

Differential effects of statins on plasma and brain cholinesterase activities in chicks

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ABSTRACT: Statins used to treat dyslipidemia may differentially modulate cholinesterase (ChE) activity impacting neuronal function. This study examines the effects of three statins (atorvastatin, fluvastatin, and simvastatin) on plasma and brain ChE activities and cholesterol levels in a chick model of 7-14 days old. Chicks were dosed orally with single doses of each statin at 50, 100, and 200mg/kg or repeated doses at 100mg/kg/day for 14 consecutive days. Plasma and whole brain ChE activities were measured electrometrically, whereas cholesterol levels were measured using a commercial colourimetric kit. *In vitro* ChE inhibition by the statins was initiated at 37°C for 10 mins. Data were statistically analysed using analysis of variance followed by the least significant difference test. Atorvastatin and fluvastatin did not significantly affect plasma ChE activities 2 hours after the oral administration, whereas simvastatin at 100 and 200mg/kg significantly increased (28% and 16%, respectively) plasma ChE activity. Repeated oral doses of the statins did not significantly affect plasma ChE activity. However, only simvastatin significantly decreased whole brain ChE activity by 33%. Repeated treatments with the three statins significantly reduced cholesterol levels in the plasma but not in the whole brain. The three statins inhibited *in vitro* plasma and whole brain ChE activities by 10-33% and 8-43%, respectively. The results suggested that the statins differentially modulated ChE activity *in vivo* and *in vitro* in chicks. Additional *in vivo* studies are warranted on statin effects on ChE activity in different brain regions of animal models.

Keywords: Atorvastatin; Fluvastatin; Simvastatin; Cholinesterase; Cholesterol; Dyslipidemia

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1.0 INTRODUCTION

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A which are widely used clinically to treat dyslipidemia, such as hypercholesterolemia which is considered a risk factor for the initiation of atherosclerosis ([Climent et al., 2021](#); [Fan et al., 2021](#)).

Many recent reports have indicated statins' possible beneficial effects in patients with Alzheimer's disease (AD) ([Sparks et al., 2006](#); [Mozayan & Lee, 2007](#); [Kandiah & Feldman, 2009](#); [McGuinness et al., 2016](#)). The mechanism of such an action still needs to be fully understood. Suggestions on the neuronal activities of

statins are related, but not limited to, modifying risk factors for AD, reductions in the oxidative stress status, involvement of cholesterol in the pathophysiology of dementia, protection of alpha7-neuronal acetylcholine nicotinic receptor function from cholinesterase (ChE) inhibitors, or inhibiting the brain ChE activity ([Sparks et al., 2006](#); [Mozayan & Lee, 2007](#); [Roensch et al., 2007](#); [Sharma et al., 2008](#); [Cibickova et al., 2009](#); [Ghodke et al., 2012](#); [Shinohara et al., 2014](#)). Furthermore, statins were found to affect the behavioural outcome in young chicks following challenges with xylazine-ketamine anaesthesia and carbaryl intoxication (unpublished data).

The possibility of ChE inhibition by statins is a vital mechanism to look at since inhibitors of ChE activity were reported to modulate the functional aspects of the brain in a manner that there would be a chance to alleviate cholinergic neuronal deficiency in certain illnesses ([Small, 2005](#); [Sharma, 2019](#); [Vecchio et al., 2021](#)). Because of the side effects associated with ChE inhibitors used in cases of AD with unequal therapeutic responses at times, statins were studied as alternative therapeutics against AD ([Small, 2005](#); [Mozayan & Lee, 2007](#); [Roensch et al., 2007](#); [McGuinness et al., 2016](#)). Some reports even suggested concurrent use of statins with ChE inhibitors to overcome some side effects of the latter therapy ([Sparks et al., 2006](#); [Roensch et al., 2007](#); [Ghodke et al., 2012](#)).

However, conflicting reports exist on the neuropharmacological effects of statins ([Small, 2005](#); [Sparks et al., 2006](#); [Roensch et al., 2007](#); [McGuinness et al., 2016](#)) and their inhibitory actions on plasma or brain ChE activities *in vitro* and *in vivo* ([Roensch et al., 2007](#); [Cibickova et al., 2009](#); [Pytel et al., 2017](#); [Husain et al., 2018](#)). Atorvastatin and rosuvastatin monotherapies were reported to decrease plasma and erythrocyte ChE activities in the blood of patients with coronary artery disease ([Pytel et al., 2017](#)). Rosuvastatin was found to inhibit the ChE activity of rats *in silico* ([Husain et al., 2018](#)). Moreover, simvastatin inhibited brain ChE activity in rats, whereas atorvastatin did not; both drugs did not affect plasma and erythrocyte ChE activities ([Cibickova et al., 2007](#)). Similarly, these two drugs did not affect brain ChE activity in rats after 15 consecutive days of treatment ([Cibickova et al., 2009](#)). In a recent study, simvastatin and fenofibrate increased brain, liver and plasma ChE activities in normolipidemic and hyperlipidemic rats ([Vukšić et al., 2019](#)).

It appears that statins differentially inhibit or increase ChE activities in the blood and possibly the brain ([Darvesh et al., 2004](#); [Cibickova et al., 2007](#); [Macan et al., 2015](#); [Vukšić et al., 2019](#)). Lovastatin and simvastatin inhibited plasma ChE activity, whereas mevastatin and pravastatin did not in patients on lipid-lowering therapy ([Darvesh et al., 2004](#)). Moreover, patients on simvastatin did not suffer from changes in serum ChE ([Zdrengeha et al., 2002](#); [Muačević-Katanec et al., 2005](#)). Based on these studies, current evidence suggests no single unified pharmacological effect of statins on blood or brain ChE activity, as the statins vary in their chemical and physical properties and pharmacological effects ([Bocan, 2002](#); [Zhang et al., 2018](#); [Hirota et al., 2020](#); [Fan et al., 2021](#)).

Therefore, more studies are warranted on the impact of various statins on ChE activity based on the notion that modulating effects of these drugs on blood and brain ChE activities are relatively unexplored, and the outcome could be beneficial in understanding the neuronal actions of these drugs ([McGuinness et al., 2016](#); [Zhang et al., 2018](#)).

The purpose of the present study was to examine the effects of three statins commonly used in clinical practice (atorvastatin, fluvastatin and simvastatin), which differ in their pharmacokinetics and pleiotropic effects, but share a similar common lipid-lowering ability ([Bocan, 2002](#); [Hirota et al., 2020](#); [Fan et al., 2021](#)), on plasma and brain ChE activities in a chick model (7-14 days old) used earlier for monitoring ChE activity *in vivo* and *in vitro* ([Mohammad et al., 2012](#); [Mohammad et al., 2014](#); [Mohammed & Mohammad, 2022a](#)).

2.0 MATERIALS AND METHODS

2.1 Animals

Day-old Cobb broiler chicks of both sexes were obtained from a local hatchery in Duhok, Iraq. They were maintained in batches of 20-30 chicks in an animal quarter with wood shavings as floor litter and 24-hour lighting at 25-30°C. Water and feed were available *ad libitum*. The age of the chicks was between 7-14 days when used in the experiments. Such a model of young chicks has been applied to ChE monitoring studies ([Mohammad et al., 2012](#); [Mohammad et al., 2014](#); [Mohammed & Mohammad, 2022a](#)). The Committee of Postgraduate Studies at the College of Medicine, University of Duhok, Iraq, has approved the present study according to the institutional regulations

on humane animal handling and use in biomedical research according to guidelines set by ARRIVE (<https://www.nc3rs.org.uk/arrive-guidelines>) and the Guide for the Care and Use of Laboratory Animals (<https://www.ncbi.nlm.nih.gov/books/NBK54050/>).

2.2 Drug treatments

The statins and their suppliers were: atorvastatin (Eczacibasi Co., Istanbul, Turkey), fluvastatin (Novartis, Basel, Switzerland) and simvastatin (Alpharma, New Jersey, USA). All drugs were prepared in aqueous solutions as suspensions and administered orally by a gavage needle in a volume of 10mL/kg body weight (Mohammad, 2010). The chicks (12 groups of 10 birds each) were dosed orally with single doses of statins (active ingredient) at the dose rates of 0 (distilled water as control), 50, 100 and 200mg/kg. In another experiment, each statin was dosed orally to chicks (n=10/statin treatment group) at 100mg/kg/day for 14 consecutive days. The statin doses were chosen based on a previous report (unpublished data) and preliminary experiments in chicks without producing overt signs of toxicosis.

2.3 Blood and brain sampling

Two hours after the single doses of the statins and one day after the last dose of the statins given repeatedly for 14 consecutive days, the chicks were bled from the jugular vein to obtain blood samples (0.5-1.0mL, not exceeding 1% equivalency of body weight) into heparinised test tubes (Mohammad et al., 2012; Kelly & Alworth, 2013). The chicks were then euthanised by cervical dislocation (Kammon et al., 2010; Mohammad et al., 2012). The blood plasma was obtained by centrifugation, and each chick's whole brain was excised (Kammon et al., 2010; Mohammad et al., 2012). Plasma and whole brain samples were stored at -20°C for ChE measurement within one week. The whole brain was homogenised with a glass homogeniser in an ice bath using barbital-phosphate buffer solution, pH 8.1 (1.237g sodium barbital, 0.163g potassium dihydrogen phosphate and 35.07g sodium chloride/L of distilled water) at 3mL for every 100mg wet weight (Mohammad et al., 2012; Mohammad et al., 2014; Mohammed & Mohammad, 2022a).

2.4 Determination of ChE activity

We used an electrometric method to measure plasma and whole-brain ChE activities reported earlier in chicks (Mohammad, 2007; Mohammad et al., 2012; Mohammad et al., 2014; Mohammed & Mohammad, 2022a). The ChE reaction mixture consisted of 3mL distilled water, 0.2mL plasma or whole brain

homogenate and 3mL of barbital-phosphate buffer as described above (pH 8.1). The pH1 of the reaction mixture was measured with the glass electrode of a pH meter (Camlab Co., Cambridge, U.K.) before adding 0.1mL of the substrate acetylcholine iodide (7.1%). After incubating the mixture in a water bath at 37°C for 30 min, the pH2 of the reaction mixture was also measured for the second time.

Plasma and the whole brain ChE activities were estimated as follows: ChE activity ($\Delta\text{pH}/30\text{ min}$) = (pH1-pH2) - ΔpH of blank (no plasma or whole brain samples).

The percentage of ChE inhibition was estimated as follows: %ChE inhibition = [ChE activity (without statin) - ChE activity (statin) / ChE activity (without statin)] X 100

2.5 Determination of cholesterol level

As a control measure for the hypolipidemic effectiveness of the statins (atorvastatin, fluvastatin, simvastatin) used in the present study, plasma and whole brain homogenate (described above) of chicks treated with each of the three statins at 100mg/kg/day for 14 consecutive days were assayed 24h after the last dosing for cholesterol level using a commercial assay kit (Biolabo SA, Maizy, France).

2.6 In vitro ChE effects of statins

Plasma samples and whole brains were obtained from each of the ten untreated chicks to detect *in vitro* effects of the statins on ChE activities. These samples were pooled separately, and duplicate plasma and whole brain homogenate aliquots were used for the *in vitro* ChE inhibition assay, incubating the assigned statin with the enzyme source described before (Mohammad, 2007; Mohammad et al., 2014; Mohammed & Mohammad, 2022b). To achieve this, an aliquot of 0.1mL of the aqueous solutions of each statin was added to the ChE reaction mixture, which contained the plasma or whole brain homogenate (Mohammad, 2007; Mohammad et al., 2014). The final concentration of each statin in the ChE reaction mixture was either 0 (baseline vehicle control), 10, 25, 50 or 100µmole/L for the plasma and the whole brain. ChE reaction mixtures of the control (baseline) or the statins were incubated at 37°C for 10 mins in a water bath for *in vitro* ChE inhibition. The residual ChE activity in each plasma or whole brain homogenate reaction mixture was measured electrometrically, as described above. The percentage of *in vitro* ChE

inhibition in the plasma or whole brain samples was estimated as described above.

2.7 Statistical analysis

Parametric data presented as multiple means were statistically analysed by the one-way analysis of variance followed by the least significant difference test (Petrie & Watson, 2013). We used the statistical software SPSS (IBM) to analyse the data. The level of statistical significance was set at $p < 0.05$.

3.0 RESULTS

3.1 Treatment effects

Oral dosing of chicks with atorvastatin and fluvastatin at 50, 100 and 200mg/kg, given once, did not significantly ($p > 0.05$) affect plasma ChE activity (mean $\pm$ standard deviation (SE) ranged between 0.65 ± 0.07 to 0.88 ± 0.04 Δ pH/30 min) when measured 2 hours after the administration and compared to the respective control values (0.72 ± 0.03 and 0.79 ± 0.04) (Table 1). The percentages of variation in plasma ChE activity induced by atorvastatin and fluvastatin treatments from respective control values ranged between -10% to 11%. However, simvastatin at 100 and 200mg/kg significantly ($p < 0.05$) increased plasma ChE activity (0.64 ± 0.05 and 0.58 ± 0.07 Δ pH/30 min) compared with those of the control group (0.50 ± 0.08) by 28% and 16%, and with the 50mg/kg dose group (0.49 ± 0.06) by 30% and 18%, respectively (Table 1).

Table 1. Effects of single doses of statins on plasma cholinesterase (ChE) activity in chicks

Treatment (mg/kg, orally)	ChE activity (Δ pH/30 min)	% change from respective control
Atorvastatin		
0	0.72 ± 0.03	0
50	0.65 ± 0.07	- 10
100	0.70 ± 0.04	- 3
200	0.75 ± 0.05	+ 4
Fluvastatin		
0	0.79 ± 0.04	0
50	0.86 ± 0.05	+ 9
100	0.88 ± 0.04	+ 11
200	0.85 ± 0.15	+ 8
Simvastatin		
0	0.50 ± 0.08	0
50	0.49 ± 0.06	- 2
100	$0.64 \pm 0.05^*$	+ 28
200	$0.58 \pm 0.07^*$	+ 16

The enzyme activity was determined 2 hours after the statin dosing. Values are mean $\pm$ SE of 10 chicks/group. *Significantly different from the respective 0 (control) and 50mg/kg dose groups, $p < 0.05$.

On the other hand, repeated oral dosing of the statins (atorvastatin, fluvastatin, simvastatin) at 100mg/kg/day for 14 consecutive days did not significantly ($p > 0.05$) affect the plasma ChE activity of the chicks (0.60 ± 0.05 , 0.60 ± 0.05 and 0.59 ± 0.02 vs the control 0.51 ± 0.04 Δ pH/30 min), as the percentages of change from the control value ranged between 16% to 18% (Table 2). However, only simvastatin significantly ($p < 0.05$) decreased whole brain ChE (0.43 ± 0.08) activity by 33% compared to that of the control group (0.64 ± 0.08) at the end of the 14-daily doses (Table 2).

Table 2. Effects of repeated daily doses (100 mg/kg, orally) of statins for 14 consecutive days on plasma and whole brain cholinesterase (ChE) activities in chicks

Treatment (mg/kg/day, orally)	Plasma ChE activity (Δ pH/30 min)	Whole brain ChE activity (Δ pH/30 min)
Control (distilled water)	0.51 ± 0.04	0.64 ± 0.08
Atorvastatin	0.60 ± 0.05	0.50 ± 0.06
Fluvastatin	0.60 ± 0.05	0.52 ± 0.06
Simvastatin	0.59 ± 0.02	$0.43 \pm 0.08^*$

The enzyme activity was determined 24 hours after the last statin dosing. Values are mean $\pm$ SE of 10 chicks/group. *Significantly different from the respective control group, $p < 0.05$.

3.2 Effects of statins on plasma and whole brain cholesterol levels

All three statins (atorvastatin, fluvastatin and simvastatin) administered to chicks for 14 consecutive days significantly ($p < 0.05$) decreased plasma cholesterol levels (90 ± 10 , 84 ± 7 , and 93 ± 9 mg/100mL, respectively) in comparison with the respective control value (170 ± 37) (Table 3). The percentages of decrease in plasma cholesterol level by the three statins were 47%, 51%, and 45%, respectively. The three statins did not significantly affect ($p > 0.05$) the whole brain cholesterol (mg/g) level (22 ± 6 , 24 ± 4 , and 20 ± 3 , respectively) when compared with that of the control group (29 ± 5) (Table 3). Nonetheless, the percentages of decrease in brain cholesterol level by the three statins were 24%, 17%, and 31%, respectively.

3.3 Effects of statins on plasma and whole brain ChE activities *in vitro*

In vitro exposure of plasma ChE to the three statins (atorvastatin, fluvastatin, and simvastatin at 10, 25, 50, and 100 μ mole/L) for 10 min at 37 °C inhibited, in a

concentration-dependent manner, the enzyme activity by 10-33%, 16-27% and 17-24%, respectively (**Figure 1a**). Using the whole brain ChE, percentages of enzyme inhibition *in vitro* by the three statins at 10, 25 and 50, and 100µmole/L were concentration-dependently 0-35%, 0-43%, and 0-41%, respectively (**Figure 1b**).

Table 3. Effects of repeated daily doses (100 mg/kg, orally) of statins for 14 consecutive days on plasma and whole brain cholesterol levels in chicks

Treatment (mg/kg/day, orally)	Plasma cholesterol (mg/100mL)	Whole brain cholesterol (mg/g)
Control (distilled water)	170 ± 37	29 ± 5
Atorvastatin	90 ± 10*	22 ± 6
Fluvastatin	84 ± 7*	24 ± 4
Simvastatin	93 ± 9*	20 ± 3

Footnotes: Cholesterol level was determined 24 h after the last statin dosing. Values are mean ± SE of 10 chicks/group.

*Significantly different from the respective control group, $p < 0.05$.

4.0 DISCUSSION

The results of no inhibitory effect of the single-dose statin treatments on ChE activity in chicks are similar to findings reported in rats ([Cibickova et al., 2007](#); [Cibickova et al., 2009](#)). However, conflicting reports exist on the effects of statins on blood or liver ChE activities, being of differential effects, no effect, or even inhibitory or incremental effects ([Darvesh et al., 2004](#); [Cibickova et al., 2007](#); [Cibickova et al., 2009](#); [Vukšić et al., 2019](#)).

The present single-dose results and those of other reports prompted us to examine the effects of repeated statin treatments on plasma and whole-brain ChE activities in chicks. The findings of the present study in a chick model investigating ChE activity demonstrated differential changes in the ChE activity of the chicks depending on the type of the statin and duration of the oral dosing (single vs repeated).

The present study determined plasma and brain ChE activities, representing the pseudo and true ChEs, respectively, excluding erythrocytes devoid of ChE activity in the avian species, including the chicken ([Donovan & Zinkl, 1994](#); [Wilson et al., 2005](#)). Such a differential effect of certain statins has been reported earlier by others ([Cibickova et al., 2007](#); [Cibickova et al., 2009](#); [Vukšić et al., 2019](#)). These results altogether need further systematic confirmation taking into

account various types of statins, especially those with potential central nervous system effects.

Within this context are suggestions in the literature for the potential use of statins in patients with AD regardless of the need for the hypocholesteremia effects ([Sparks et al., 2006](#); [Kandiah & Feldman, 2009](#); [Butterfield et al., 2011](#); [McGuinness et al., 2016](#); [Zhang et al., 2018](#)). Based on the present results regarding the effects of statins on ChE activity following drug type and duration of therapy as well as on the results of other investigators ([Darvesh et al., 2004](#); [Cibickova et al., 2007](#); [Roensch et al., 2007](#); [Vukšić et al., 2019](#)), caution should be practised in generalising this notion on all statins, which differ considerably in their pharmacokinetic, pharmacodynamics, and even neurotoxic profiles ([Bocan, 2002](#); [Butterfield et al., 2011](#); [Zhang et al., 2018](#); [Hirota et al., 2020](#)). The statins vary greatly when pseudo and true ChE are considered for any activity modulation ([Zdrenghea et al., 2002](#); [Darvesh et al., 2004](#); [Muačević-Katanec et al., 2005](#); [Cibickova et al., 2007](#); [Cibickova et al., 2009](#); [Macan et al., 2015](#); [Vukšić et al., 2019](#)). Therefore, extensive preclinical studies are needed on different types of statins, considering their anti-ChE and potential neurotoxic effects.

Furthermore, in the present study and support of the notion of the differential effects of the statins on plasma ChE, we noticed that all the statins administered to chicks for 14 consecutive days significantly decreased plasma cholesterol levels by 45-51% (**Table 3**). Brain cholesterol levels decreased, though non-significantly, by 17-31%. Statins, however, may affect brain cholesterol levels and other cerebrovascular events by a mechanism not yet defined but could be indirectly through reducing plasma lipid levels ([Motti et al., 2000](#); [Butterfield et al., 2011](#); [Cibickova, 2011](#)). Additional studies are needed on statins taking into account their potential anti-ChE activity in association with the well-known hypocholesteremia effects.

Several factors appear to be involved in the reported differential effects of statins on ChE activity. These include but are not limited to the inherent structural properties of the statins, the dosage and duration of therapy, species variation, and the existence of cholesterolemia ([Cibickova et al., 2007](#); [Cibickova et al., 2009](#); [Husain et al., 2018](#); [Vukšić et al., 2019](#)). It is reasonable to assume, additionally, based on our findings, that repeated simvastatin treatment modulates brain (true) ChE activity in chicks. This

situation is close to clinically prolonged daily therapy with statin (Sirtori, 2014; Zhang et al., 2018).

considerations (Bocan, 2002; Sirtori, 2014; Zhang et al., 2018; Hirota et al., 2020).

The limitations of the present study may lie in the fact that the neurotoxic potentials of the statins were not examined in the young chick model, and true ChE activity and cholesterol level should have been determined in different regions of the brain, as the present results indicated potential effects on the brain. Future experimental studies in laboratory animals may consider these suggestions. Further, we used the avian species as an animal model (young chicks) in the present study, and any extrapolation to the mammalian species (e.g., rodents) should be under extreme scientific scrutiny.

5.0 CONCLUSIONS

Overall, the results of the present study, taken together in the context of those of others, suggest the possibility of modulating the effects of cholesterol-modifying statins on ChE activity, which in turn might affect the outcome of brain function. Examining ChE inhibition *in vitro* could be helpful for any initial screening and assessment of statins intended to be administered for non-dyslipidemia. Despite the limitations of direct extrapolations of animal studies (chicks in the present study) to humans, additional *in vivo* studies on statin effects on ChE activity in different brain regions of animal models are certainly warranted.

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Conflicts of Interest: The authors declare no conflict of interest.

In vitro exposure of plasma and whole brain ChEs to the three statins (atorvastatin, fluvastatin, simvastatin) inhibited, in a concentration-dependent manner, the activities of both enzymes of the chicks (Figure 1). This finding supports the reported *in vitro* inhibitory effects of simvastatin and lovastatin on human plasma ChE activity (Roensch et al., 2007). The *in vitro* results also confirm that statins can be examined for their inhibitory action against pseudo (plasma) and true (brain) ChE activities without any interference with drug metabolism and other pharmacokinetic

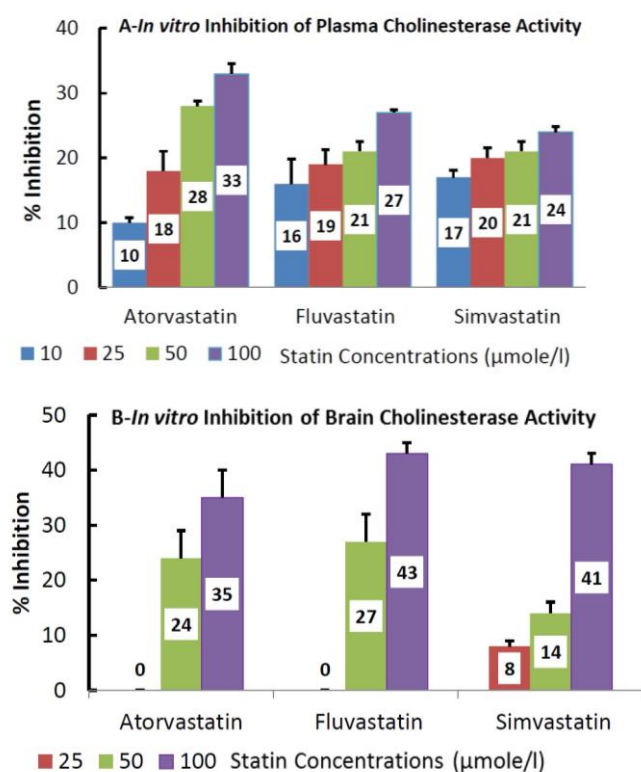


Figure 1. Inhibition of chick (a) plasma and (b) whole brain cholinesterase activities after *in vitro* incubation at 37°C for 10 minutes with statins. Values are mean ± SE.

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