al., 1995). Statins are among the most widely used prescription drugs and exert their lipid-lowering actions by reversible and competitive inhibition of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting step in the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, a precursor of cholesterol (McTaggart et al., 2001). It has been suggested that statins appear to have therapeutic benefits in diseases that are unrelated to elevated serum cholesterol levels such as rheumatologic diseases and ischemic stroke (McCarey et al., 2004). Statins are also known for their pleiotropic effects which are independent of their lipid-lowering properties. Among the effects of statins, the most relevant are anti-atherosclerotic and

anti-inflammatory actions, improvement of endothelial dysfunction, anti-thrombosis and anti-oxidant actions, prevention of Alzheimer's disease and antineoplasic actions (Goncalves *et al.*, 2011). Although such clinical benefits are mediated in part through lipid modulation, recent studies demonstrate broader properties for statins, particularly in modifying inflammatory pathways ongoing within the atherosclerotic lesion (Ross, 1999). Statins might exert beneficial effects beyond cholesterol reduction include improving endothelial function, decreasing vascular inflammation, inhibiting smooth-muscle proliferation and immunomodulation. Most of these effects are mediated through inhibition of isoprenoid synthesis with subsequent effects on multiple downstream signaling pathways (Wang *et al.*, 2008).

Immunomodulatory activities of statins could be beneficial in treatment of various inflammatory conditions. This observation prompted subsequent *in vitro* studies that demonstrated that statins interfered with production of several important proinflammatory mediators (Pahan *et al.*, 1997; Stuve *et al.*, 2002). Most of the reports point to the anti-inflammatory effects of statins focused on cardiovascular diseases and atherosclerosis and about orally administration of these drugs (Garjani *et al.*, 2008; Goncalves *et al.*, 2011; Kumar *et al.*, 2010). Also, studies showed that statins has a biphasic pro- and anti-inflammatory effect (Garjani *et al.*, 2008). Therefore, the aim of this study was to determination of the effect of intraperitoneally injection of different doses of simvastatin on pain and inflammatory response induced by formalin in mice.

MATERIALS AND METHODS

Animals: Experiments were performed on 22-25 g adult NMRI male mice in their 8-9 weeks (n = 8 for each group), purchased from Razi Institute (Iran). Animals were housed 8 cage^{-1} in the Animal House of Veterinary Faculty of Tabriz Branch Islamic Azad University in a temperature (20-22°C) and humidity (50±10%) controlled environment under a 12 h light/dark cycle (lights on at 7 a.m.). Food and water were available *ad libitum*. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Research Affairs of Tabriz University of Medical Sciences, Tabriz, Iran. All efforts were made to minimize the number of animals which were used and their suffering degree. Animals were divided into 9 groups randomly, the 1st group received saline normal (ip) (saline group); the 2nd group received Carboxymethylcellulose (CMC) 0.5% (ip) (vehicle group) and the next groups received respectively different doses of simvastatin (1, 5, 10, 20, 40, 80 and 100 mg/kg/daily) for 4 days (ip) before formalin test.

Chemicals: Formalin was purchased from Merck Company. Simvastatin was provided from Sigma-Aldrich. All other reagents were of analytical grade. Simvastatin was prepared by being dissolved in 0.5% Carboxymethylcellulose (CMC) and the vehicle group was given an equal volume of vehicle.

Formalin Test: Behavioral experiments were done in a quiet, temperature-controlled (20-22°C) room between 10 a.m. and 4 p.m. Formalin Test was preformed based on Dubission and Dennis Method (Modified Formalin Test: characteristic biphasic pain response). After being weighted, the animals were placed in observation container for 15 min to get used to the new environment and then 20 µL of attenuated formalin (5%) was injected in hind paw skin (intradermal route) with insulin syringe in restrainer.

After injection, animals were returned to observation container immediately and were viewed. The time of hind paw biting and licking time measured in 5 min interval for an hour (Abad *et al.*, 2011).

Data analysis: Group data are presented as mean±SEM and analyzed statistically using Student Test. Time course data were analyzed using one-way ANOVA followed by Tukey's Post Hoc Test. The level for statistical significance was set at a $p < 0.05$.

RESULTS AND DISCUSSION

Formalin injection to hind paw after intra peritoneal injection of normal saline induced significant ($p < 0.05$) pain response (Time of licking and biting of injected paw) in the 1st and 4-8th, 5 min in comparison with other 5 min (Fig. 1) as results show formalin induces biphasic pain response (the 1st phase: 0-5 min and the 2nd phase 15-40 min after injection).

In vehicle group, CMC have not shown significant change on formalin induced biphasic pain response compared with saline group (Fig. 1).

Intraperitoneal injection of simvastatin before hind paw injection of formalin showed significant ($p < 0.05$) decrease of pain responses (Time of licking and biting of injected paw) in the 1st phase (1st 5 min) and the 2nd phase (4th, 5th, 6th, 7th and 8th 5 min) in a dose dependently manner compared with saline and vehicle groups (Fig. 1).

Formalin Test has two phases; early or 1st phase (neurogenic pain) and delayed or 2nd phase (inflammatory pain). It seems that early phase induced by C fiber activation and peripheral stimulates. But inflammatory reactions in peripheral tissues and action change in dorsal root of spine are the main reasons of delayed phase.
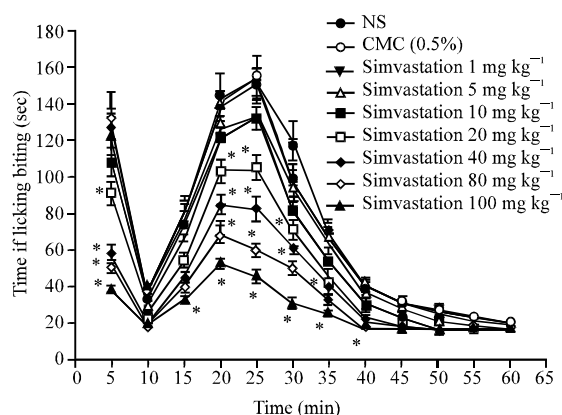
Fig. 1: Time course of paw licking and biting response to formalin injection. Effect of intraperitoneally administration of different doses of simvastatin on 1st and 2nd phase of pain responses. Each line represents mean±SEM of 8 mice. *p<0.05 compared with saline and vehicle groups

The main reason of pain in the 2nd phase is the inflammatory reactions (Abad *et al.*, 2011). Results of present study have shown that simvastatin decreased pain and inflammatory response in Formalin Test, in a dose dependently manner in both neurogenic and inflammatory origin.

Anti-nociceptive and -inflammatory effects of statins have been shown in different experimental animal models of pain and inflammatory and clinical studies but effects of intraperitoneally injection of different doses of statins have not shown. Studies showed that lovastatin has a potent anti-inflammatory activity, since it inhibits carrageenan-induced peritonitis and paw edema. Although, these findings are consistent with studies showing these effects of statins in many inflammation models most of them were carried out with other statins mainly simvastatin (Dantas *et al.*, 2010; Massaro *et al.*, 2010; Nezic *et al.*, 2009b).

Studies demonstrate that simvastatin provides a highly effective therapy in treating murine Collagen-Induced Arthritis (CIA), a Surrogate Model for human Rheumatoid Arthritis (RA). Previous studies of thioglycolate peritonitis or carrageenan-induced paw pad inflammation suggested that statins modify acute inflammation (Sparrow *et al.*, 2001; Weitz-Schmidt *et al.*, 2001). Histological examination of paw lesions confirmed that simvastatin inhibits the acute inflammation induced by carrageenan. These investigators concluded that simvastatin reduced the polymorphonuclear leukocyte infiltration, dose-dependently and similarly to indomethacin (Nezic *et al.*, 2009a). Furthermore, atorvastatin was shown to be even more effective than

diclofenac in decreasing joint inflammation and hyperalgesia in a Rat Model of arthritis (Mandosi *et al.*, 2010) and in reducing TNF-α production in lipopolysac charideactivated monocytes from diabetic patients (Trocha *et al.*, 2010). In both experimental and clinical studies, statins have been shown to suppress acute and chronic inflammation. Their anti-inflammatory effect is brought about through inhibition of oedema formation, leukocyte-endothelial adhesion and production of inflammatory mediators (Kumar *et al.*, 2010). Besides, they have been shown to afford protection in various experimental models of arthritis by reducing articular inflammation, preventing bone loss and preserving bone mineral density (Funk *et al.*, 2008; Yamagata *et al.*, 2007). Another statin, simvastatin was reported to prevent the increase of iNOS concentration after ischemia/reperfusion injury in the rat liver. As well as in those studies, these effects of statins were mediated, at least in part by the reduction of cytokines such as TNF-α and by the decrease of iNOS production. Besides being involved in inflammatory processes, iNOS has also been implicated in neuropathic pain (Bonnefous *et al.*, 2009).

Hasegawa *et al.* (2003) reported that 6 weeks treatment with cerivastatin (1 mg/kg/day) significantly attenuated the expression of pro-inflammatory cytokine Interleukin 1b (IL-1b) in Dalt salt sensitive rats with left ventricular hypertrophy and chronic heart failure. Lovastatin partially suppressed acute Experimental Autoimmune Encephalomyelitis (EAE) in rats (Stanislaus *et al.*, 1999). Atorvastatin one of the longer acting and clinically used statins has been demonstrated to attenuate inflammatory hyperalgesia in a rat model of monoarticular arthritis. Atorvastatin has been documented to relieve hyperalgesia and to improve joint functions in the arthritic rats (Wahane and Kumar, 2010). Michele *et al.* (2005) have reported anti-inflammatory and anti-hypernociceptive effects of atorvastatin in chronic arthritis induced by immunization of rats with adjuvant. Clinical studies such as the WOSCOPS (West of Scotland Coronary Prevention Study Group, 1998), a randomized placebo controlled study, provided evidence that the anti-inflammatory effect of pravastatin treatment is probably independent of changes in the lipid profile. Statins have been used to reduce inflammation, tame immune cell activation or arrest degenerative processes. Because of their widespread use and long-term safety record some physicians prescribe statin therapy for non approved indications.

A number of case reports describe the dramatic effects of statins added to standard therapies but beneficial effects need to be confirmed in controlled studies. For example in three patients with lupus

glomerulonephritis who were not responding to the standard of therapy, adding high doses of simvastatin brought about a dramatic reduction in disease activity. There are randomized, placebo controlled studies but two issues cloud the results: the particular statin used and the dose.

Clearly the anti-inflammatory properties of statins vary whereas the cholesterol-lowering properties are similar. Six clinically used statins were examined *in vitro* for their ability to affect NF-kB, phosphorylation of IkB and activation of tissue factor, the 1st step in coagulation. There was a distinct difference between the statins: cerivastatin, atorvastatin and simvastatin were more effective in reducing these parameters than fluvastatin, lovastatin or pravastatin (Dinarello, 2010). Also, other clinical studies demonstrated that in a small group of patients with Heart Failure (HF) and normal cholesterol levels that administration of atorvastatin 10 mg for 4 weeks led to a significant reduction in IL-6, TNF-alpha and VCAM-1 levels that confirmed that the anti-inflammatory action of statin in this high risk group of patients (Tousoulis *et al.*, 2005). Nevertheless, there seems to be no dearth of case reports and small uncontrolled trials that show the benefits of statins for reducing inflammation.

In the case of rheumatoid arthritis where the anti-inflammatory effects of blocking cytokines have been shown repeatedly, there have been >10 trials of statins resulting in a moderate reduction in joint inflammation associated with a fall in C-Reactive Protein (CRP) and red cell sedimentation rate and decreased cytokine production by circulating monocytes. As many patients with long-standing rheumatoid arthritis are also at high risk for cardiovascular disease, adding statin therapy to the standard of care is highly cost effective. Adding statins to the regimen of cyclosporine and sirolimus for kidney transplant patients lowered the rate of organ rejection. In patients with relapsing-remitting multiple sclerosis, statin therapy for 6 months significantly lowered the number of brain lesions detected by gadolinium, an established marker of disease activity. Examination of peripheral blood cells revealed no suppression in T cell responses but did reveal an increase in production of IL-10, an anti-inflammatory cytokine (Dinarello, 2010). Nevertheless, new and completely studies must be carried out in order to determine in more detail the beneficial actions of statins regarding the reduction of pro-inflammatory mediators.

CONCLUSION

According to the results, simvastatin has analgesic and anti-inflammatory effects on mice. Nevertheless, new studies must be carried out in order to determine the beneficial effects of statins in treatment of pain and inflammatory.

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