PROTECTIVE EFFECT OF LOVASTATIN ON NEUROTOXICITY OF EXCESSIVE FLUORIDE IN PRIMARY HIPPOCAMPAL NEURONS

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ABSTRACT: The protective role of lovastatin against neurotoxicity induced by fluorosis was investigated by using primary hippocampal neurons. The cholesterol content, activity of superoxide dismutase (SOD), and content of malondialdehyde (MDA) were measured by biochemical assays. The cell viability was assessed by examining the rate of apoptosis by flow cytometry. The results showed that high fluoride inhibited activity of SOD and increased levels of MDA and apoptosis in the primary neurons. Interestingly, certain dosages of lovastatin, without changing the cholesterol level, attenuated these neurotoxicities resulting from high exposure to fluoride in the primary cultured neurons. The results suggest that lovastatin may play a protective role against the neurotoxicity induced by an excessive amount of fluoride.

Key words: Apoptosis; Cholesterol; Fluorosis; Hippocampus; Lovastatin; Malondialdehyde (MDA); Oxidative stress; Primary hippocampal neurons; Superoxide dismutase (SOD).

INTRODUCTION

The excessive accumulation of fluoride over a long period has been proposed to cause a vast array of symptoms and pathological changes in many tissues and organs, in addition to its well-known effects on the skeleton and teeth. Numerous investigations have shown that the underlying mechanism(s) may involve elevated levels of free radicals and inhibited antioxidant defenses, i.e., a high level of oxidative stress.[1-3]

Exposure to excess fluoride can severely damage different systems, especially the nervous system which is a matter of increasing concern.[4] Epidemiological investigations provide compelling evidence that the intelligence quotient of children in areas characterized by endemic fluorosis is significantly reduced.[5] In addition, we previously showed that fluoride exposure impaired learning and memory in both adult and newborn rats.[6,7]

Statins, which play an important role as a therapeutic agent for hypercholesterolemia, reduce plasma cholesterol levels by inhibiting the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the cholesterol biosynthetic pathway.[8] Interestingly, besides lowering the blood cholesterol, many studies show that statins have many pleiotropic effects, e.g., protecting neurons from oxidative stresses, excitotoxins, and apoptosis; suppressing inflammatory responses; and promoting synaptogenesis,[9] and these effects may occur through a cholesterol-independent mechanism.[10]

In the present study, we treated primary cultured hippocampal neurons from the brains of new postnatal rats with excessive fluoride and lovastatin, and

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subsequently examined the toxicity induced by fluoride by assessing oxidative stress and apoptosis. We also addressed the potential mechanism by which lovastatin may attenuate the neurotoxicity of fluoride.

MATERIALS AND METHODS

Materials: Lovastatin, poly-L-lysine hydrobromide, Cholesterol Quantitation Kit (MAK043), Lipid Peroxidation Assay Kit for detecting malondialdehyde (MDA), Superoxide Dismutase (SOD) Determination Kit, as well as other general chemicals were purchased from Sigma (USA). Cell Counting Kit-8 (Dojindo, Japan); the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson Biosciences, USA); sodium fluoride (NaF) with analysis purity (Sigma-Aldrich, USA); Neurobasal-A Medium, Hibernate-E Medium, B-27 Serum-Free supplement, GlutaMAX Supplement (Life Technologies, USA); mouse anti-NeuN antibody (Merck Millipore, Germany); rabbit anti-GFAP antibody (Dako, Denmark); anti-mouse IgG labeled with CY-3, and anti-rabbit IgG labeled with 488 (Thermo scientific, USA) were purchased from the sources indicated.

Cell cultures: Primary hippocampal neurons were prepared from the brains of neonatal Sprague-Dawley (SD) rats according to a published procedure with slight modifications.[11] Briefly, the hippocampal regions were separated from the brains of neonatal rats within 2–3 min after sacrifice and maintained in Hibernate A Medium chilled on ice. After removing the meninges, the hippocampus was washed 3 times with Hank's buffered saline solution and then digested with 0.25% trypsin for 10 min in 37ºC. Subsequently, the incubation medium was discarded and DMEM containing 10% FBS was added to terminate digestion. After washing twice more with Hank's buffered saline solution, the digested tissue was resuspended in 2 mL of Neurobasal/B27 complete medium (Neurobasal A Medium with 2% B27, 1% GlutaMAX Supplement, 100 U/mL penicillin and 100 mg/mL streptomycin) and broken apart with fire-polished glass pipettes. The upper single-cell suspension was transferred into a new tube and the cells were counted by trypan blue exclusion, and thereafter placed on 96-, 12- or 6-well PLL-coated plates at a cell density of approximately $5.0 \times 10^4/cm^2$. The neurons were maintained in a humidified atmosphere containing 5% CO_2 at 37ºC, with half-replacement of the medium every 3 days. The purity of the primary neurons is determined by immunofluorescent double staining with mouse anti-NeuN antibody (diluted 1:50) and anti-mouse IgG labeled with CY-3 (red), and with rabbit anti-GFAP (glialfibrillary acidic protein) antibody (diluted 1:300) and anti-rabbit IgG labeled with 488 (green). After 10 days of incubation, the medium was replaced with neurobasal medium without B27, and the neurons then were prepared for different treatments.

Ethical approval: All experiments described here were approved by the regional ethical committee of Guizhou Province of China.

Treatments by fluoride and lovastatin in the cultured neurons: These primary neurons were seeded onto 96-, 12- or 6-well PLL-coated plates. B27 in the culture medium was withdrawn for 2 hr prior to the treatments.

To find the optimal conditions for the cells' survival in starving media, a toxicity test was performed on the cultured cells by Cell Counting Kit-8 (CCK-8 test) when exposed to different concentrations of fluoride (10–80 ppm) and lovastatin (0.01–1 μM) for 48 hr. For the cell viability analysis, 10 μL CCK-8 solution was added to each well and the incubation continued for 1 to 4 hr. The absorption values at a wavelength of 450 nm were determined by spectrophotometer. From the results obtained from the CCK-8 test, suitable concentrations and incubation times for the cultured cells with lovastatin and fluoride were selected.

Analysis of cholesterol contents: The content of cholesterol was measured according to the Cholesterol Quantitation Kit. In brief, cholesterol in neurons was extracted with 200 μL of chloroform:isoporpanol:IGEPAL CA-630 (7:11:0.1) in a micro-homogenizer and the mixture was centrifuged at 13,000×g for 10 min. The organic phase after separation was transferred to a new tube, dried by air at 50ºC, and then put under vacuum for 30 min. Thereafter, the sample containing dried lipids was dissolved with the Cholesterol Assay Buffer and mixed with vortex until homogenous. The content of cholesterol in the sample was detected by adding Cholesterol Probe, Cholesterol Enzyme Mix, and Cholesterol Esterase, and calculated according to the standard curve.

Examinations of activity of superoxide dismutase (SOD) and content of malondialdehyde (MDA): SOD was assayed with the SOD Determination Kit-WST. In brief, the neurons were homogenized on ice in the lysis buffer and then centrifuged at 13,000×g for 10 min to remove insoluble material. The supernatant, enzyme assay solution, and WST buffer were added in the order indicated by the instructions for the kit. Following incubation at 37ºC for 20 min, the absorbance at 450 nm was determined using a microplate reader and SOD activity (% inhibition) calculated.

The content of MDA, a major product of lipid peroxidation, was detected with thiobarbituric acid-reactive substance (TBARS) assay employing the Lipid Peroxidation Assay Kit. In brief, 200 μL of the supernatant prepared as described above were placed into a micro-centrifuge tube and 600 μL of the TBARS solution then added. This mixture was incubated at 95ºC for 60 min and cooled to room temperature in an ice bath for 10 min. Finally, 200 μL was pipetted into each well of a 96-well plate and the absorbance at 532 nm measured.

Characterization of apoptosis: The cultured cells were washed twice with PBS before suspension in 1×binding buffer. FITC-labeled annexin V (10 μL) was mixed with 100 μL suspension containing 2×10^5 cells, followed by incubation at RT for 5 min. Thereafter, 5 μL PI solution (10 μg/mL) was added to the cells, followed by an additional 5-min incubation. Finally, the scatter parameters of the cells (10,000 cells per experiment) were analyzed using the FACS Calibur® system (Becton Dickinson, USA).

Statistical analysis: The results are expressed as the mean±SD of values from different groups. The data were calculated by employing the analysis of variance

(ANOVA) followed by Student-Newman-Kenl's test or the two-paired Student's *t* test in the SPSS17.0 software (SPSS Inc., USA).

RESULTS

The purity of primary cultured neurons: Immunostaining of the primary cultured neurons originating from the hippocampal region of the brains of newborn rats with an antibody directed towards the neuronal marker (NeuN) and an antibody against GFAP, a marker for astrocytes revealed that about 90% of these cells were positively identified as neurons (Figure 1).

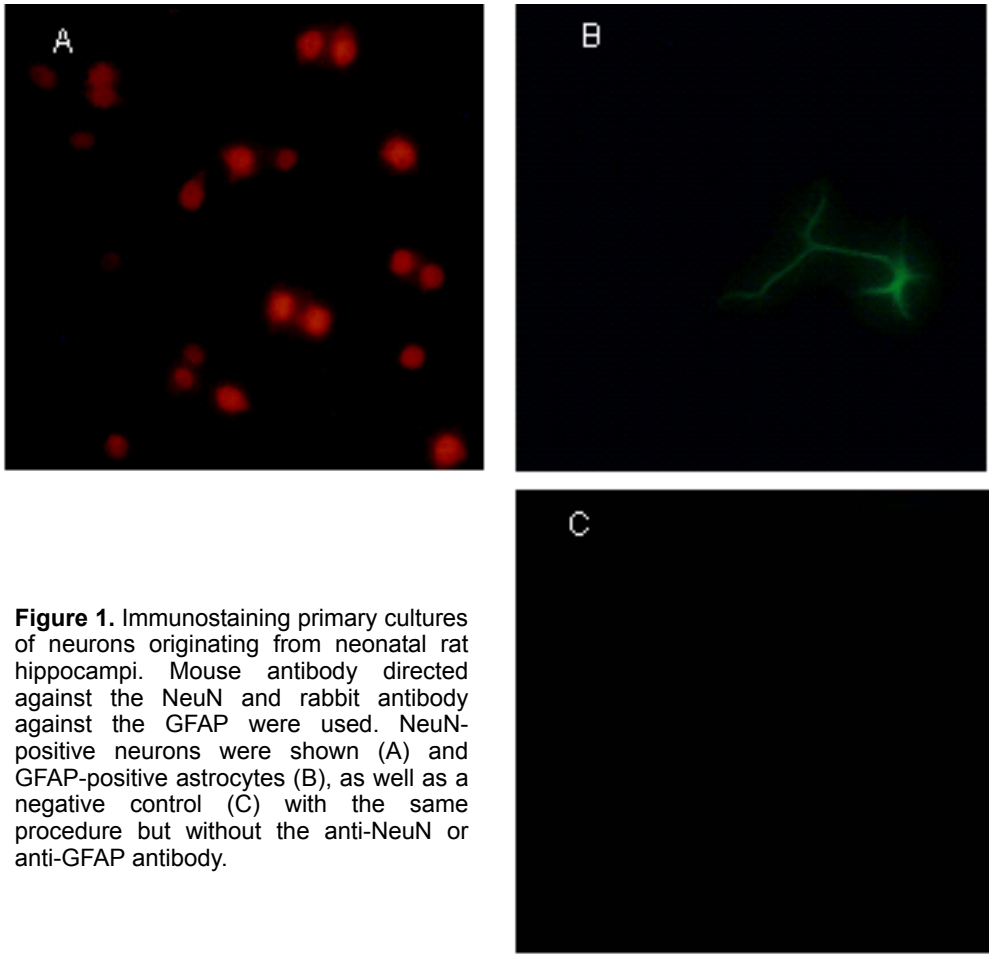

Figure 1. Immunostaining primary cultures of neurons originating from neonatal rat hippocampi. Mouse antibody directed against the NeuN and rabbit antibody against the GFAP were used. NeuN-positive neurons were shown (A) and GFAP-positive astrocytes (B), as well as a negative control (C) with the same procedure but without the anti-NeuN or anti-GFAP antibody.

Cell viability and cholesterol level in the cultured neurons exposed to lovastatin or/and fluoride: When the cells were exposed to 0.01, 0.1, and 1.0 μM of lovastatin, the cellular viability, detected by the CCK-8 test, was significantly decreased by 40% in primary hippocampal neurons with the treatment of 1.0 μM lovastatin (Figure 2A). The level of cholesterol also declined significantly with the same concentration of lovastatin exposure (1.0 μM) (Table 1). Therefore, 0.01 or 0.1 μM of lovastatin were selected for the exposure without significantly inducing cytotoxicity and changing the level of cholesterol in the study. A significant decline in CCK-8 reduction was also observed in the primary neurons with

exposure of the cells to 20 ppm fluoride for 48 hr (Figure 2B). Interestingly, the decline of the primary neurons induced by fluoride (20 ppm) can be attenuated by pre-treating the neurons with 0.1 μM lovastatin (Figure 3).

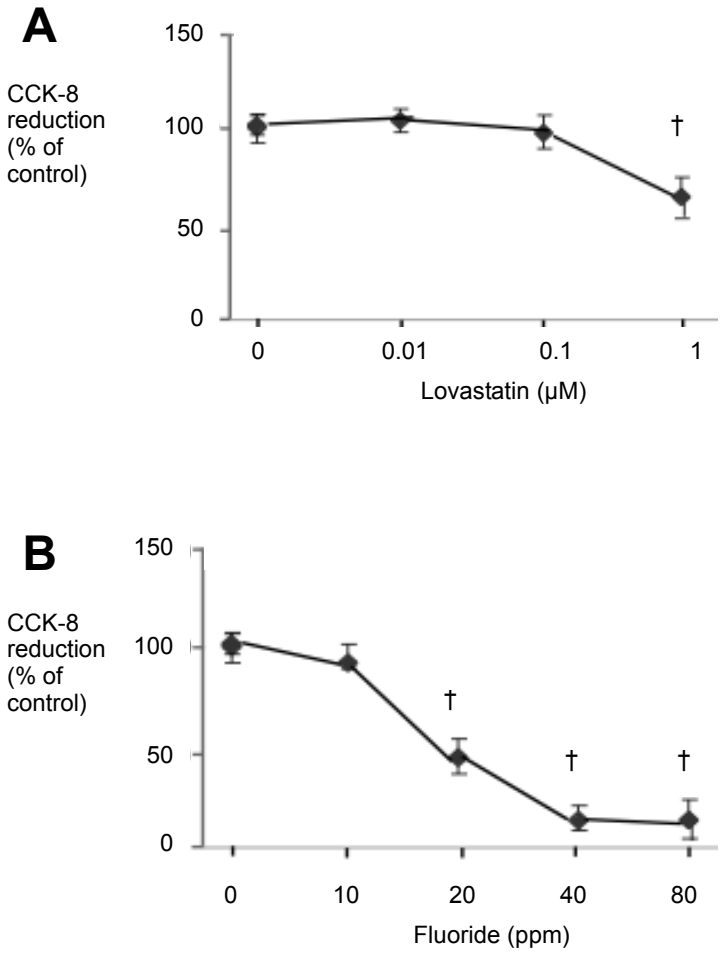

Figure 2. CCK-8 reduction in primary neurons treated with lovastatin or fluoride. CCK-8 reduction (A) in the primary neurons exposed to lovastatin; CCK-8 reduction (B) in the primary neurons treated with fluoride. Results are expressed as mean±SD of three independent experiments. †p<0.01 compared with untreated cells (control) as determined by employing analysis of variance(ANOVA), followed by Student-Newman-Keul's test.

Table 1. Cholesterol level in primary neurons exposed to lovastatin

	Group			
	Control group	0.01 μM lovastatin	0.1 μM lovastatin	1 μM lovastatin
Cholesterol (nmol/μL)	0.01127±0.004	0.01034±0.005	0.01121±0.006	0.00839±0.002†

Results are expressed as mean±SD of three independent experiments. †p<0.01 compared with untreated cells (control group) as determined by employing analysis of variance(ANOVA), followed by Student-Newman-Keul's test.

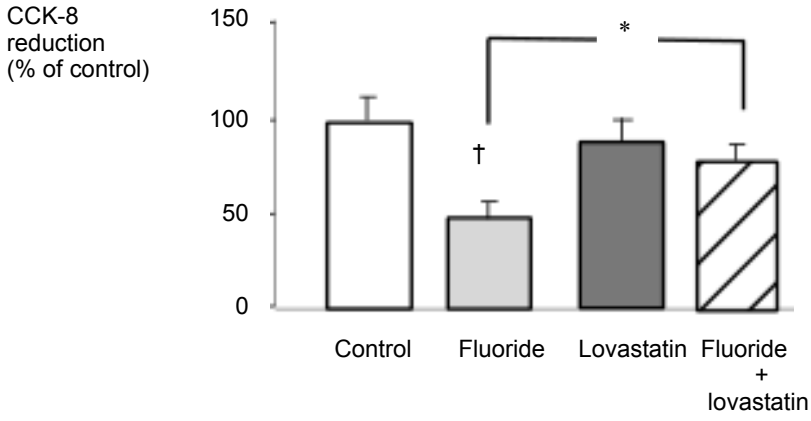

Figure 3. CCK-8 reduction in primary neurons treated by fluoride or/and lovastatin. Results are expressed as mean±SD of three independent experiments as determined by employing analysis of variance(ANOVA), followed by Student-Newman-Keul's test.
*p<0.05 compared with primary neurons treated with fluoride.
†p<0.01 compared with untreated cells (control).

Changed SOD activity and MDA content in the primary neurons exposed to fluoride or/and lovastatin: A significant decrease in SOD activity by 20% and an increase in MDA content by 40% were observed in the primary hippocampal neurons after exposure to 20 ppm fluoride for 48 hr (Table 2).

Table 2. SOD activity and MDA content in primary neurons exposed to fluoride, lovastatin, or the combination of fluoride and lovastatin

Group	SOD activity (inhibition rate %)	MDA contents (nmol/μL)
Control	81.02±6.13	0.02665±0.0045
Fluoride (20 ppm)	65.80±4.25*	0.03728±0.0099†
Lovastatin (0.1 μM)	76.90±3.40	0.02927±0.0023
Fluoride (20 ppm) + lovastatin (0.1 μM)	79.68±4.23‡	0.02717±0.0068§

The values are shown as the mean±SD by 3 independent tests. *p<0.05, †p<0.01 compared to the control and ‡p<0.05, §p<0.01 compared to the fluoride treatment group as determined by employing analysis of variance(ANOVA), followed by Student-Newman-Keul's test.

However, the changes in SOD and MDA due to fluoride can be prevented by pre-treating the primary neurons with 0.1 μM lovastatin for 24 hr before the treatment with fluoride (Table 2).

Rate of apoptosis in the primary neurons exposed to fluoride or/and lovastatin: An increased percentage of apoptotic death rate in a time-dependent manner was

observed in the cultured cells incubated with 20 ppm fluoride (Figure 4). In contrast, in the cells treated only with lovastatin, no change in the apoptotic death rate was observed. Furthermore, the increased apoptotic death rate induced by fluoride can be attenuated by pre-treating the primary neurons with 0.1 μM lovastatin for 24 hr before the treatment of fluoride (Figure 4).

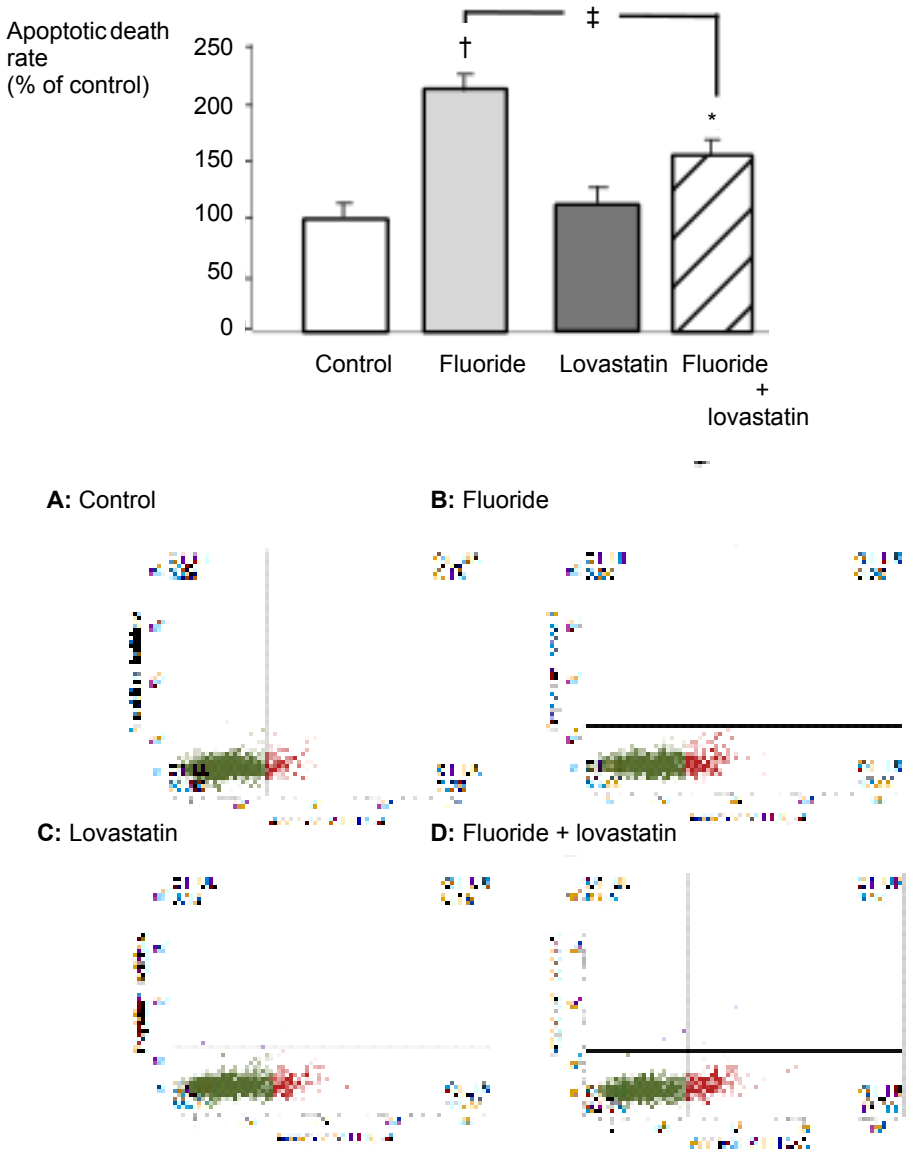

Figure 4. Apoptotic death rate of primary neurons treated by fluoride or/and lovastatin. A (control), B (fluoride), C (lovastatin), and D (fluoride + lovastatin) present the apoptotic cells for the four groups, respectively. The results are expressed as mean±SD of three independent experiments as determined by employing analysis of variance (ANOVA), followed by Student-Newman-Keul's test. *$p<0.05$ and †$p<0.01$ compared with untreated cells (control); ‡$p<0.05$ compared with primary neurons treated with fluoride.

DISCUSSION

With prolonged exposure, excessive fluoride accumulates in the brains of experimental animals and damages the CNS.[12] Both the impaired learning and memory of rats with chronic fluorosis, and the reduced intelligence quotient of children living in areas with endemic fluorosis are particularly noteworthy.[3,7,13,14] Earlier, we proposed that oxidative stress is an important factor in the multiple systemic damage associated with chronic fluorosis, including in the central nervous system[1-3] and several studies support this hypothesis.[13,14]

In recent years, more investigations concern interventions in chronic fluorosis using anti-oxidative stress compounds or drugs.[15-18] The results obtained indicate that using Vitamin C, Vitamin E, selenium, melatonin, Ginseng, epigallocatechin gallate, quercetin, spirulina platensis, and silymarin, etc., can inhibit the toxicity induced by high-amounts of fluoride through attenuating the level of oxidative stress.

Generally, statins are used as cholesterol-lowering agents worldwide for their action in inhibiting HMG-CoA reductase, resulting in the inhibition of cholesterol biosynthesis. Their strong cholesterol-lowering action contributes to the beneficial effects of statins with large clinical trials demonstrating that they significantly reduce cardiovascular risk.[19]

In addition to reducing cholesterol levels, statins have been shown to affect several important physiological and systemic processes such as endothelial functioning, inflammation, atherosclerosis, oxidative stress, blood coagulation, cellular proliferation, and differentiation in a favorable manner through a cholesterol-independent mechanism.[19,20] Many of these pleiotropic effects of statins are mediated by their ability to block the synthesis of important isoprenoid intermediates, which serve as lipid attachments for a variety of intracellular signaling molecules.

It was found that statins suppressed inflammation and oxidative stress, thereby exerting a protective effect.[21,22] Lovastatin may reduce excitotoxicity during epileptogenesis induced by pilocarpine by increasing the synthesis of IL-10 and decreasing proinflammatory cytokines in the hippocampus.[23] Lovastatin inhibited proliferation of malignant B cells by decreasing the levels of membrane exogenous cholesterol, intracellular reactive oxygen species (ROS), transient receptor potential canonical 6 (TRPC6), and intracellular Ca^{2+}.[24] A high fat diet (HFD), in Sprague-Dawley rats, significantly increased the QTc interval recorded in the electrocardiogram (ECG), heart rate (HR), total cholesterol (TC), triglycerides (TG), low density lipoproteins (LDL), alanine (ALT) and aspartate (AST) transaminases, alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), liver TG, and cardiac and hepatic lipid peroxidation, but decreased antioxidants and high density lipoproteins (HDL); while simvastatin decreased HR, liver TG, serum TC, TG, and LDL and increased HDL in the HFD rats. Vitamin E alone had no effect. Furthermore, simvastatin and vitamin E decreased the QTc interval, serum ALT, AST, ALP, GGT, and cardiac and hepatic lipid peroxidation and increased antioxidants in HFD rats. Simvastatin and vitamin

E slow progression of hypercholesterolemia-induced oxidative stress in liver and heart and improve their functions.[25] Statins may lower intra-mitochondrial ionized calcium that decreases mitochondrial nitric oxide synthase (mtNOS) activity, lowers oxidative stress, prevents the mitochondrial membrane permeability transition (MPT) opening, and prevents the release of cytochrome c from the mitochondria. These results provide a novel framework for understanding the antioxidative properties of statins and their effects on mitochondrial functions.[26] A study was undertaken to evaluate the antioxidant property of lovastatin against H_2O_2 induced oxidative stress in rats. The results indicated that lovastatin can induce reduction in thiobarbituric acid reactive substances and an increase in the activity of the antioxidant enzymes, catalase, and glutathione peroxidase in the liver and heart of rats, suggesting that lovastatin possesses antioxidant activity and reduces oxidative stress.[27]

In the present study, the exposure of primary neurons to a high amount of fluoride resulted in neurotoxicity, including decreased cell viability, inhibited activity of SOD, increased lipid peroxidation, and enhanced apoptosis. Interestingly, pre-treatment of lovastatin attenuated all the cytotoxic parameters induced by fluoride, while the amount employed of the drug did not influence the level of cholesterol in the cells. The results obtained here provide evidence for the protective effect of the lovastatin on fluoride-induced neurotoxicity by a mechanism involving a cholesterol independent pathway.[28]

Many investigations have shown that statins can reduce the level of cellular apoptosis and play an important protective role. Lovastatin protected NRK-52E cells from the cytotoxicity of high doses of the platinating agent cisplatin (CisPt) and reduced the level of kidney DNA damage and apoptosis triggered by CisPt treatment of mice.[29] A study reported that lovastatin significantly decreased the apoptosis rate in lovastatin-pretreated bone marrow stromal cell-derived neural stem cells and suggested that this drug may protect neural stem cells against oxidative stress-induced cell death, and therefore, might be a candidate for treatment of oxidative stress-mediated neurological diseases.[30] In addition, simvastatin can improve cardiac function after myocardial infarction and reduce apoptosis of myocardial cells, possibly by decreasing Bax and Caspase-3 expression and increasing the expression level of Bcl-2.[31] Simvastatin is also crucial in counteracting tumor necrosis factor-α (TNF-α)-induced apoptosis of endothelial progenitor cells (EPCs) and this protection may involve the actions of silent information regulator type-1 (SIRT1).[32] However, some controversial reports indicate that statins may promote apoptosis in tumor growth suppression, by the mechanisms of cell killing and oxidative stress improvement.[33,34]

In the present study, we found that the exposure of neurons to excessive fluoride caused an increased level of apoptosis and that the pre-treatment of the neurons with lovastatin attenuated the fluoride-induced apoptotic rate.

In conclusion, lovastatin may play a protective role against the neurotoxicity induced by excessive amounts of fluoride and may potentially have a role in the treatment of chronic fluorosis.

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