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College of Veterin ary Medicine

Assessment of neurobehavior, oxidative stress status and cholinesterase activity in male mice treated with some statins

Rawnaq Faris Abdulqader Hasan

Ph.D. Dissertation

Veterinary Medicine / Veterinary Pharmacology

Supervised by

Professor

Dr. Fouad Kasim Mohammad

2024 A.D. **1446 A.H.**

Assessment of neurobehavior, oxidative stress status and cholinesterase activity in male mice treated with some statins

A Dissertation submitted by

Rawnaq Faris Abdulqader Hasan

To

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In

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For the Degree of Doctor of Philosophy

In

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Professor

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صدق الله العظيم

سورة الاسراء

الاية: ٨٥

Supervisor Certification

I certify that this dissertation entitled (**Assessment of neurobehavior, oxidative stress status and cholinesterase activity in male mice treated with some statins**) was prepared under my supervision at the College of Veterinary Medicine / University of Mosul in partial fulfillment of the requirements for the degree of Ph.D. in Veterinary Medicine/Veterinary pharmacology.

Signature:
Name: **Prof. Dr. Fouad Kasim Mohammad**
Date: `17 / 9 /2024

Linguist Certification

I certify that this dissertation entitled (**Assessment of neurobehavior, oxidative stress status and cholinesterase activity in male mice treated with some statins**) has been linguistically reviewed and corrected for language and expression mistakes; therefore, it becomes ready for the defense as far as the integrity of the writing and expression.

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Based on the supervisor and linguistics recommendations, I forward this dissertation for the defense.

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Postgraduate Committee Director Certification

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Examination Committee Decision

We, the members of the Evaluation and Discussion Committee, have reviewed this dissertation and discussed the student **Rawnaq Faris Abdulqader Hassan** in its contents on 25 / 11 / 2024, and certify that it deserves the degree of Ph.D. in Veterinary Medicine/Veterinary Pharmacology.

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Member	Member
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Professor	Professor
Dr. Mahmood Basher Mahmood	**Dr.Banan Khalid Abdulrahman**
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Professor	Professor
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Chairman	Member and Supervisor

College Council Decision

The Council of the College of Veterinary Medicine, University of Mosul has convened for the meeting on / / 2024 and decided to award **Rawnaq Faris Abdulqader Hassan** a Ph.D. degree in Veterinary Medicine/Veterinary pharmacology.

Prof.Dr. Raad AbdulghanyAl-Sanjary	**Prof.Dr. DhaferMohammad Aziz**
Assistant Dean for Scientific Affairs	Dean of the College
/ / 2024	/ / 2024

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Abstract

The hypolipidemic statins have been associated with various side effects in humans and laboratory animals. There is limited information on the neurobehavioral cholinergic oxidant/pro-oxidant effects of statins in mice. The present study aimed to examine changes in neurobehavioral performance, cholinesterase (ChE) activity and oxidative stress in mice treated with atorvastatin, simvastatin, and rosuvastatin.

Materials and Methods: Male mice were treated orally with various doses of statins (atorvastatin, simvastatin, and rosuvastatin). Control mice were treated with distilled water. Section1: Two hours after vehicle (control) or statin dosing at 250, 500, 750, or 1000 mg/kg, each mouse was subjected to 5 min open-field activity, negative geotaxis at an angle of 45°/60 s, 5 min head pocking, and forced swimming endurance. Plasma, erythrocyte, and brain ChE activities were determined spectrophotometrically at 2 and 24 h after oral dosing of statins at 500 and 1000 mg/kg. Section 2: Mice were dosed with statin single doses at 500 or 1000 mg/kg. Mice were also dosed with repeated daily doses of each statin at 200 mg/kg/day for 14 or 28 days. Brain and plasma glutathione (GSH) and malondialdehyde (MDA) levels, alanine transaminase (ALT) and aspartate transaminase (AST), were determined. Section 3: Mice were treated with each statin (200 mg/kg/day) for 28 days. 24 h after the last statin or control dosing, mice were subjected to a pharmacological challenge with propofol at 100 mg/kg, intraperitoneally or to a toxicological challenge with dichlorvos at 150 mg /kg, orally. Propofol anesthesia and dichlorvos cholinergic toxidrome and brain ChE activity were monitored. Section 4: Mice were dosed with each statin at 200 mg/kg/day for 14- and 28 days. 24 h after the last 14- or 28-day dosing, each mouse was tested for neurobehavioral performance and ChE

determination mentioned above. Section 5: In vitro aqueous concentrations of each statin were used on the plasma and whole brain homogenate at concentrations of 0 (baseline-control), 10, 25, 50 or 100 μM. The inhibition of plasma and brain ChE activity was determined electrometrically and spectrophotometrically in the brain together with the determination of generation of oxidative stress (brain MDA).

Results: section 1: The statins variably, but dose-dependently and significantly delayed the latency to move in the open-field area, decreased locomotion and rearing, reduced head pocking, and delayed negative geotaxis performance. Statins significantly increased the duration of forced swimming and reduced the duration of swimming immobility.

Statins significantly and dose-dependently decreased plasma, erythrocyte, and brain ChE activity 2 and 24 h after dosing. Section 2: Single statin doses at 500 or 1000 mg/kg significantly and dose-dependently reduced the GSH level in the plasma and the whole brain. Statins at 200 mg/kg/day for 14 or 28 days significantly and time-dependently reduced plasma and brain GSH levels. MDA levels significantly increased in the plasma and brain following single or repetitive dosing. Activities of ALT and AST were also increased in the plasma. Section 3: Statins significantly decreased the onset of propofol sleep and reduced its duration. Statins significantly increased the onset of signs of poisoning, delayed the onset of death and decreased signs of poisoning, toxicity score and death. Statins significantly reduced whole brain ChE activity. Section 4: Repeated statin treatments significantly decreased open-field activities and head pocking, and increased the durations of negative geotaxis and forced swimming endurance with reduction of immobility duration. Plasma, erythrocyte and whole brain ChE activities were significantly reduced. Section 5: Statins at 10 to 100

μM in vitro significantly inhibited plasma and brain ChE activity (determined electrometrically) in a concentration-dependent manner.

Hydrogen peroxide significantly increased brain MDA level In Vitro. Statins at 25 to 100 μM significantly increased MDA level in whole brain homogenates in a concentration-dependent manner. Brain ChE activity, determined spectrophotometrically, by in vitro statins (25 to 100 μM) was significantly inhibited. Hydrogen peroxide also decreased brain homogenate ChE activity by 66.3, 68.7 and 69.8%, respectively.

Conclusion: Single and repeated doses of statins differentially modulate neurobehavioral outcomes in mice in association with reduced plasma, erythrocyte, and brain ChE activity. Plasma or erythrocyte ChE may be used for biomonitoring of the adverse/therapeutic effects of statins. Statins induced oxidative stress as adverse effects on the brain and plasma of mice, which suffered from the additional burden of liver injury. The results support pharmacological and toxicological challenges in statin-treated mice to uncover changes in responses to propofol anesthesia and dichlorvos intoxication. These effects could be the basis of in-depth explorations of statin adverse effects in experimental animals and to find an animal model, probably the mice, for the induction of adverse effects of statins that target the brain, as well as to shed light on potential statin intolerance outcomes in this species. As in vitro results complemented the in vivo effects, anti-ChE and oxidative stress effects of statins may be involved in their adverse/pleiotropic actions.

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List of Abbreviations

1	HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
2	LDL	Low Density Lipoprotein
3	AD	Alzheimer's Disease
4	ChE	Cholinesterase
5	AChE	Acetylcholinesterase
6	BuChE	Butyrylcholinesterase
7	PUFA	Polly Saturated Fatty Acid
8	NO	Nitric Acid
9	GSH	Glutathione
10	MDA	Malondialdehyde
11	ALT	Alanine aminotransferase
12	AST	Aspartate aminotransferase
13	I.p.	Intraperitoneal
14	Bpb	Barbital-phosphate buffer

Chapter One

Introduction

Chapter One

Introduction

Statins are a category of drugs with different pharmacokinetic, pharmacodynamic, and pleiotropic characteristics that are clinically used in humans mainly for the treatment of hypercholesterolemia (Hirota *et al*., 2020; Climent *et al*., 2021). They lower blood lipids by inhibiting the activity of hydroxyl-methyl-glutaryl-CoA reductase, a rate-limiting enzyme in cholesterol synthesis found in the liver (Climent *et al*., 2021; Arvanitis and Lowenstein,2023:Mbou, 2024) . These statins vary in their structures and chemical and physical properties (Hirota *et al*., 2020; Climent *et al*., 2021). Numerous studies have shown intriguing aspects of statins that are not related to their hypolipidemic effects, such as actions on the central nervous system and peripheral neuromuscular functions (McGuinness *et al*., 2016; Hirota *et al*., 2020; Attardo *et al*., 2022). These actions include modulation of the central and peripheral cholinergic system (Cibickovà *et al*., 2007; Roensch *et al*., 2007; McGuinness *et al*., 2016). The type of cholinesterase (ChE) in the plasma is pseudo ChE (EC 3.1.1.8), whereas those of the brain and erythrocytes are true ChE (EC 3.1.1.7). The enzyme is usually monitored for inhibitory effects of medications such as those used against Alzheimer's disease (Reuben *et al*., 2024), and organophosphate or carbamate toxicants used as insecticides (Wilson, 2014). Statins have been shown to have different effects on blood or brain ChE activity (Roensch *et al*., 2007; Macan *et al*., 2015). These actions could be pertinent to the type of statin in use, dosage applied, response of the animal species vs. humans, and the type of ChE in the blood or brain tissue-true vs. pseudo-ChE (Cibickova *et al*., 2007; Husain *et al*., 2018; Rashid and Mohammad, 2023a). Interestingly, the effects of different statins on ChE activity do not appear to be consistent

in both in vitro (Roensch *et al*., 2007; Fong, 2014; Rashid and Mohammad, 2023a) and in vivo studies (Cibickova *et al*., 2007; Husain *et al*., 2018; Rashid and Mohammad, 2023a). The conflicting effects of statins on plasma, erythrocyte, and brain ChE activities have been reported in the form of either an increase, decrease, or no effect (Cibickova *et al*., 2007 ; Husain *et al*., 2018; Rashid and Mohammad, 2023a).

Statins are characterized by a wide margin of safety; however, side effects or even adverse outcomes in association with acute and chronic treatment regimens have been reported clinically (Sakaeda *et al*., 2011; Hirota *et al*., 2020; Attardo *et al*., 2022). These adverse effects can also be considered as undesirable effects, and they have been found to be associated with repeated uses of these statins, such as myotoxicity, liver injury, kidney dysfunction as well as alterations in blood and tissue biochemical indices (Darvesh *et al.*, 2004; Tatley and Savage, 2007; Sakaeda *et al.*, 2011; Sirtori, 2014; Pal *et al.*, 2015; Attardo *et al.*, 2022). Additionally, studies have reported statin intolerance, which is a condition not related to drug dosage or its duration of therapy (Alonso *et al.*, 2019; Bytyçi *et al.*, 2022). Within this context, and in the light of pleiotropic effects of statins and their apparent wide margin of safety (Profumo *et al.*, 2014; Sitori, 2015; Sørensen *et al.*, 2019), several animal models have been used to address undesirable effects of various statins that differ pharmacokinetically and pharmacodynamically (Hirota *et al*., 2020; Climent *et al*., 2021; Mbou, 2024).

Considering that the anti-ChE effects of statins were not consistent across the statins/tissues or species examined (Cibickovà *et al*., 2007; Husain *et al*., 2018), another aspect of the pleiotropic effects of statins is behavioral modulation in patients and laboratory animals receiving statin therapy. Many studies in experimental animals have also supported and

documented adverse effects of statins, such as neurobehavioral and locomotive changes (Vukšić *et al.*, 2019; Hai-Na *et al*., 2020; Xu *et al*., 2021; Rashid and Mohammad, 2023a), impairment of neuromuscular function (Bouitbir *et al*., 2011), and alterations in the cholinergic system (Vukšić *et al.*, 2019; Rashid and Mohammad, 2023b). Specifically, satins were reported to cause changes in the cholinergic system and reduce blood or brain cholinesterase (ChE) activity in rats (Cibicková *et al.*, 2007; Vukšić *et al.*, 2019) and chicks (Rashid and Mohammad, 2023a). These compounds also cause neuromuscular dysfunction in rats (Bouitbir *et al*., 2011). Others have reported increased plasma ChE activity and decreased malondialdehyde (MDA) level in statin-treated rats (Macan *et al.*, 2015). Furthermore, examining the neurobehavioral functional aspects of experimental animals treated with statins has shown various behavioral performance alterations such as cognitive impairment in rats (Husain *et al.*, 2018), memory dysfunction in mice (Ghodke *et al.*, 2012), depressive behaviors in mice (Hai-Na *et al.*, 2020) reductions in general locomotion, changes in swimming performance and epilepsy (Oliveira *et al.*, 2018) in mice, as well as modulation of social behavior in rats (Durankuş *et al.*, 2023). Behavioral effects also included aggression, depression, memory loss, and confusion (Rahola, 2012; Leppien *et al*., 2018). These neurobehavioral experimental studies have been reported in laboratory animals, such as mice, rats, and young chicks treated with different statins (Ghodke *et al*., 2012; Durankuş *et al*., 2023). Additionally, it was also reported, recently, that statins reduce the duration of anaesthetic action of xylazine-ketamine in chicks, with reductions of the toxicity outcome of the reversible ChE inhibitor carbaryl (Rashid and Mohammad, 2023b). This latter approach of pharmacological and toxicological challenges has been used when there are no overt actions of drugs in experimental animals, but rather

changes appear when they are subjected to challenges with drugs or toxicants (Frankel *et al.*, 2007; Rashid and Mohammad, 2023b). These effects collectively suggest the involvement of the CNS in the adverse effects of statins.

Various mechanistic investigations have indicated the involvement of several exacerbated biochemical events in the induction of statin intolerance, adverse effects, and toxicity. This is in the light of current evidences that show statins to modulate cognitive functions, reduce the risk of dementia and modulate depression, possibly through multiple neuronal mechanisms that involve monoamines, amino acids, neuropeptides and the cholinergic associated pathways with the inhibition of brain ChE activity (Jasińska *et al.*, 2007). For example, adverse/toxic outcomes of statins may include reduced blood or tissue ChE activities (Vukšić *et al.*, 2019; Rashid and Mohammad, 2023b), hyperkalemia (Deska and Nowicki, 2017), impaired mitochondrial function (Kwak *et al.*, 2012), and generation of reactive oxygen species causing oxidative stress (Liu *et al.*, 2019).

The oxidative stress was found to be an additional burden on the CNS with statin adverse effects following in vivo treatment with these drugs (Bouitbir *et al.*, 2011; Ahmadi *et al.*, 2020). Nevertheless, the antioxidant effects of statins have been reported as well (Beltowski, 2005; Macan *et al.*, 2015; Mansouri *et al.*, 2022). It also appears, however, that such antioxidant/pro-oxidant effects of statins, which vary considerably, could be dose-dependent and/or tissue specific (Beltowski, 2005; Bouitbir *et al.*, 2011; Liu *et al.*, 2019). Within this context, the current evidences consider oxidative stress of statins as part of their tissue-induced damage (Beltowski, 2005; Bouitbir *et al.*, 2011; Liu *et al.*, 2019; Ahmadi *et al.*, 2020), which might have contribution to the reported statin-intolerance (Meza-Contreras *et al.*, 2023; Tsushima and

hatipoglu, 2023). While the in vitro ChE inhibitory effects of statins matched their in vivo effects (Husain *et al.*, 2018; Vukšić *et al.*, 2019; Rashid and Mohammad, 2023a), discrepancies have been reported though, in the sense that in vitro oxidative effects of statins present therapeutic as well as adverse effects challenges that could be specific to tissues and/or the type of statin involved (Darvesh *et al.*, 2003; Cibickovà *et al.*, 2007; Roensch *et al.*, 2007; Macan *et al.*, 2015; Vukšić *et al.*, 2019; Rashid and Mohammad, 2023a). The in vitro oxidative effects of statins have been demonstrated using the heart (Sodha *et al.*, 2008), oral squamous cells (Biselli-Chicote *et al.*, 2019), as well as other internal organs such the liver and kidneys and skeletal muscles (Bouitbir *et al.*, 2011; Kwak *et al.*, 2012; Liu *et al.*, 2019). However, by contrast, other sets of in vitro studies reported antioxidant properties of statins without involving the brain tissue per se (Acheampong *et al.*, 2007; Schupp *et al.*, 2008). Hepatocellular liver injury with concomitant elevations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase have been seen during various therapeutic applications of statins irrespective of the duration of therapy (Averbukh *et al.*, 2024) . However, it has been argued that the therapeutic benefits of statins could be related to their antioxidant actions, which are also implicated in the pleiotropic effects or tissue damage of these drugs (Profumo *et al.*, 2014; Sørensen *et al.*, 2019).

Limited information is available on statins regarding possible collective behavioral changes in laboratory animals with alterations in blood and brain ChE activities as well as oxidative/anti-oxidative effects. Within this context, examining statins' toxicity or adverse effects became warranted in light of the withdrawal of one statin from clinical use (Tobert, 2003) and the actual clinical or perceived statin adverse effects (Sakaeda *et al*., 2011; Hirota *et al*., 2020; Attardo *et al*., 2022;), as

well as statin intolerance among certain hyperlipidemic patients (Alonso *et al*., 2019; Bytyçi *et al*., 2022). Further, these studies cited above have shown that various neuronal and functional-behavioral aspects of statins in experimental animals are necessary to be explored to understand possible undesirable effects of these drugs. This is important in the light of suggestions to further characterize and explore undesirable effects or even pleiotropic effects of statins in animal models, because statin-induced intolerance is being implicated in various adverse outcomes of these medications (Alonso *et al.*, 2019; Bytyçi *et al.*, 2022). It appears that complex contradictory features of statins' effects involve the ChE activity and oxidative stress, whether they are of therapeutic or adverse outcomes (Cibickovà *et al*., 2007; Liu *et al* ., 2019; Sørensen *et al*., 2019; Vukšić *et al*., 2019; Rashid and Mohammad, 2023a). This is in connection with statins' cholinergic and/or prooxidant-antioxidant impacts (Liu *et al*., 2019; Vukšić *et al*., 2019; Shokoples, 2022; Rashid and Mohammad, 2023a), and the search for animal models of statins adverse effects and possibly intolerance phenomenon (Ghodke *et al.*, 2012; Ludka *et al.*, 2014; Husain *et al.*, 2018; Vukšić *et al.*, 2019; Okudan and Belviranli, 2020). Therefore, in depth experimental explorations of statin effects become warranted.

Aim of the study

The present study in mice aimed to collectively examine and ascertain possible adverse neurobehavioral changes induced by three statins (atorvastatin, simvastatin, and rosuvastatin), commonly used in clinical practice, by considering changes in blood and brain ChE activities, as well as the levels of brain and plasma malondialdehyde (MDA, an index of lipid peroxidation), a metabolic biomarker of

oxidative stress, and the antioxidant peptide glutathione (GSH) with concurrent evaluation of liver enzymes ALT and AST.

Objectives of the study

Specifically, the present study examines the following points of evaluations of the impacts of the three statins (atorvastatin, simvastatin, and rosuvastatin) given in single or repeated doses in mice:

1. Neurobehavioral outcome.
2. Blood and brain ChE activity.
3. Blood and brain MDA and GSH levels.
4. Plasma ALT and AST activities.
5. Pharmacological and toxicological challenges.
6. Plasma and brain cholesterol levels.
7. In vitro brain ChE inhibition by statins.
8. In vitro pro-oxidant action of statins on the brain.
9. In vitro action of hydrogen peroxide, as a positive pro-oxidant agent, on brain ChE activity and MDA level for comparison with statins.

Chapter Two

Review of Literatures

Chapter Two

Review of Literatures

2-1 : Lipid Lowering Statins

These compounds are structural analogs of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA reductase). Lovastatin, atorvastatin, fluvastatin, pravastatin, simvastatin, and rosuvastatin belong to this class (Katzung, 2015; Tall *et al*., 2022; Salih and Al-Khashab, 2023). The 3-hydroxy 3-methylglutaryl-coenzyme A reductase inhibitor or statins have been a primary force in the management of hypercholesterolemia for many years and are important in the primary and secondary prevention of heart disease (Brunton *et al*., 2018). However, statins vary considerably in their pharmacodynamics and pharmacokinetic characteristics, with marked pleiotropic effects that differ from the known actions targeting hypercholesteremia (Hirota *et al*., 2020; Climent *et al*., 2021).

2-1-1 : Chemistry of statins

The chemical structures of statins vary, with some being derived from fungi or synthetics such as Lovastatin, pravastatin and simvastatin and others being fully synthetic such as pravastatin, pitavastatin and rosuvastatin (Weng *et al*., 2010 ; Riste *et al*., 2015; Di Bello *et al*., 2020).

Lovastatin, pravastatin and simvastatin are fungal derived inhibitors of HMG-CoA reductase, while atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are fully synthetic compounds (Riste *et al*., 2015; Belo *et al*., 2020) .

The statins can be broken down into three main components: an analogue of the target enzyme substrate HMG-CoA, a hydrophobic ring structure covalently linked to the substrate analog, and side groups on the rings. These components play key roles in how the statins interact with

the reductase enzyme (Belo *et al*., 2020; Climent *et al*., 2021)Lovastatin and simvastatin are inactive lactone prodrugs that are hydrolyzed in the gastrointestinal tract to the active β-hydroxyl derivatives, whereas pravastatin has an open, active lactone ring (Murphy *et al*., 2020; Xu *et al*., 2021). Atorvastatin, rosuvastatin, and fluvastatin are fluorine-containing congeners that are active as given (Katzung, 2018; Althanoon *et al*., 2020)(Figer 2-1).

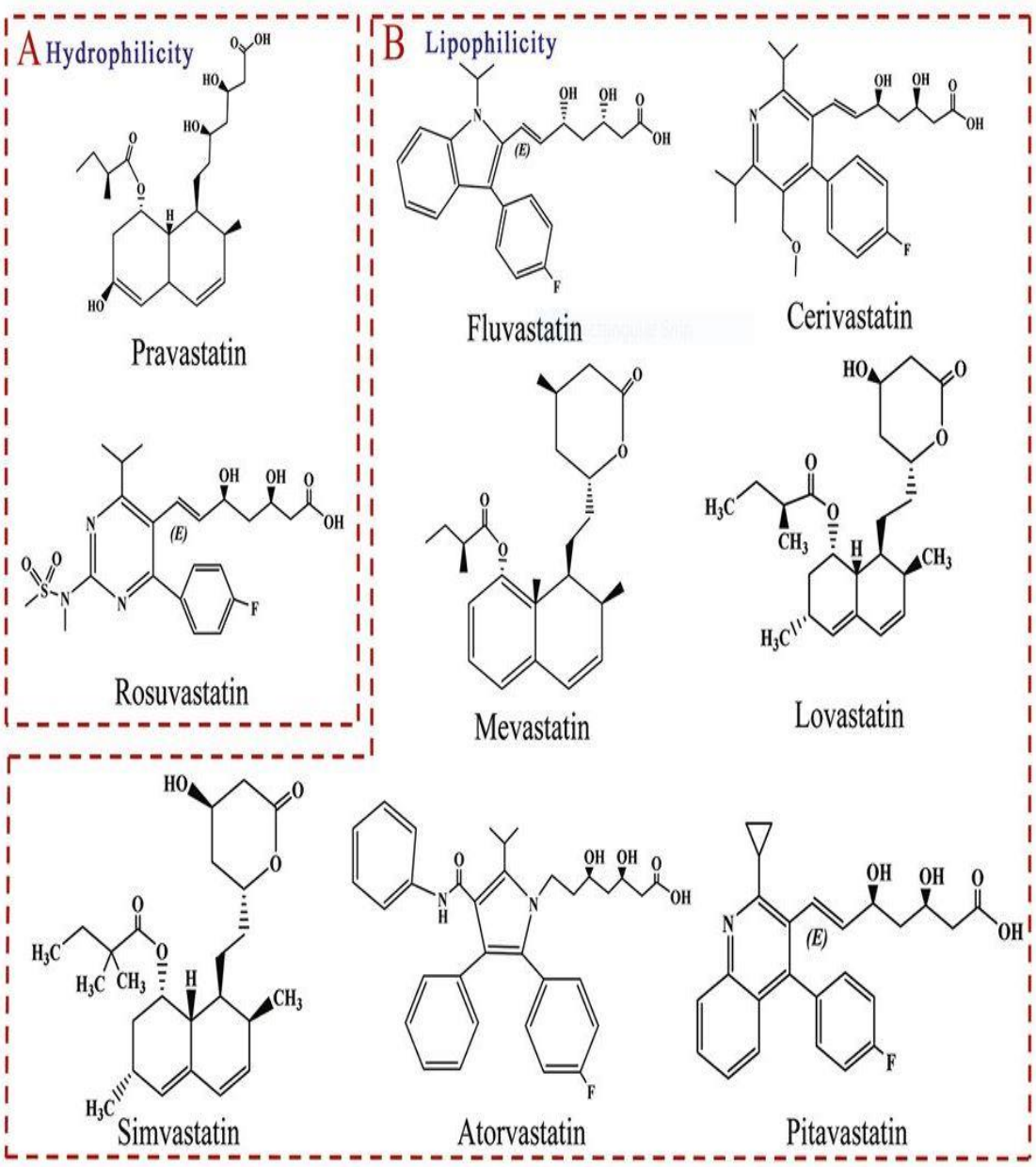

Figure (2-1) The structure of statins. Adapted from Liu *et al*., (2019).

2-1-2 : Mechanism of action of statins

Statins, also known as 3-Hydroxy-3-methylglutaryl (HMG) coenzyme A reductase inhibitors, are analogs of 3-hydroxy-3-methylglutarate, which is a precursor of cholesterol (Alonso *et al*., 2019).

A- Statins have a strong affinity for the enzyme HMG CoA reductase and effectively compete to inhibit its function (Tashjian and Armstrong, 2011). HMG-CoA reductase is the enzyme that limits the rate of cholesterol formation in the liver and other tissues (Su *et al* ., 2024), by inhibiting this enzyme, statins decrease the cholesterol content in hepatocytes, stimulate the expression of LDL receptors, and ultimately enhance the removal of LDL-C from circulation (Luo *et al*., 2022) (Figure 2-2) .

B- Increase in LDL receptors

Statins lead to an increase in LDL receptors, which results in the depletion of intracellular cholesterol (Piper *et al*., 2019), this causes cells to increase the number of specific cell-surface LDL receptors, leading to the binding and internalization of circulating LDLs. Ultimately, this process results in a reduction of plasma cholesterol levels due to lowered cholesterol synthesis and increased catabolism of LDL (Luo *et al*., 2022; Tall *et al*., 2022).

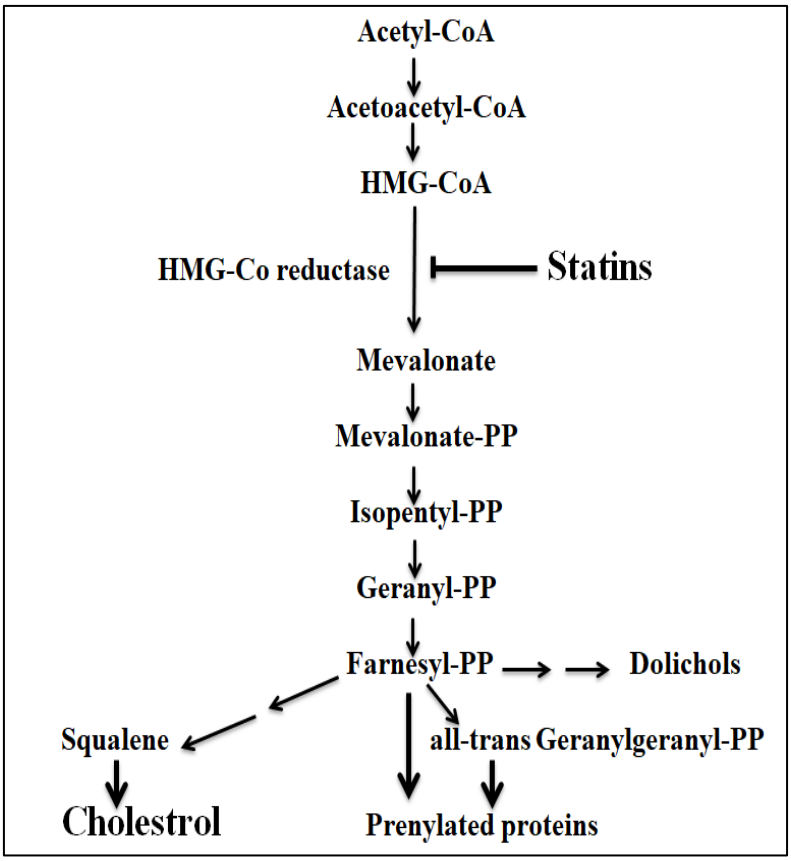

Figure (2-2) The mammalian mevalonate pathway; pp ,pyrophosphate. Adapted from Coradini, (2022).

2-1-3 : Pharmacokinetics of statins

The various statins share a common mechanism of action, but they differ in their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy (Hirota *et al*., 2020). These differences in chemical structures impact their water solubility, which plays a role in their absorption, distribution, metabolism, and excretion (Filppula *et al*., 2021).

Statins come in two types: lipophilic, which includes atorvastatin, simvastatin, fluvastatin, and lovastatin, and hydrophilic, which includes pravastatin and rosuvastatin as a result of a polar hydroxyl group and methane sulphonamide group, respectively (Sung *et al*., 2021; Hirota and Ieiri, 2024). Lipophilic compounds are relatively more fat-soluble, while hydrophilic compounds are more water-soluble. All statins are quickly

absorbed after being taken, reaching their highest concentration in the blood within 4 hours. The absorption of most statins ranges from 40% to 75%, except for Fluvastatin, which is almost completely absorbed (Brunton *et al*., 2018; Sung *et al*., 2021; Yasamineh *et al*., 2024).

Lovastatin is better absorbed when taken with meals, compared to other drugs in the same class. This is because all statins have low systemic bioavailability due to a significant first-pass effect at the intestinal and/or hepatic level, which can be advantageous since the liver is the target organ for statins (Sharpton and loomba, 2023; Hirota and Ieiri, 2024; Prieto *et al*., 2024). Simvastatin and lovastatin are drugs that are inactive in their original form and activated by first-pass metabolism in liver cytochrome P450(CYP3A)(Hardjo and Kadir, 2021). They undergo extensive first pass metabolism in the liver (Makhlouf *et al*., 2021), it is primary site of action. Less than 5% of oral dose has been reported to reach the circulation as active metabolites (Sharpton and loomba, 2023; Chaurasiya *et al*., 2024). This process of change happens mainly in the liver, and only a small percentage of the original drug reaches the bloodstream in its active form. Both drugs are also highly attached to proteins in the blood, except for pravastatin, which is only 50% attached (Ricci *et al*., 2019).

Atorvastatin undergoes first-pass metabolism, partly due to active derivatives (Guan *et al*., 2019). Plasma half-lives of statin drugs typically range from 1 to 3 hours. However, atorvastatin has a half-life of 14 hours, and rosuvastatin has a half-life of 19 hours (Althanoon *et al*., 2020).

Fluvastatin and pravastatin are the most thoroughly researched statins from a clinical pharmacokinetic perspective (Warden, *et al*., 2023). The key distinction between these two compounds is the higher liver extraction of fluvastatin during the absorption phase compared with

pravastatin, at 67% and 45% respectively (Hirvensalo *et al*., 2019), within the same dose range (Xiang *et al*., 2020).

Statins differ mainly in their metabolism and the number of active and inactive metabolites (Clemente *et al*., 2021). All statins have highly active metabolites, and their pharmacological activity depends on the kinetic profile of both the parent compound and active metabolite (Adil and Asif, 2023). Pravastatin is a hydrophilic drug that is mainly eliminated by the kidneys (Dróżdż *et al*., 2023). Most of the absorbed dose of statins is excreted in the bile, with about 5-20% being excreted in the urine (Filppula *et al*., 2021; Sharaf *et al*., 2021).

2-1-4 : Uses and indications

Statins have proven to be an effective treatment for hypercholesterolemia, a potential benefit factor for both vascular dementia and Alzheimer's disease (AD) (Chen *et al*., 2018). These drugs effectively lower plasma cholesterol levels in various types of hyperlipidemia (Kapelouzou *et al*., 2021). With their increasing use for cardiovascular indications, statins also show promise in providing protective effects against dementing illnesses (Zahedi *et al*., 2023).

The main clinical focus is on plasma LDL-cholesterol, with a reduction typically observed 4 to 6 weeks after the initiation of statin treatment (Shiomi, 2020).

2-1-5 : Multiple effects of statins

A. Lipid- dependent effect

Statins inhibit HMG-CoA reductase, which is crucial to cholesterol metabolism, leading to a reduction in LDL concentration. This results in decreased mortality when used for the primary and secondary prevention of cardiovascular disease (Khan *et al*., 2018; Takeda *et al*., 2020).

B. Lipid- independent effects

The findings from randomized trials consistently highlight the significant impact of statins in reducing the risk of cardiovascular events (Jasinska *et al*., 2007; Bonaterra *et al*., 2020; Takeda *et al*., 2020) surpassing the expected reduction based solely on the decrease in LDL cholesterol levels (Khan *et al*., 2018). Additionally, these benefits are observed earlier than the actual reduction in LDL levels, attributed to the pleiotropic effects of statins, including their anti-inflammatory, vasodilatory, and antithrombotic properties (Cianflone *et al*., 2020).

It has been widely documented in numerous studies that statins offer significant benefits beyond just lowering lipid levels (Shiomi, 2020). These benefits include improving endothelial function, reducing oxidative stress, and having direct anti-inflammatory, antithrombotic, and plaque-stabilizing effects (Flores-Castillo *et al*., 2019). These beneficial effects may contribute to cardiovascular protection by statin therapy beyond low-density lipoprotein (LDL) cholesterol lowering (Chen *et al*., 2018; Alvarez-Jimenez *et al*., 2023). Furthermore, statins exhibit anti-proliferative, and immunomodulatory actions (McGregor *et al*., 2016) , as well as improving endothelial dysfunction and increasing the availability of nitric oxide (NO) (Chen *et al*., 2018; Bonaterra *et al*., 2020). This is achieved by inhibiting HMG-CoA reductase, the enzyme responsible for the rate-limiting step in cholesterol synthesis, which converts HMG-CoA to mevalonate (Kapelouzou *et al*., 2021).

2-1-6 : Statins and specific diseases

Statins have been associated with a range of diseases, including cancer (Haciseyitogˇlu *et al*., 2024), infectious diseases, Alzheimer's disease, respiratory diseases, cardiovascular diseases, stroke, osteoporosis, arthritis, diabetes, dementia, multiple sclerosis, organ

rejection, kidney disease, sepsis, and age-related maculopathy (Rezakhani *et al*., 2023). This information helps to better understand the potential impact and risks associated with statin use (Atef *et al*., 2023).

2-1-7 : Adverse effect of statins

The HMG–CoA reductase inhibitors (statins) are generally well tolerated (Riste *et al*., 2015), however they are associated with two uncommon but important side effects:

1. Liver: Biochemical abnormalities in liver function have occurred with HMG CoA reductase inhibitors (Mueller *et al*., 2021).
2. Muscle: Asymptomatic elevation in liver enzymes and skeletal muscle abnormalities, which can range from benign myalgia to myopathy (10-fold elevation in creatine kinase with muscle pain or weakness) and life-threatening rhabdomyolysis (Zulfahmidah *et al*., 2021; Ibrahim and Shaheen, 2023). Both of these have been reported with concurrent cyclosporine , gemfibrozil, erythromycin and nicotinic acid (Ko *et al*., 2017).
3. The pattern of statin side effects resembles the pathology of selenium deficiency (Abo-zalam *et al*., 2023).
4. Headache, nausea, bowel upset ,sleep disturbances (with lipophilic drugs), and rashes, (Kosowski *et al*., 2021) Statin use is contraindicated during pregnancy and in nursing mothers. They should not be used in children or teenagers (Spoiala *et al*., 2024).
5. Statins are characterized by a wide margin of safety, however, side effects or even adverse outcomes in association with acute and chronic treatment regimens have been reported clinically (Sakaeda *et al*., 2011; Hirota *et al*., 2020; Attardo *et al*., 2022). Many studies, in experimental animals, have also supported and documented adverse effects of statins, such as neurobehavioral and locomotive changes

(Hai-Na *et al*., 2020; Rashid and Mohammad 2023a), impairment of neuromuscular function (Bouitbir *et al*., 2011) and alterations in the cholinergic system (Vukšić *et al*., 2019; Rashid and Mohammad 2023b). Within this context, examining statins toxicity or adverse effects became warranted in the light of withdrawal of one statin from clinical use (Tobert, 2003) and the actual clinical or perceived statin adverse effects (Sakaeda *et al*., 2011; Hirota *et al*., 2020; Attardo *et al*., 2022), as well as the statin intolerance among certain hyperlipidemic patients (Alonso *et al*., 2020; Bytyçi *et al*., 2022).

2-1-8 : Drug interactions and statins

Statin interactions with CYP450 inhibitors

According to the study "Statin interactions with CYP450 inhibitors" by (Patel *et al*., 2022), the induction or inhibition of CYP450 isoenzymes plays a significant role in these interactions. Competitive inhibition between drugs at the enzymatic level is common and may impact the way statins are metabolized in the body, potentially leading to higher levels in the bloodstream and an increased risk of adverse events (Patel *et al*., 2022; Zhang *et al*., 2022) (Table 2- 1).

Table (2- 1): Inhibitors and Inducers of the cytochrome P450 Enzymatic pathway

Inhibitors and Inducers of the cytochrome P450 Enzymatic Pathway		
CYP Substrates (Statins)	**Inducers**	**Inhibitors**
CYP3A4		
Atorvastatin, Lovastatin, Simvastatin	Phenytoin,Phenobarbital, Rifampin, Barbiturates, Carbamazepine, Dexamethasone, troglitazone, omeprazole Cyclophosphamide	Ketoconazol,Itraconazole, flucanazole,erythromycin, flucanazole,erythromycin, nefazodone,venlafaxine, fluvoxamine, fluxetine, sertraline,cyclosporine A, Xtacrolimus,ditiazem,verapamil, Protease inhibitors,midazolam,corticosteroids, grapefruit juice,tamoxifen,amiodarone
CYP2C9		
Fluvastatin,Rosuvastatin (2C19)	Rifampin,Phenobarbital, Phenytoin,troglitazone	Ketoconazol,Fluconazole, Sulfaphenazole

Adapted from Balasubramanian and Maideen, (2021).

Pharmacokinetic interactions, such as increased bioavailability, have been reported to result in myopathy and rhabdomyolysis after concurrent use of statins and various classes of drugs (Ibrahim, 2020) Table (2-2). Selected drugs that may increase the risk of myopathy and rhabdomyolysis when used Concomitantly with statins (Mollazadeh *et al*., 2021; Ibrahim and Shaheen, 2023).

Table (2- 2): Inhibitors/Substrates of CYP3A4

CYP3A4 Inhibitors/Substrates	Others
Cyclosporine,tacrolimus	Digoxin
Macrolides(azithromycin, Clarithromycin,erythromycin)	Fibrates(gemfibrozil)
Azole antifungals (itraconazole, Ketoconazole)	Niacin
Calcium Antagonists(mibefradil,diltiazem, Verapamil)	
Nefazodone	
Protease inhibitors(amprenavir, indinavir,nelfinavir,ritonavir)	
Sildenafil	
Warfarin	

Adapted from Iacopetta *et al*., (2023).

The following are important interactions to ensure safe medication use

- Antibacterials: When taking antibiotics such as rifampicin, the metabolism of fluvastatin is accelerated, resulting in reduced effectiveness. Additionally, clarithromycin and erythromycin increase the risk of myopathy when taken with simvastatin (Hennessy *et al*., 2016**).**
- Anticoagulant: HMG CoA reductase inhibitors increase warfarin levels.(La Mura *et al*., 2022).
- Antifungal :itraconazol,ketokonazol,increase the risk of myopathy with simvastatin (De Oliveira Neto *et al*., 2021).
- Antiviaral : protease inhibitors increase the risk of myopathy with simvastatin (Koike *et al*., 2021).

- Cardiac glycosides : Atorvastatin may possibly increase plasma digoxin concentration(Ren *et al* ., 2019).
- Immunosupressent : cyclosporin increase the risk of rhabdomyolysis (Lee *et al*., 2023).
- Other lipid regulating drugs : There is an increased risk of myopathy when fibrates and nicotinic acid are taken (Dubińska-Magiera *et al*., 2021).

2-2 : Neurobehavioral effects of statins

Numerous studies have shown intriguing aspects of statins that are not related to their hypolipidemic effects, such as actions on the central nervous system and peripheral neuromuscular functions (McGuinness *et al*., 2016; Hirota *et al*., 2020; Attardo *et al*., 2022; Rahsid and Mohammad 2023a). These actions produced by single or repeated doses of statins include modulation of the central and peripheral cholinergic system (Roensch *et al*., 2007; Cibickova *et al*., 2009; McGuinness *et al*., 2016), which were evidenced by statins having different effects on blood or brain ChE activity (Roensch *et al*., 2007; Macan *et al*., 2015). These actions could be pertinent to the type of statin in use, The dosage applied, The response of the animal species versus humans, and the type of ChE in the blood or brain tissue-true versus pseudo-ChE (Cibicková *et al*., 2007; Husain *et al*., 2018; Rashid and Mohammad 2023a). Within this context, another aspect of the pleiotropic effects of statins is behavioral modulation in patients and laboratory animals receiving statin therapy. Behavioral effects included aggression, depression, memory loss, and confusion (Rahola, 2012; Leppien *et al*., 2018; Jamshidnejad-Tosaramandani *et al*.,2022). Several experimental studies have reported neurobehavioral changes in laboratory animals, such as mice, rats, and young chicks treated with different statins (Ghodke *et al*., 2012;

Durankuş *et al*., 2023). These behavioral changes induced by statins included modulation of memory function (Ghodke *et al*., 2012), antidepressant action (Hai-Na *et al*., 2020), alterations of general activity, swimming behavior, and epilepsy in mice (De Oliveira *et al*., 2018), reduction of addiction risk (Chauvet *et al*., 2016), and social behavior (Durankuş *et al*., 2023) in rats, and reduced xylazine-ketamine anesthetic duration with reductions of anti-ChE toxicity in chicks (Rashid and Mohammad, 2023b).

However, limited information is available on possible behavioral changes in laboratory animals with changes in blood and brain ChE activities. In accordance with experimental findings in laboratory animals treated with statins, the reported adverse behavioral effects of statins in humans include episodes of aggression, depression, loss of memory, or confusion (Rahola, 2012; Leppien *et al*., 2018), and stressing the fact that in laboratory animals, statins cause memory alteration, changes in locomotor, social, and swimming activities, modulation of drug addiction, and antidepressant effects (Ghodke *et al*., 2012; Durankuş *et al*., 2023).

Furthermore, the neurobehavioral alterations of experimental animals treated with statins could be the results of CNS effects of the drugs employed or due to their neuromuscular action (Bouitbir *et al*., 2011; Ghodke *et al*., 2012; Husain *et al*., 2018; De Oliveira *et al*., 2018; Hai-Na *et al*., 2020; Hirota *et al*., 2020; Durankuş *et al*., 2023). It was also recently reported, that statins reduce the duration of anaesthetic action of xylazine-ketamine in chicks, with reductions in the toxicity outcome of the reversible ChE inhibitor carbaryl (Rashid and Mohammad, 2023b). This latter approach of pharmacological and toxicological challenges has been used when there are no overt actions of drugs in experimental animals, but rather changes appear when they are

subjected to challenges with drugs or toxicants (Frankel *et al.*, 2007; Rashid and Mohammad, 2023b).

Overall, these studies cited above reflect various neuronal and functional-behavioral aspects of statins in experimental animals that could be classified as potential undesirable or pleiotropic effects of these drugs. Several mechanistic explorations of the CNS effects of statins have shown that statins might modulate monoamines, amino acids, neuropeptides and the cholinergic associated pathways with the inhibition of brain cholinesterase (ChE) activity (Aminifar *et al.*, 2023; Rahola, 2012).

2-3 : Esterase

Esterases are a large group of enzymes found in plasma (or serum) and tissues, which hydrolyze a several of xenobiotics. Some of these enzymes have not yet been measured in tropical domestic animals (Ozmen *et al*., 2023) (table 2-3).

Example of B-Esterase:

The type of cholinesterase (ChE) in the plasma is pseudo ChE (EC 3.1.1.8), whereas those of the brain and erythrocytes are true ChE (EC 3.1.1.7). The enzyme is usually monitored for inhibitory effects of medications such as those used against Alzheimer's disease (Reuben *et al*., 2024) and organophosphate or carbamate toxicants used as insecticides (Wilson, 2014; Bilen *et al*., 2022) .

Table (2- 3): Esterase Classes

Esterase Classes
A- Esterases
Hydrolyze OPs to inactive products
Found in liver and HDL in plasma
High activity in mammals
Lower activity in birds
Examples: Paraoxonase and DFPase
B- Esterases
Widely distributed in cells and tissues
Inhibited by OPs and CBs
Slow hydrolysis of OP-enzyme complex
Relatively rapid hydrolysis of CN- enzyme complex
Examples: AChE, BuChE, CaE, and NTE
OP, organophosphate ester; HDL, high-dencity lipoprotein, CB,carbamate; AChE, actylcholinestrase; BuChE, butyrylcholinestrase; CaE, carboxylesterase; NTE, neuropathy target esterase.

Adapted from Žnidaršič *et al* (2023)

2-3-1 : Cholinesterases

Cholinesterase (ChE) is a generic term used for a family of closely related enzymes responsible for breaking down choline esters at a faster rate compared to other esters under optimal conditions. These enzymes, which belong to the esterase family(Pan *et al* 2019), are serine hydrolases found in higher eukaryotes (Halder and Lal, 2021). In the mammalian brain, two major forms of cholinesterases exist :

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Pohanka, 2020). The two forms differ genetically ,structurally and in their kinetics. Butyrylcholine is not a physiological substrate in mammalian brains which makes the function of BuChE difficult to interpret (Severi *et al*., 2023). In human brains, BuChE is found in neurons and glial cells as well as in neuritics plaques and tangles in

patient with Alzheimers disease(AD) (Jamshidnejad-Tosaramandani *et al*.,2022; Severi *et al*., 2023). While AChE activity decreases progressively in the brains of Alzheimer's disease patients, BuChE activity show some increase (Scheiner *et al*., 2021) (Table 2- 4).

Table (2- 4): Cholinesrase properties

Cholinestrase Properties
All
Hydrolyze ACh and other choline esters
AChE
Prefers ACh, inhibited by excess substrates
Found atneural junctions and in mammal RBCs , plasma and platelets of some vertebrates
BuChE
Prefers butyrylcholine, proplonylcholine
Widely distributed in vertebrate tissues and plasma

Adapted from Pan *et al* (2019).

2-3-2 : Acetylcholinesterase

Acetylcholinesterase is an enzyme that specifically cleaves acetylcholine to acetate and choline and ,thus, terminate its action. Its located both pre-and post-synaptically in the nerve terminal, where it is membrane bound (Horio *et al*., 2020).

Additionally known as true, specific, genuine, or type 1 ChE, this enzyme is present in erythrocytes, nerve endings, lungs, spleen, and throughout the brain. It exists in several molecular forms and is a membrane-bound glycoprotein (Akhtar and Humaira, 2022; Zieliński *et al*., 2021).

Acetylcholinesterase and Butyrylcholinesterase are both types of serine hydrolases belonging to the esterase family found in higher eukaryotes (Alias and Mohammad, 2005). These enzymes act on various carboxylic esters (Pan *et al*., 2019). The primary biological role of acetylcholinesterase is to rapidly hydrolyze the neurotransmitter acetylcholine, which helps terminate impulse transmissions at cholinergic synapses within the nervous system (De Boer *et al*., 2021).

2-3-3 : Structure of Acetylcholinesterase

AChE exists in tow general classes of molecular form: simple homomeric oligomers of catalytic subunits (i.e. monomers, dimmers, and tetramers) and heteromeric associations of catalytic subunits with structural subunit (Krunić *et al*., 2023; Luque and Muñoz-Torrero).

2-3-4 : Function of cholinesterase

1. Specific or true cholinesterase (Neural CH.E.)

The primary function of neural cholinesterase is to hydrolyze acetylcholine neurotransmitter at the nerve endings (Krunić *et al*., 2023).

2. Butyrylcholinesterase (BuChE)

Butyrylcholinesterase, also known as acylcholine acyl-hydrolase, is a significant enzyme found in mammalian blood plasma, with over eleven isoenzyme variants (Horn *et al*., 2024). It is also present in the liver, smooth muscle, pancreas, intestinal mucosa, and the white matter of the central nervous system (Dingova *et al*., 2024; Severi *et al*., 2023). BuChE hydrolyzes butyrylcholine four times more rapidly than acetylcholine and does not hydrolyze D-β-methyl acetylcholine, unlike AChE. It exhibits higher activity with butyryl and propionylcholine than with acetylcholine (Horn *et al*., 2024).

BuChE, an enzyme significantly linked to coronary artery disease (CAD), has unknown substrates and biological functions, but its activity has been observed to increase in patients with hyperlipoproteinemia and diabetes (Ju and Tam, 2020).

2-3-5 : Cholinesterase roles in the brain during health and disease

The cholinergic hypothesis proposes that decreased levels of acetylcholine (ACh) in the brain may contribute to deficits in learning, memory, and behavior in dementia (Fallon *et al*., 2023; Žnidaršič *et al*., 2023). Recent evidence suggests that cholinesterase inhibitors (ChEs) may have broader functions in the central nervous system (CNS) beyond their traditional enzymatic activities, including modulating glial activation, cerebral blood flow, the amyloid cascade, and tau phosphorylation (Sridhar and Gumpeny, 2024). It has been speculated that these actions could impact the underlying disease processes in Alzheimer's disease (AD), and that using ChE inhibitors may affect long-term disease progression (Silman, 2021; Hughes *et al*., 2022). Recent research on butyrylcholinesterase (BuChE) has expanded our understanding of the roles of ChEs in health, disease, and aging (Silman, 2021).

2-3-6 : Pseudocholinesterase and disease

Pseudocholinesterase, also known as plasma cholinesterase (Plasma CH.E.), It seems to have its most significant impact in vitro on longer chain cholinesters (such as butyrylcholine) and plays a role in breaking down the action of acetylcholine, a neurohumoral agent (Cheng *et al*., 2024). However, it is important to carefully consider any direct association between plasma cholinesterase and neural transmission before making assumptions (Hefter *et al*., 2022; Cheng *et al*., 2024).

2-3-7 : AChE and alzheimer’s disease

Alzheimer’s disease (AD) is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory deterioration (Xu *et al*., 2021).

Progressive impairment of activity of daily living, and a variety of neuropsychiatric symptoms and behavioral disturbances (Ihendu *et al*., 2024).

Alzheimer's disease (AD) is the most common type of dementia(Zhang *et al*., 2022). The beta-amyloid peptide (Abeta) is the major constituent of senile plaques, which together with atrophy and neurofibrillary tangles, is the main neuropathological finding in Alzheimer's disease (Feng *et al*., 2020). It is a widely accepted theory that aggregation of A beta into plaques is an initial event in the pathogenesis of AD (Belo *et al*., 2020).

Cholinergic loss is the most common neurotransmitter deficiency in Alzheimer's disease (AD) and has resulted in the use of acetylcholinesterase inhibitors (AChE-Is) and unselective cholinesterase inhibitors (ChE-Is) as the primary treatment (Zueva *et al*., 2019). This is particularly important because AChE activity is reduced in the AD brain, leading to decreased ACh levels, while BuChE activity is increased (Ekundayo *et al*., 2022).

Initial pharmacologic approaches for AD aimed to enhance cholinergic transmission in the brain based on the "cholinergic hypothesis" of memory dysfunction (Rapaka *et al*., 2022). Among the various strategies used to increase synaptic levels of acetylcholine (ACh), inhibiting the breakdown of ACh by inhibiting acetylcholinesterase has been the most successful (Fallon *et al*., 2023). Inhibiting the enzyme butyrylcholinesterase, which is a minor component in normal brains but is increased in the brains of AD patients about to plaques and tangles (Feng

et al., 2020), may also improve cholinergic transmission (Rapaka *et al*., 2022).

2-3-8 : Effects of statins on cholinesterase

The impact of statins on cholinesterase activities in the plasma or the brain is not fully understood (Chamani *et al*., 2024). Some studies have shown that simvastatin and alendronate, which can cross the blood-brain barrier, can significantly decrease the activity of acetylcholinesterases in the frontal cortex of rats when administered orally for seven days (Zahedi *et al*., 2023). However, no significant changes in AChE activity were observed after administration of atorvastatin. The activities of acetylcholinesterase and butylcholinesterase in the blood were not affected by any of the drugs (simvastatin, atorvastatin, and alendronate) (Rashid and Mohammad, 2023a).

On the other hand (Anjum, 2023), reported that lovastatin and simvastatin significantly inhibited human butyrylcholinesterase, without impacting acetylcholinesterase. They suggested that some statins inhibit butyrylcholinesterase, potentially protecting against dementia.

Notably, a statistically significant decrease in butylcholinesterase activity in patients with hyperlipoproteinemia type 2a and 2b during treatment with simvastatin was not observed (Al-hashemi *et al*., 2023). Furthermore, acetylcholinesterase activity in rats remained unaffected by simvastatin (10 or 20 mg/kg b.wt.) and atorvastatin (10 or 20 mg/kg b.wt.) (Verma *et al*., 2022).

Rashid and Mohammad, 2023a reported that the effect of simvastatin on serum pseudocholinesterase in chicks remained unchanged after a 14 days treatment of simvastatin. Furthermore, the results indicated that both lipophilic and hydrophilic statins decreased cholinesterase activities, suggesting a potential neuroprotective role of

statins in Alzheimer's disease (AD) treatment. Additionally, there is evidence (Abdel Wahab *et al*., 2023) supporting the potential benefits of atorvastatin therapy in the treatment of mildly to moderately affected AD patients and its potential to slow the disease's progression, thus improving the quality of life for those affected (Degiorgi, *et al*., 2023). On the other hand, (Fatima *et al*., 2020) concluded that there is currently insufficient evidence to recommend statins for reducing the risk of Alzheimer's disease. However, there is a growing body of biological, epidemiological, and non-randomized clinical evidence suggesting that lowering serum cholesterol may slow the progression of Alzheimer's disease (Barros *et al*., 2022). While case-control studies indicate a lower likelihood of statin use among AD patients (Degiorgi, *et al*., 2023) longitudinal epidemiology studies have not confirmed a reduced risk of AD among statin users (Ibrahim, 2020). Despite these mixed results, there is still a possibility that statin therapy could play a role in reducing the risk of dementia and AD (Fatima *et al*., 2020).

Furthermore, observational studies have suggested that receiving statin therapy in midlife may be associated with a decreased risk of AD. This potential reduction in risk could be linked to statins' ability to reduce β-amyloid formation and deposition, as well as their anti-inflammatory effects (Husain *et al*., 2018).

Additionally, recent epidemiological evidence points to the potential protective effects of statins against Alzheimer's disease and other forms of dementia (Gangoda *et al*., 2023).

Recent epidemiological studies have shown a promising reduction in the occurrence of Alzheimer's disease among patients treated with cholesterol- lowering statins (Climent *et al*., 2021) suggest that cholesterol-lowering medications may have a positive impact on cognitive decline in Alzheimer's disease and could potentially offer

neuroprotective benefits (Fatima *et al*., 2020). However, there is ongoing debate regarding the effect of statins on cholinesterase activity, and further research aims to provide clarity on this matter (Husain *et al*., 2018).

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2-4 : Oxidative stress

Oxidative stress is the result of an imbalance between the production of Reactive Oxygen Species (ROS) and the body's ability to eliminate them (Banerjee *et al*., 2023). This imbalance leads to damage in various body tissues, causing the peroxidation of polyunsaturated fatty acids (Ahsan *et al*., 2020; Salama *et al*., 2024).

To counteract these harmful compounds, the body employs various mechanisms, including antioxidants, which are substances that inhibit oxidation processes caused by free radicals (Machado *et al*., 2023). Antioxidants are classified into different categories based on their basic chemical nature (Gabbia *et al*., 2023; Linillos-Pradillo *et al*., 2023).

2-4-1 : Antioxidants

1. Enzymatic Antioxidants

The body produces important compounds including the enzymes catalase, superoxide dismutase (SOD), glutathione reductase (GSH-rd), and glutathione peroxidase (GSH-px) (Allameh *et al*., 2023; Banerjee *et al*., 2023).

2. Non-Enzymatic Antioxidants

Metabolic antioxidants originate within the body due to metabolic processes and include important substances such as uric acid, lipoic acid, transferrin, L-arginine, and glutathione (Igbayilla *et al*., 2021; Algefare, *et al*., 2024). Non-enzymatic dietary antioxidants are obtained from food and include compounds such as omega-3, carotenoids, flavonoids, vitamin C, vitamin E, and methionine (Gabbia *et al.*, 2023).

2-4-2 : Reduced Glutathione (GSH)

Glutathione, a crucial non-enzymatic metabolic antioxidant, is primarily synthesized in the liver (Bosch *et al*., 2020) ; Birdane *et al*., 2024) and red blood cells (Bosch *et al*., 2020; Watanabe *et al*., 2020) . It plays a vital role in reductive and oxidative reactions, protecting against oxidative stress (Algefare, *et al*., 2024). Composed of three amino acids (L-glutamyl, L-cysteinyl, glycine) with a molecular weight of 307.32 daltons (Bosch *et al*., 2020; Watanabe *et al*., 2020). Glutathione is synthesized through the enzymes γ-glutamyl cysteine synthetase and GSH-synthase, using two molecules of ATP (Bosch *et al*., 2020). Under normal conditions, glutathione exists in a reduced state (GSH), but it converts into the oxidized form (GSSH) when exposed to oxidative processes or stress (Ahsan *et al*., 2020; Igbayilla *et al*., 2021). With numerous vital functions in the body, glutathione directly neutralizes the effect of free radicals and active oxygen compounds (Banerjee *et al*.,

2023), and crucially inhibits the initial reactions in the lipid peroxidation chain (Salama *et al*., 2024).

2-4-3 : Malondialdehyde (MDA)

Malondialdehyde is formed when polyunsaturated fatty acids (PUFA) in cells, particularly cellular membranes, undergo oxidation (Choghakhori *et al*., 2024). These acids, containing multiple double bonds, are highly prone to free radical reactions. Oxidation of these acids, due to various pathological conditions, results in a loss of selective permeability in cellular membranes, leading to uncontrolled passage of fluids and substances through the membranes (Ahsan *et al*., 2020; Althanoon *et al*., 2020). Lipid peroxidation can impact most lipids, including those in platelets and epithelial cells. It can also occur in cell organelle membranes, such as lysosomes and mitochondria, which release enzymes and intensify the destructive actions of free radicals (Tekeli *et al*., 2024; Nassef *et al*., 2024). This process disrupts the balance between the production of active oxygen species and the ability of antioxidant defense systems to neutralize them or eliminate their by-products (Birdane *et al*., 2024).

2-4-4 : Alanine aminotransferase (ALT)

Alanine aminotransferase is an important enzyme found in the liver, kidneys, heart, and muscles (Li *et al*., 2024; Hasan *et al*., 2024). It catalyzes the transamination reaction and only exists in cytoplasmic form. Any kind of liver injury can cause a rise in ALT and increase free fatty acids and triglycerides (Ahsan *et al*., 2020; Köroğlu *et al*., 2024). In general, high levels of ALT may be a sign of liver damage from hepatitis, infection, cirrhosis, liver cancer, or other liver diseases, reduced blood flow to the liver, or certain medications or poisons (Hasan *et al*., 2024).

Monitoring ALT levels can provide valuable insights into liver health and possible underlying conditions (Cristina *et al*., 2024).

2-4-5 : Aspartate aminotransferase (AST)

Aspartate aminotransferase is an enzyme that is predominantly found in the liver, followed by the heart, muscle, kidney, brain, pancreas, and lungs (Osuchukwu *et al*., 2021). Despite being present in various organs, AST is considered a less specific indicator of liver damage compared to ALT (Rabelo *et al.*, 2024). AST exists in two forms: the mitochondrial form and the cytoplasmic form. An elevation of mitochondrial AST in the blood strongly indicates tissue necrosis in myocardial infarction and chronic liver disease (Yoo *et al*., 2024). The majority of liver AST activity is attributed to the mitochondrial form, whereas the circulating AST in the blood is contributed by the cytoplasmic form (Osuchukwu *et al*., 2021; Haque *et al*., 2024). Elevated AST levels are particularly notable in individuals with liver cirrhosis (Parsa *et al*., 2024). Also known as serum glutamic-oxaloacetic transaminase (SGOT), AST is responsible for protein breakdown for energy (Osuchukwu *et al*., 2021; Haque *et al*., 2024). Release of AST into the blood occurs when any of these tissues are damaged or diseased (Parsa *et al*., 2024).

2-4-6 : Statin effect on oxidative stress

Statins are primarily used to lower the concentration of low-density lipoprotein cholesterol. Additionally, research has indicated that statins can also reduce oxidative stress by modulating redox systems (Arnesdotter *et al*., 2021; Wang *et al*., 2024). Notably, statins have demonstrated antioxidant properties in certain cardiovascular (Khatiwada and Hong, 2024) and atherosclerotic conditions (Mason, 2000). Moreover, studies have highlighted that the antiatherogenic effects of

statins are associated with their diverse activities, particularly their antioxidant effects (Liu *et al*., 2019).

For instance, the vital role of statins as antioxidants in cardiovascular and atherosclerotic diseases. It analyzes various studies that showcase the antiatherogenic effects of statins and details how these drugs effectively reduce oxidative stress by inhibiting the generation of reactive oxygen species (ROS) and balancing the NAD+/NADH ratio. Moreover, it delves into the impact of statins on nitric oxide synthase, lipid peroxidation, and adiponectin levels, underscoring their potential to deliver protective cardiovascular benefits beyond their lipid-lowering functions, primarily through their antioxidant properties (Parsa *et al*., 2024).

However, It is important to note that oxidative stress could potentially trigger adverse effects associated with statin use, such as diabetic complications, myopathy, (Sathya, *et al*., 2022; Reith *et al*., 2024) and fatty liver development (Wei *et al*., 2023; Salama, *et al*., 2024). For example, statins may impact glucose homeostasis through the redox system. While statins act as antioxidants in atherosclerotic tissues, they can lead to oxidative stress-related issues in liver, kidney, and muscle cells (Wei *et al*., 2023; De Liyis *et al*., 2024). Statins cause hepatotoxicity, nephrotoxicity, and muscle toxicity by oxidative stress (Wei *et al*., 2023). Therefore, the effects of statins on oxidative stress are different in different organs or tissues (Sathya, *et al*., 2022; Hasan *et al*., 2024).

There is mounting evidence suggesting a close link between statin toxicity and oxidative stress (Algefare, *et al*., 2024; Hasan *et al*., 2024). Deficient antioxidant defense or excessive free radical production can culminate in oxidative stress, (Verma, *et al*., 2022; Vieira *et al*., 2024)

often initiated by reactive oxygen species such as superoxide anion, hydroxyl radical, and perhydroxy radical($HOO^{\cdot}$) (Wang *et al*., 2024).

The metabolism of statins has been found to generate ROS, leading to oxidative stress and different levels of toxicity (Zakaria *et al*., 2024). This includes skeletal muscle toxicity, as well as hepatic and renal damage (Pal, *et al*., 2015; Igbayilla *et al*., 2021). Since statins are widely used around the world, it is important to give more attention to a thorough analysis of their side effects (Igbayilla *et al*., 2021). Recent investigations have looked into the impact of various side effects, such as oxidative stress and ROS, on statin-associated myotoxicity, photosensitivity disorders, hepatotoxicity, and nephrotoxicity (Pal, *et al*., 2015; Wang *et al*., 2024). Both in vitro studies in primary and passage cells or mitochondria, and in vivo studies in human beings, guinea pigs, rats, and mice have revealed that oxidative stress plays a crucial role in the toxic effects induced by statins (Tabassum *et al*., 2020; Wei *et al*., 2023; Kang, *et al*., 2024).

Chapter Three

Materials and Methods

Chapter Three

Materials and Methods

3-1: Ethical approval

This study was reviewed and approved by the Departmental Scientific Committee on Research and Animal Care and Use. The Committee of Postgraduate Studies at the College of Veterinary Medicine, University of Mosul, Iraq approved (No. 2144, November 2, 2022) the present study according to the institutional regulations and ethics on the use of laboratory animals and their handling in biomedical research in compliance with the arrive guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and the guide for the care and use of laboratory animals (National Research Council, 2011). The University of Mosul also granted the permission (No. 4S/29927, October 30, 2022). The present study was conducted from November, 2022 to July, 2024 at the Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Mosul, Iraq.

3-2: Chemicals and kits used

1. Atorvastatin powder (calcium salt), The State Company for Drugs Industry and Medical Appliances, Samarra, Iraq.
2. Simvastatin powder (sodium salt), The State Company for Drugs Industry and Medical Appliances, Samarra, Iraq.
3. Rosuvastatin powder (calcium salt), The State Company for Drugs Industry and Medical Appliances, Samarra, Iraq.
4. Propofol Solution (Diprivan 1%, 10 mg/ml), Cordin Pharma S.P.A., Italy.

5. Dichlorvos Solution (Nicoz, 50% EC, 150 mg/kg) Royal Company, India.
6. Acetylcholine iodide, Direvo Industrial Biotechnology, Germany.
7. Dihydrogen potassium phosphate (KH_2PO_4), E-Merck Darmstadt, Germany.
8. Sodium Chloride (NaCl), B.D.H, England.
9. Sodium Barbital, B.D.H, England.
10. Hydrochloric acid (HCl), B.D.H, England.
11. Sodium hydroxide (NaOH), B.D.H., England.
12. Disodium hydrogen phosphate (Na_2HPO_4), 99.5% B.D.H., England.
13. Heparin Sodium (25000 IU/ml), B/Braun Melsungen AG, Germany.
14. Potassium chloride (KCl), Bios Europe, England.
15. Hydrogen Peroxide (30%), Sharlau, Spain.
16. Physiological Saline Solution, Aqua Life Health Care Pharmaceutical Solutions Manufacturer, India.
17. Colorimetric ChE activity kit (Catalog No. E-BC-K052-S, Elabscience Biotechnology Inc., Houston, TX, USA).
18. MDA ELISA kit (Catalog No. E-EL-0060, Elabscience Biotechnology Inc., Houston, TX, USA).
19. Reduced glutathione colorimetric assay kit (Catalog No: E-BC-K030-S, Elabscience Biotechnology Inc., Houston, TX, USA).
20. Alanine aminotransferase colorimetric method kit (REF. 92027), BIOLABO, France.
21. Aspartate aminotransferase colorimetric method kit (REF. 92025), Biolabo, France., BIOLABO, France.
22. Total cholesterol colorimetric assay kit (REF. K2106), BIOLABO, France.

3-3: Devices and Equipment used

1. Water bath, T and B, England.
2. Sensitive balance, A and D Company Ltd, England.
3. Weighing scale, Great River Company, China.
4. Centrifuge, Chalice, England.
5. Spectrophotometer, Emclab, Germany.
6. Homogenizer, Omni Bead Ruptor 24 USA.
7. pH meter, Hanna, Romania .
8. ELISA reader device, Biosin, Latvia.
9. ELISA dishwasher, Biosan. Latvia.
10. Micropipettes, Dragon Company, China.

3-4: Animals

Male Swiss-origin mice (age, 100–120 days; weight, 30–35 g; total No., 628) were used throughout the study. The rodents were housed at temperatures of 20–24°C and a 12-h light/dark cycle, with free access to laboratory food and water. All experiments were conducted between 10 a.m. and 2 p.m. The study comprised five sections as described below in details.

3-5 Section one

3-5-1 Drugs

Atorvastatin, simvastatin, and rosuvastatin were used. The required drug concentrations were freshly prepared using distilled water as a vehicle for oral dosing by a gavage needle at dose rates of 250, 500, 750, and 1000 mg/kg of body weight in a volume of 10 mL/kg of body weight on the experimental day. These statin doses were predetermined in preliminary experiments in mice and were selected for use in the present study because they did not induce overt signs of toxicosis; however,

being sufficiently high, they allowed us to delineate neurobehavioral alterations in animal behavioral paradigms.

3-5-2: Animal treatment and behavioral measurements

Mice were randomly divided into groups of 10/group to be orally administered atorvastatin, simvastatin, and rosuvastatin at the dose rates mentioned above (250, 500, 750, and 1000 mg/kg of body weight) with distilled water (control) (Figure 3-1). Two hours after vehicle or drug administration, each mouse was separately subjected to the neurobehavioral performance that included 5-min open-field activity in an open box (25 cm x 25 cm x 35 cm with 25equal squares arena), which involved time to initiate movement, the numbers of squares crossed and rearing activity in the arena (Figure 3-2) (Mohammad *et al*., 2006), negative geotaxis performance at an angle of 45° to complete 180° after placing the mouse in a head down position within 60 seconds (Mohammad and St. Omer, 1986) (Figure 3-3), 5-min head pocking behavior in a 30-cm diameter circular arena which contained 8 holes (2 cm in diameter each) (Figure 3-4) (Soni and Parle , 2017) and a single session of forced swimming endurance at a temperature of 24 ± 1 °C, which is an indication of despair test, to measure the durations of initial swimming attempts and immobility in a cylindrical water tank (30 cm in height and 15 cm in diameter with water level at the 20 cm mark) (Castagné *et al*., 2010) (Figure 3-5). Thereafter, the mice were allowed to rest in their home cages for about one hour before the start of blood sampling.

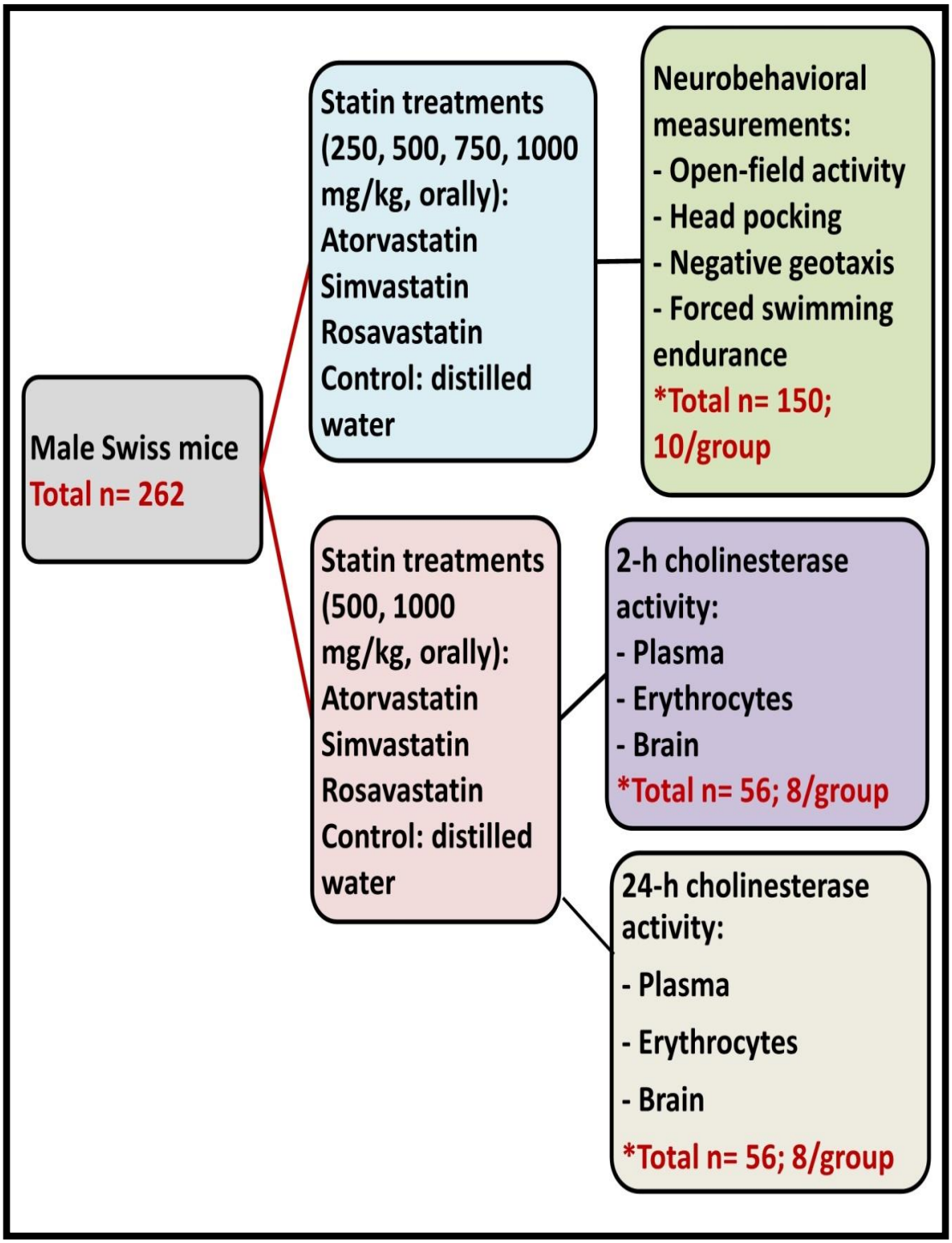
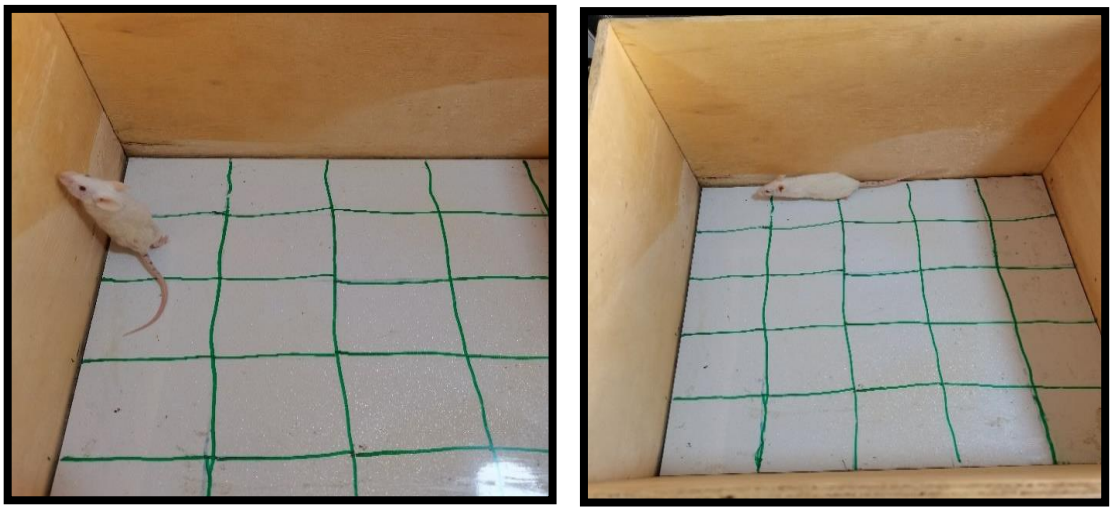
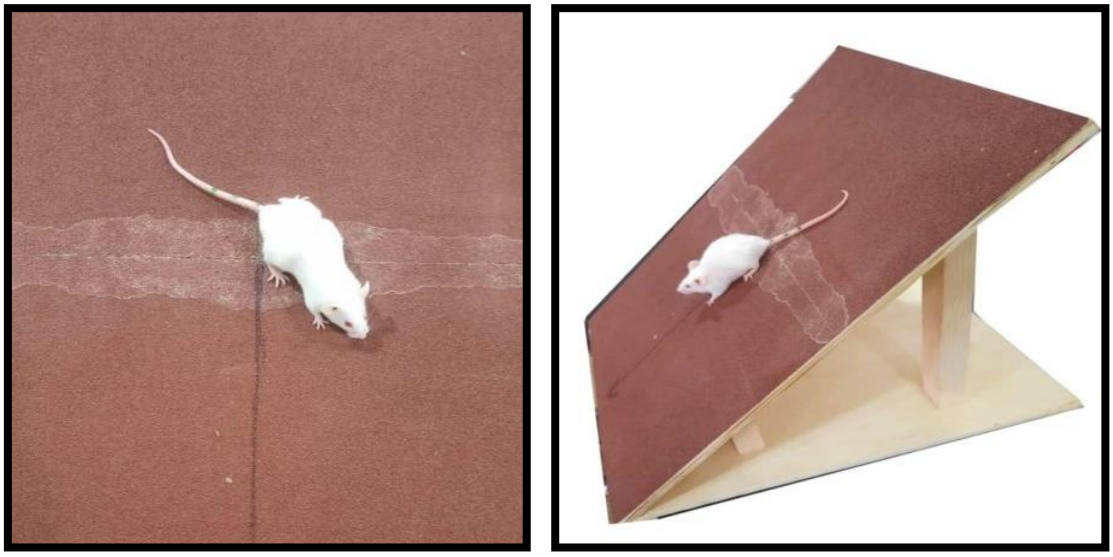


Figure (3-1): Section one of the study design, statin treatments and neurobehavioral measurements work flow



a- Rearing **b- Squares crossed**

Figure (3-2): open-field activity box



(a) **(b)**

Figure (3-3): Negative geotaxis test at an angle of 45º

(a) (b)

Figure (3-4): Head pocking test

Figure (3-5): Forced swimming test

3-5-3: Determination of ChE activity

In this experiment (Figure 3-1), mice were randomly divided into 8/group (2 h or 24 h) for oral dosing with distilled water (control) and atorvastatin, simvastatin, and rosuvastatin at dose rates of 500 and 1000 mg/kg each. Two and 24 h after the dosing of distilled water or statin, blood samples were obtained from the retro-orbital plexus under terminal ether anesthesia into heparinized capillary tubes. Blood samples were centrifuged at 3000 rpm for 15 min to separate the plasma; erythrocytes were washed with physiological saline solution. The whole brain was dissected out and homogenized with sodium chloride-barbital-phosphate buffer solution, pH 8.1, which consisted of 1.237 g sodium barbital, 0.163 g potassium dihydrogen phosphate and 35.07 g sodium chloride/L of distilled water (1:9) (Mohammad *et al*., 2006; Mohammad *et al*., 2014) using a homogenizer at a speed of 400 rounds/second. Cholinesterase activity in the plasma, erythrocytes, and whole brain was determined spectrophotometrically using a commercial kit.

According to the kit manual, colorimetric method of ChE determination is based on the reaction of acetylcholine that is not hydrolyzed by ChE in the reaction mixture with basic hydroxylamine to form acetylhydroxamic acid. As the hydroxamate-iron complex evolves at 37 °C within 20 min, the developing red-brown color intensity is directly proportional to residual acetylcholine that is not hydrolyzed (Miao *et al*., 2010). The optical density of the developed color was measured spectrophotometrically at 520 nm. Details and steps of the procedure according to the ChE kit manual are shown in appendix 1.

3-6: Section Two

3-6-1: Drugs

Atorvastatin, simvastatin, and rosuvastatin were used. The required drug concentrations were freshly prepared in distilled water as a vehicle for oral dosing by a gavage needle at a volume of 10 mL/kg of body weight. Figure 3-6 outlines the experimental protocol and allocation of 128 mice to different statin treatment regimens (n= 96) and their appropriate control counterparts treated with distilled water (n= 32). The doses and treatment regimens of the three statins were as follows: Single-dose treatments consisted of oral dosing of 8 mice / group with each of the three statins at 500 or 1000 mg/kg of body weight. Eight control mice were treated with distilled water concurrently with each statin dose level. Repeated doses with each of the statins were at 200 mg/kg of body weight/day for 14 or 28 consecutive days (8 mice/dose group of each statin/14 days or /28 days), and two control groups of eight mice each were used for each period. According to the results of section one and our preliminary experiments, these doses of statins did not produce overt toxicity in mice within the time frame allocated for each experiment. The mice were randomly assigned to treatment groups.

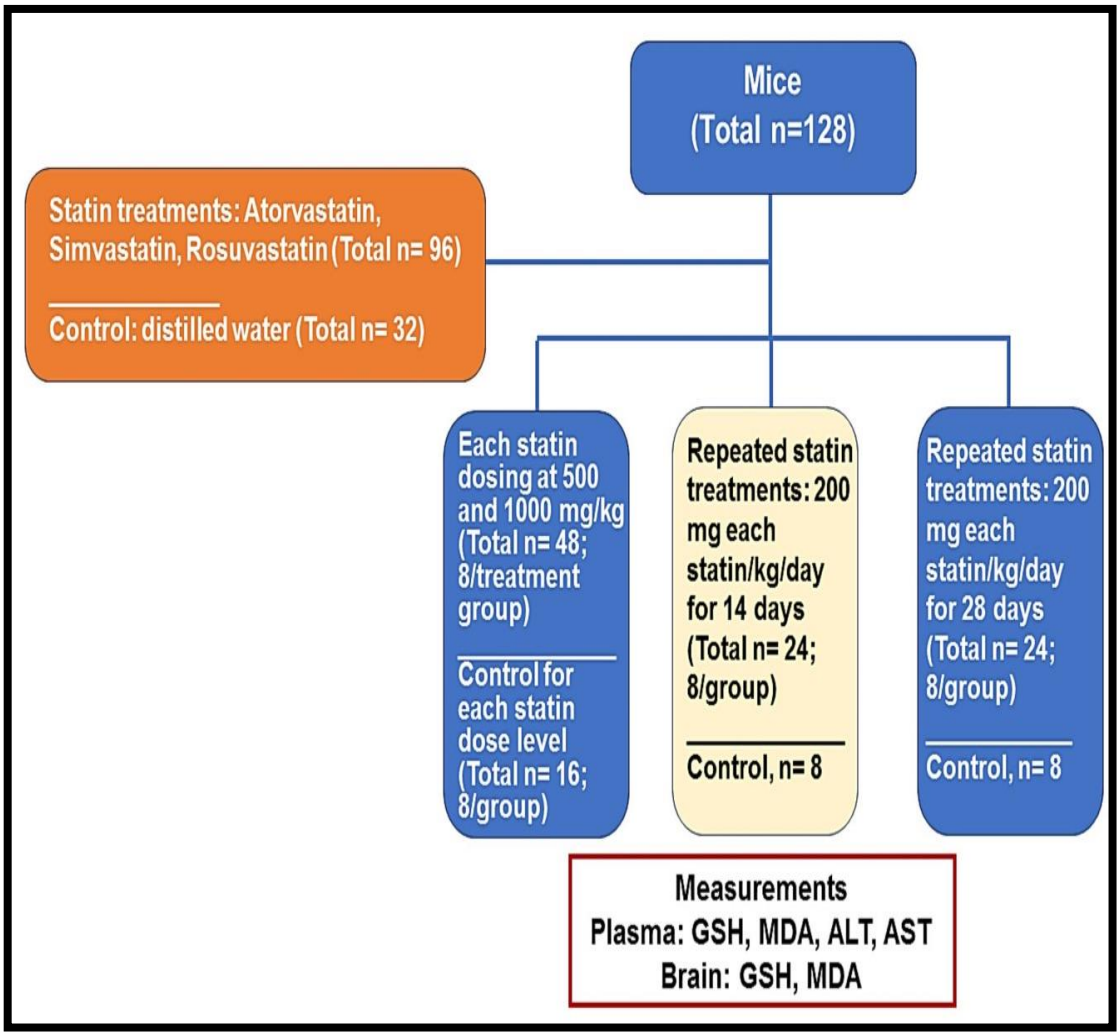


Figure (3-6): The integrated experimental design of section two of the study and allocation of mice to single or repeated doses of three statins, while distilled water treatment groups were used as controls. n: numbers of mice used within each treatment regimen or control group; GSH: glutathione; MDA: malondialdehyde; ALT: Alanine aminotransferase; AST: aspartate aminotransferase.

3-6-3: Samples and measurements

Blood samples were withdrawn from the retro-orbital plexus under terminal anesthesia with ether into heparinized capillary tubes two hours after the single-dose treatments with the three statins or with distilled water (control), as well as 24 hours after repetitive statin or distilled water (control) dosing for 14 and 28 days. Thereafter, blood samples were

centrifuged at 3000 rpm for 15 minutes to separate the plasma. The whole brain was dissected out and homogenized with the sodium chloride-barbital-phosphate buffer solution, pH 8.1 (1:9) (Mohammad *et al*., 2006; 2014) the samples put in ice glass, using a homogenizer at a speed of 400 rounds/second.

Brain and plasma GSH levels were determined by the GSH colorimetric assay kit.

The method is based on the reaction of reduced form of GSH with chromogen dithionitrobenzoic acid (DTNB) to produce dithionitrobenzoic acid (TNB) and glutathione disulfide (GSSH). The optical density of the yellow color produced by nitromercaptobenzoic acid is measured vs. GSH standard spectrophotometrically at 420 nm:

GSH + DTNB → GSSH + TNB

The GSH level was calculated as follows according to the kit manual:

For plasma GSH

GSH activity (mg GSH/L)

= [OD Sample−OD Blank/OD Standard−OD Blank] × Concentration of standard (20×10^{-3} mmol/L)

×GSH molecular weight (307) × Dilution factor (2) × Dilution factor of sample before testing

For brain homogenate GSH

GSH activity (mg GSH/g protein)

= [OD Sample−OD Blank/OD Standard−OD Blank] × Concentration of standard (20×10^{-3} mmol/L)

× GSH molecular weight (307) × Dilution factor (2) × Dilution factor of sample before testing

÷ Concentration of protein in hemoglobin (g protein/L)

Notes:

OD= Optical density

Concentration of standard: 20 µmol/L= 20×10^{-3} mmol/L

Dilution factor = Dilution factor of preparation of supernatant (2 times)

Details and steps of the procedure according to the GSH assay kit manual are shown in Appendix 1.

The levels of MDA as a product of oxidative stress in the plasma and brain were determined by MDA ELISA kit. Principally, MDA in the sample or standard competes with that on the solid phase for MDA-specific sites. Avidin-Horseradish Peroxidase conjugate are added to each micro plate well and incubated, then followed by the addition of the substrate. The optical density of the enzyme-substrate reaction is measured spectrophotometrically at a wavelength of 450 nm. The concentration of MDA in samples was calculated by extrapolation from a standard MDA curve. Details and steps of the procedure according to the MDA assay kit manual are shown in Appendix 2.

Plasma ALT and AST activities were determined using the commercial kits. The principle of the ALT method is based on the following reaction:

L-Alanine + 2-Oxoglutarate →(ALT)← Pyruvate + L-Glutamate

Where pyruvate reacts with 2,4 DNPH to form 2,4 Dinitrophenylhydrazones the absorbance of which can be measured spectrophotometrically at 505 nm in an alkaline solution. On the other hand, the principle of the AST method is based on the following reaction:

L-Aspartate + 2-Oxoglutarate →(AST)← Oxaloacetate + L-Glutamate

Where oxaloacetate reacts with 2,4 DNPH to form 2,4 Dinitrophenylhydrazones the absorbance of which can be measured

spectrophotometrically at 505 nm in an alkaline solution. Details and steps of the procedure according to the AST and AST assay kit manuals are shown in appendix 2.

3-7: Section Three

3-7-1: Drugs

The statins used were, atorvastatin, simvastatin and rosuvastatin. Each statin dosage (200 mg/kg of body weight/day for 28 consecutive days) was prepared in distilled water as a vehicle for oral administration using a gavage needle at a volume of 10 ml/kg of body weight. These dose rates of the three statins were found to produce oxidative stress in mice as found in section two, but without overt signs of toxicosis. As shown in Figure (3-7), 92 mice were randomly allocated to statin or distilled water (control) treatment groups (n= 8 or 10/group). The time of daily drug administration was between 9 a.m to 10 a.m (Rashid and Mohammad, 2023a).

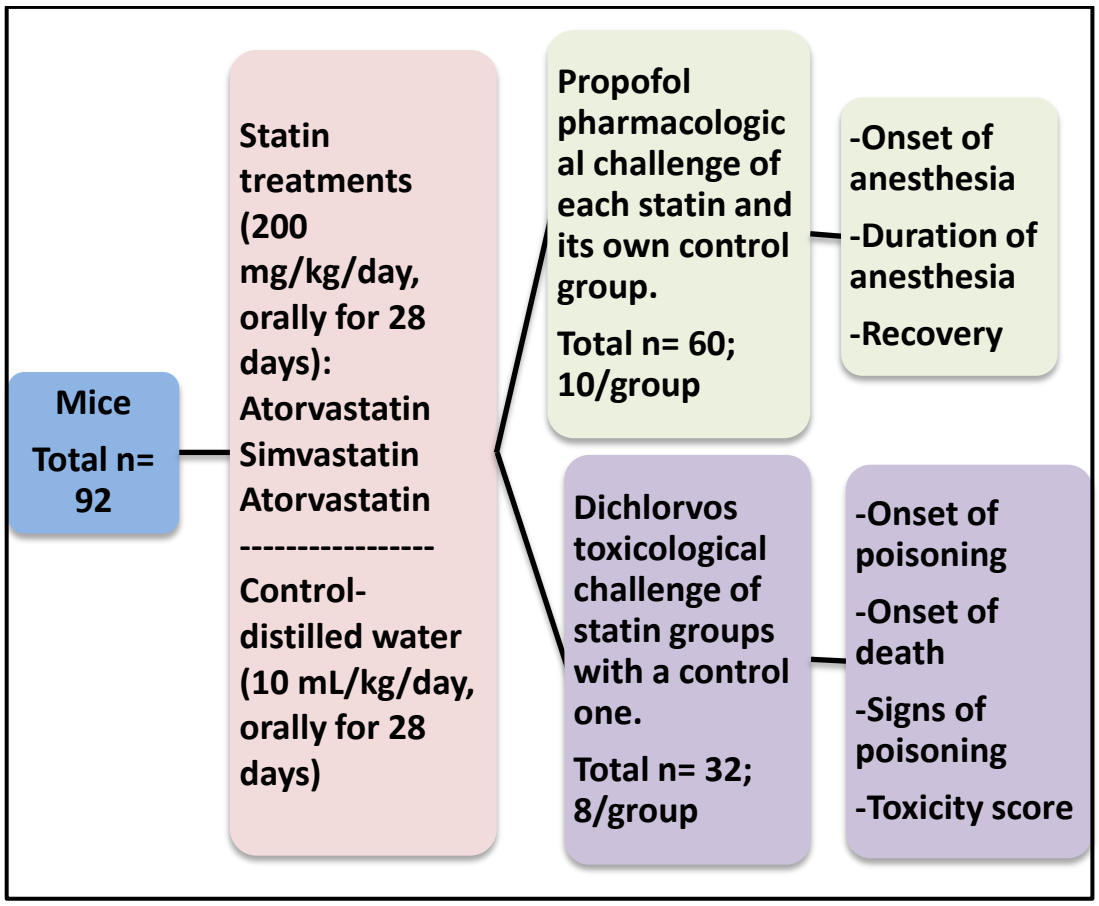


Figure (3-7): The integrated experimental design of section three of the study and allocation of male mice treated with statins to propofol pharmacological and dichlorvos toxicological challenges.

3-7-2: Pharmacological challenge with propofol

Twenty four hours after each statin or distilled water (control) repetitive dosing for 28 days, each mouse was injected intraperitoneally (i.p.) with propofol (Diprivan 1%, Corden Pharma SpA, Caponago, Italy) at 100 mg/kg of body weight (Luo *et al.*, 2022) as a pharmacological challenge (n=10 mice/group). The lag time of 24 hours for the pharmacological challenge was based on the literature in order to avoid possible acute statin effect that might result from the last day dosing on the challenge outcome (Rashid and Mohammad, 2023b). The latency to onset of loss of the righting reflex (sleep as an index of anesthesia) and the duration of anesthesia were recorded for each mouse (Rashid and Mohammad, 2023b).

3-7-3: Toxicological challenge

As with the pharmacological challenge, a toxicological challenge was introduced 24 hours after each statin- or distilled water (control) repetitive dosing for 28 days. Mice were dosed orally with dichlorvos (Nicoz, 50% EC Royal Brand, India) at 150 mg-active ingredient/10 mL distilled water/kg of body weight (n= 8 mice/group) (Mohammad *et al.*, 1989). After the dichlorvos dosing, we observed each mouse for the appearance of signs of acute organophosphate toxidrome which was characterized by excessive salivation, frequent defecation and tremors, as well as any 4- and 24 h lethality (Mohammad *et al.*, 2023). The latencies to onset of signs of acute poisoning and death were recorded. The severity of dichlorvos poisoning was rated by the toxicity score from the grades of one to four allocated to the percentage of occurrence of signs of organophosphate poisoning as well as 4- and 24 h lethality (Mohammad *et al.*, 2023). Briefly, the percentages of occurrence of each sign of poisoning, and the 4- and 24 h lethality were scored as follows: 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). The highest toxicity score would be $6 \times 4 = 24$ in intoxicated mice within a group, when all the signs of poisoning and death would occur.

3-7-4: Determination of whole brain ChE activity

Twenty four hours after repetitive doses statin or distilled water (control) dosing for 28 days, mice were sacrificed by cervical dislocation. The whole brain was dissected out and homogenized in the sodium chloride-barbital-phosphate buffer solution, pH 8.1 (1:9) using a homogenizer at a speed of 400 rounds/second (Mohammad *et al.*, 2014). The ChE activity in the whole brain was determined spectrophotometrically , as outlined in section one.

3-8: Section Four

3-8-1: Drugs

Atorvastatin, simvastatin, and rosuvastatin were used. The required dose of each statin was freshly prepared on the day of administration as 2% solution in distilled water to be administered orally by a gavage needle at a volume of 10 ml/kg of body weight (200 mg/kg of body weight/day for 14 and 28 consecutive days). Mice treated with distilled water at 10 mL/kg of body weight accompanied all statin treatments as control counterparts. Figure 3-8 outlines the experimental protocol and distribution of a total of 80 mice (age 100-120 days and body weight 30-35 g) randomly into 8 groups of statin and control (distilled water) treatment regimens. The final number of mice for behavioral measurements was 10 per statin or control group, whereas it was 8 per statin or control group (because it involved animal sacrifice) for the determination of ChE activity and cholesterol level. According to section two of the present study, the repetitive dose of each statin (200 mg/kg of body weight) we applied in the present section was not obviously toxic in mice

3-8-2: Neurobehavioral measurements

Twenty four hours after the last repetitive 14-day or 28-day dosing of each statin and distilled water (Figure 3-8), each mouse was separately tested for the neurobehavioral performance that included 5-min open-field activity, negative geotaxis performance at an angle of 45°, 5-min head pocking behavior and a single session of forced swimming endurance at a temperature of 24 ± 1 °C, as mentioned in section one of the study. Thereafter, the mice were dried off and allowed to rest in their home cages for about one hour before the start of blood sampling.

Samples obtained:

After conclusion of the behavioral tests, a blood sample was withdrawn from retro-orbital plexus of each statin or control mouse under anesthesia with ether into heparinized capillary tubes. Blood samples were centrifuged at 3000 rpm for 15 min to separate the plasma and erythrocytes. The whole brain was dissected out and homogenized with an electric homogenizer at a speed of 400 rounds/s using the sodium chloride-barbital-phosphate buffer solution, pH 8.1 (1:9).

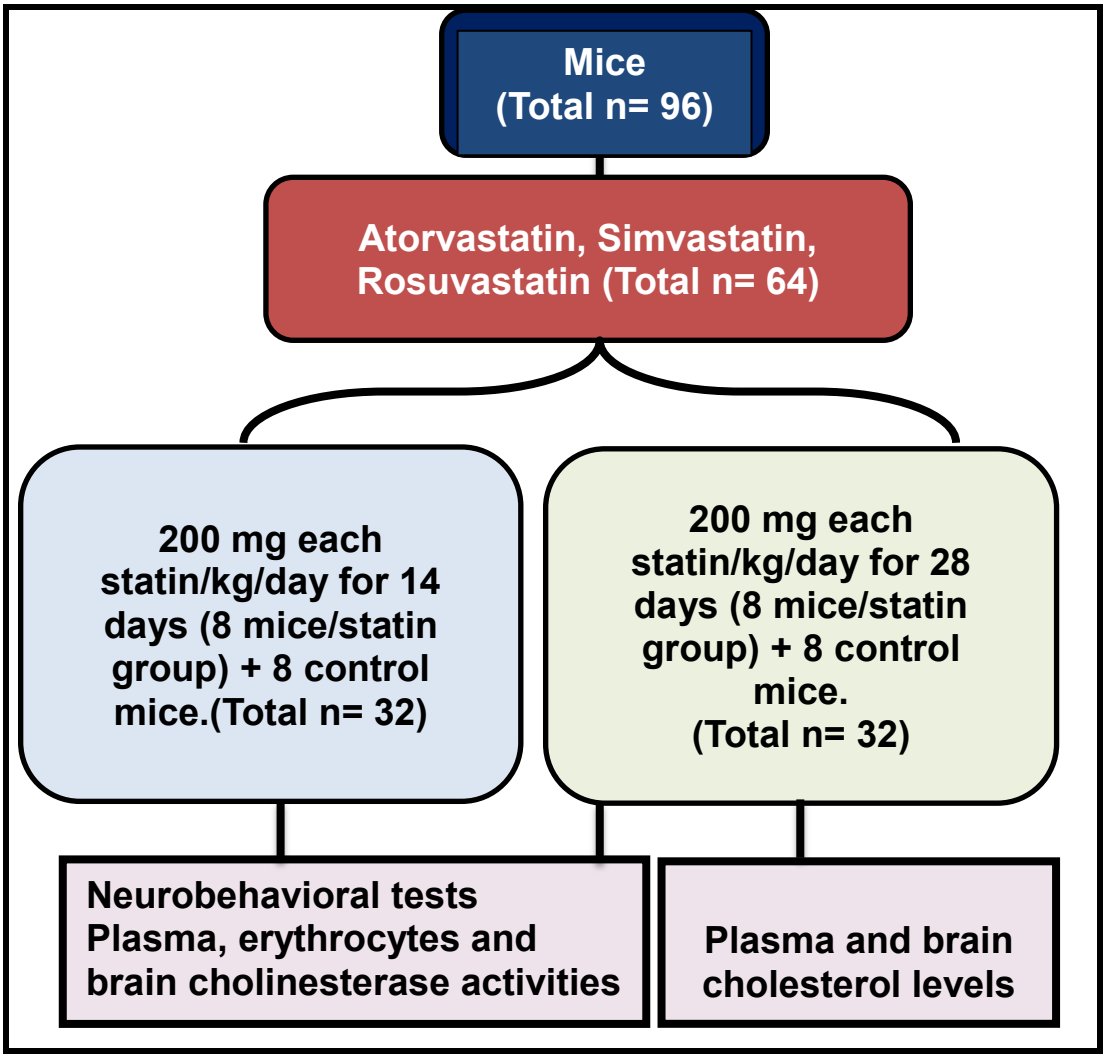


Figure (3-8): Section four of the study design, repeated statin treatments, and allocation of mice into groups for neurobehavioral measurements and determinations of cholinesterase activity and cholesterol level.

3-8-3: Determination of ChE activity and cholesterol level

ChE activities in the plasma, erythrocytes, and whole brain were determined, 24 h after the last repetitive 14-day or 28-day dosing of each statin and distilled water, spectrophotometrically as outlined before in section one of the study. Plasma and brain cholesterol levels were determined, 24 h after the 28th dosing (taken as a target treatment endpoint) of each statin and control group, using a commercial spectrophotometric kit as outlined above (figure 3-8).

3-9: Section Five

3-9-1: Drugs

Atorvastatin, simvastatin and rosuvastatin were used. The required in vitro aqueous concentrations of the three statins were freshly and separately prepared before each in vitro experiment, so that the final concentrations in the reaction mixtures of ChE inhibition and generation of oxidative stress would be at 0 (baseline-control), 10, 25, 50 or 100 µM. These concentrations were chosen from preliminary in vitro experiments we conducted and from the literature (Kwak *et al*., 2012; Liu *et al*., 2019; Hacıseyitoğlu *et al*., 2024).

Experimental protocol

A total of 50 drug-free male mice of Swiss-origin (age 100-125 days and body weight 30-35 g) were used in the present study (Figure 3-9).

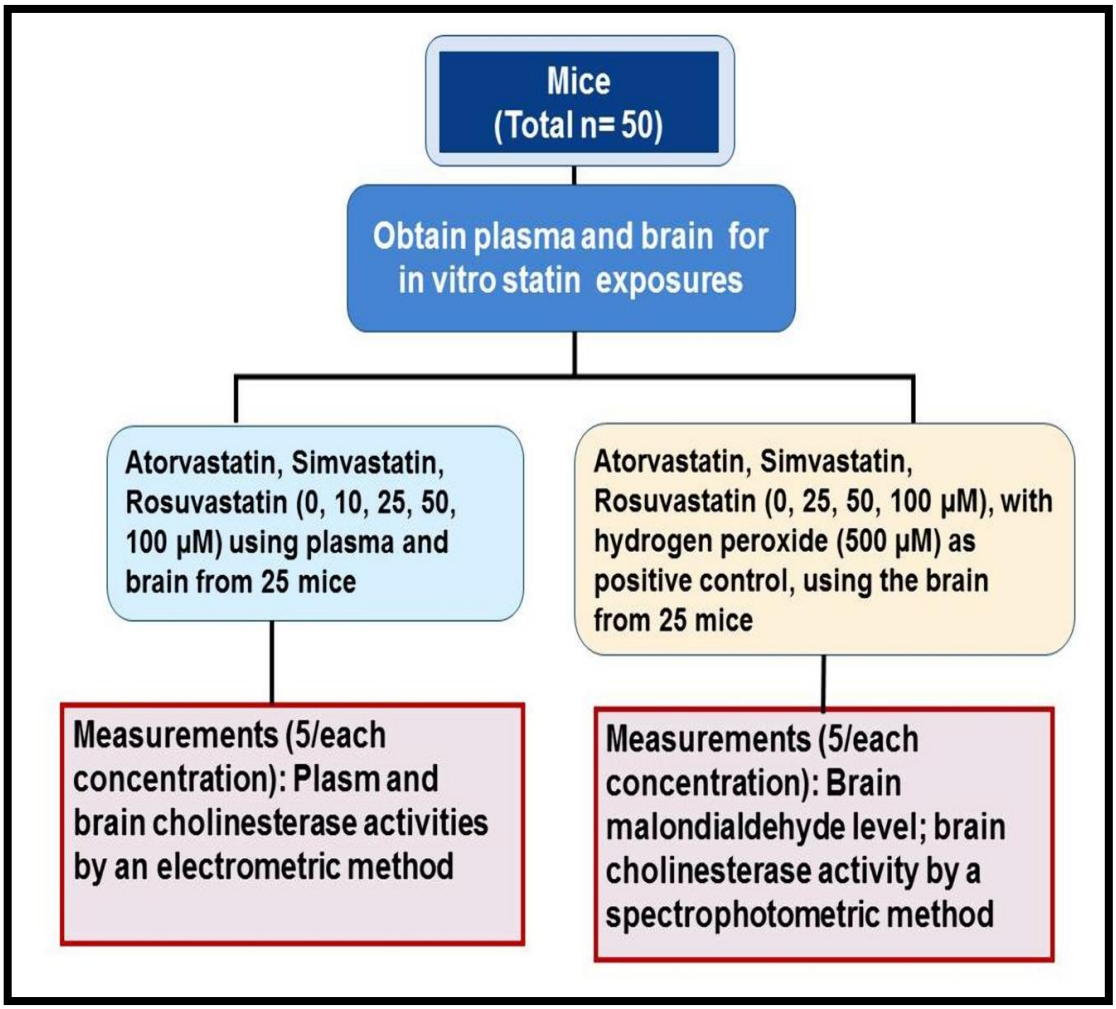


Figure (3-9): The integrated section five of the study design for the in vitro effects of statins on cholinesterase activity and generation of malondialdehyde by using the plasma and whole brain homogenates of mice.

3-9-2: In vitro ChE inhibition by statins and measurement of the enzyme activity by an electrometric method

Mice (n= 25) under ether anesthesia were used to obtain at first blood samples, which were withdrawn from the retro-orbital plexus into heparinized capillary tubes (Figure 3-9). Blood samples were centrifuged at 3000 rpm for 15 min to separate the plasma aliquots which were pooled for the homogeneity of the enzyme source (Mohammad *et al*., 2014; Rashid and Mohammad, 2023a). The whole brain was dissected out and

subjected to homogenization using an electric homogenizer at a speed of 400 rounds/s with the sodium chloride-barbital-phosphate buffer solution, pH 8.1 at 100 mg weight/3 ml (Mohammad *et al*., 2014; Rashid and Mohammad, 2023a). Brain homogenates were also pooled.

Aliquots (0.2 ml) of pooled plasma samples and whole brain homogenates were separately used for the in vitro ChE inhibition by incubating them with selected concentrations of each statin (0, 10, 25, 50, 100 µM), 3 ml of the sodium chloride-barbital-phosphate buffer solution and 3 ml of distilled water at 37 °C for 10 min (Mohammad *et al*., 2014; Rashid and Mohammad, 2023a). Thereafter, the ChE activity in the plasma or brain homogenate mixtures were determined (5 times/each concentration) by an electrometric method reported earlier (Mohammad *et al*., 2014; Rashid and Mohammad, 2023a). The procedure included a reaction mixture in a 10-ml glass beaker, which contained 3 ml of distilled water, 0.2 ml plasma or whole brain homogenate and 3 ml of sodium chloride-barbital-phosphate buffer solution, pH 8.1. The initial pH of the enzymatic reaction mixture (pH1) was measured with a glass electrode using a pH meter. Thereafter, 0.10 ml of 7.1% acetylcholine iodide, as a substrate, was added to the reaction mixture, which was then subjected to 30 min incubation at 37 ºC. At the end of the 30-min incubation period, the pH of the reaction mixture (pH2) was measured again. The enzyme activity was estimated as follows:

ChE activity (ΔpH/30 min.) = (pH1 – pH2) - Δ pH of blank

The percentage of ChE inhibition was estimated using the formula:

% ChE inhibition= [ChE activity (without statin) – ChE activity (with statin) / ChE activity (without statin)] ^{x} 100

To double check in vitro effects of statins as well as that of hydrogen peroxide (500 µM) on brain ChE activity and to avoid the relatively long incubation period of 30 min of the electrometric method

mentioned above, aliquots of brain homogenates were used for the determination of ChE activity using a commercial kit.

3-9-3: In vitro pro-oxidant property of statins using whole brain homogenates

In another experiment using 25 mice, whole brain homogenates were obtained as described above, and they were pooled (Figure 3-9). As outlined in Table 3-1, aliquots (0.25 ml) of the brain homogenate were used for the in vitro determination of MDA generation under the influences of selected concentrations of each statin (0, 25, 50, 100 µM) or 0.13 ml hydrogen peroxide (500 µM) added separately to the reaction mixture, as a source of oxidative stress (positive control) with 0.15 ml of distilled water; the mixture was then incubated at 37 °C for two hours (Nakamura *et al*., 2003; Karam and Mohammad, 2024).

Table (3-1): Steps of in vitro evaluation of oxidative stress with statins

Contents	Tube No.1	Tube No.2	Tube No.3	Tube No.4	Tube No.5
Statin (µM)	25/0.1 mL	50/0.1 mL	100/ 0.1 mL	-ve control (no statin), 0.1 mL H_2O	+ve control (H_2O_2),0.1 mL H_2O
Brain homogenate (mL)	0.25	0.25	0.25	0.25	0.25
First incubation 37 °C (h)	1	1	1	1	1
H_2O_2 (500 µM) (0.13 mL)	0	0	0	0	0.13
H_2O (Distilled water) (0.1 mL)	0.15	0.15	0.15	0.15	0.02
Second incubation 37 °C (h)	1	1	1	1	1
Total volume, mL	0.5	0.5	0.5	0.5	0.5

3-10: Statistical Analysis

Section one

Data were statistically analyzed using the statistical software package Past 4.13 (https://www.nhm.uio.no/english/research/resources past/). Parametric data consisting of multiple means were statistically analyzed using analysis of variance followed by least significant difference test. Frequency data were analyzed using Fisher's exact probability test, and non-parametric data were analyzed using the Kruskal–Wallis test, followed by Dunn's test. The level of statistical significance was set at $p \leq 0.05$.

Section Two

The data were statistically analyzed by analysis of variance followed by the least significant difference test, using IBM SPSS Statistics for Windows, Version 20 (Released 2011; IBM Corp., Armonk, New York). The level of statistical significance was at $p < 0.05$

Section Three

The statistical package SPSS-version 20 (IBM) was used to analyze data statistically. Parametric data were subjected to the one way analysis of variance followed by the least significant difference test, whereas non-parametric frequency data were analyzed by the Fisher's exact probability test, and the scores of organophosphate poisoning were subjected to the Kruskal-Wallis test followed by the Dunn's test. The level of statistical significance was at $p \leq 0.05$.

Section Four

The data were presented whenever appropriate as mean ± standard error (SE). They were statistically analyzed by the analysis of variance

followed by the least significant difference test, using the statistical software package SPSS version 20 (IBM). Linear regressions analyses were also performed to correlate and report r^2 values of brain and blood ChE activities. The level of statistical significance was at $p \leq 0.05$.

Section Five

Experimental results were presented whenever appropriate as mean ± standard error (SE). They were subjected to one way analysis of variance followed by the least significant difference test, using the statistical software package SPSS version 20 (IBM). Linear regressions and correlation analyses were also performed to find out the correlation coefficient (r) between values of brain MDA levels and ChE activities under in vitro statin exposures. The level of statistical significance was at $p \leq 0.05$.

Chapter Four

Results

Chapter Four

Results

Section one

4-1 :Neurobehavioral effects of statins

As shown in Table 4-1, the three statins (atorvastatin, simvastatin, and rosuvastatin) variably but dose-dependently and significantly ($p < 0.05$) changed open-field activities of mice 2 h after oral dosing compared with respective control values (vehicles). Significant delays in the latency to move from the central square in the open-field arena, decreases in locomotor activity (squares crossed for 5 min), and the frequency of rearing for 5 min were observed. In line with the reduced open field activities, statin-treated mice in a dose-dependent manner showed significant reductions in head pocking behavior and delayed negative geotaxis performance at an angle of 45°. However, statins significantly increased the duration of initial forced swimming and decreased the duration of immobility in the swimming tank (Table 4-2).

Table (4-1): Open-field activity in mice two hours after statin treatments

Treatment groups (mg/kg, body weight, orally)	Open-field activity		
	Latency to move (sec)	Squares crossed/5 min	Rearing/5 min
Atorvastatin			
0	0.19±0.03	128.4±0.7	24.6±.0.6
250	0.30±0.03	122.7±1.1*	23.9±0.5
500	0.52±0.02*a	100.8±0.9*a	13.9±0.6*a
750	0.60±0.03*ab	66.6±2.1*ab	3.0±0.4*ab
1000	0.78±0.02*abc	42.2±6.7*abc	1.0±0.3*abc
Simvastatin			
0	0.13±0.02	129.9±1.9	20.5±0.5
250	0.17±0.02	125.6±1.8	19.8±0.6
500	0.40±0.03*a	115.7±1.3*a	12.7±0.4*a
750	0.58±0.03*ab	85.1±2.2*ab	4.7±0.4*ab
1000	0.68±0.02*abc	52.3±2.4*abc	2.1±0.2*abc
Rosuvastatin			
0	0.14±0.02	128.5±1.1	24.3±0.5
250	0.22±0.02	125.6±1.2	21.7±0.6*
500	0.42±0.02*a	112.1±1.6*a	15.0±0.9*a
750	0.57±0.04*ab	87.3±1.8*ab	5.1±0.6*ab
1000	0.68±0.03*abc	36.7±2.04*abc	3.5±0.3*abc

Values are mean ± SE of 10 mice/group.

The 0 treatment group is the control group treated with distilled water (10 ml/kg, body weight).

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

[a]Significantly different from the respective value of the 250 mg/kg treatment group, $p < 0.05$.

[b]Significantly different from the respective value of the 500 mg/kg treatment group, $p < 0.05$.

[c]Significantly different from the respective value of the 750 mg/kg treatment group, $p < 0.05$.

Table (4-2): Neurobehavioral outcome in mice two hours after statins treatment

Treatment groups (mg/kg, body weight, orally)	Head pocking/5 min	Negative geotaxis (sec)	Forced swimming (6min)	
			Duration of initial swimming (min)	Duration of immobility (sec)
Atorvastatin				
0	23.2±0.7	2.4±0.4	2.22±0.05	35.5±0.8
250	20.6±0.7*	2.7±0.6	2.36±0.11	34.2±1.2
500	6.8±0.3*a	11.1±0.8*a	3.38±0.07*a	18.0±0.6*a
750	4.8±0.4*a	31.3±0.8*ab	4.01±0.08*ab	3.8±0.5*ab
1000	2.1±0.2*abc	47.4±2.1*abc	4.46±0.08*abc	1.6±0.2*abc
Simvastatin				
0	24.2±0.8	1.8±0.2	2.34±0.08	34.2±0.6
250	17.8±0.4	3.2±0.2	2.66±0.05*	34.1±0.5
500	9.8±0.6*a	29.4±1.3*a	3.28±0.90*a	21.5±1.3*a
750	6.5±0.4*ab	51.3±1.2*ab	4.36±0.80*ab	5.1±0.5*ab
1000	3.1±0.4*abc	55.5±1.0*abc	5.25±0.10*abc	2.2±0.2*abc
Rosuvastatin				
0	24.0±0.8	1.4±0.2	2.35±0.06	34.2±0.6
250	21.1±0.5*	4.5±0.6*	2.25±0.05	32.8±0.5
500	10.6±0.7*a	31.9±1.4*a	3.13±0.05*a	24.1±1.0*a
750	7.0±0.4*ab	50.8±1.3*ab	4.34±0.08*ab	11.1±0.8*ab
1000	3.7±0.4*abc	50.5±0.9*abc	4.82±0.09*abc	4.6±0.5*abc

Values are mean ± SE of 10 mice/group.

The 0 treatment group is the control group treated with distilled water (10 ml/kg, body weight).

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

[a]Significantly different from the respective value of the 250 mg/kg treatment group, $p < 0.05$.

[b]Significantly different from the respective value of the 500 mg/kg treatment group, $p < 0.05$.

[c]Significantly different from the respective value of the 750 mg/kg treatment group, $p < 0.05$.

4-2: Effects of statins on ChE activity

Plasma ChE activity was significantly and dose-dependently decreased 2 h post-treatment by 25%–34% and 37%–51%, respectively, compared with the respective control values in mice orally dosed with the three statins atorvastatin, simvastatin, and rosuvastatin, each at 500 and 1000 mg/kg, respectively (Table 4-3). Twenty-four hours after statin dosing, plasma ChE activity decreased by 19%–30% and 24%–34%, respectively, compared with the respective control value (Table 4-3). When erythrocyte ChE activity was determined in statin-treated mice, the 2 h decrease in activity was significant by 11%–12% and 24%–25%, respectively, and the 24 h reduction values were 14%–16% and 25%–30%, respectively, relative to the respective control values (Table 4-4). Similarly, the 2 h brain ChE activity was significantly reduced in statin-treated mice by 13%–17% and 27%–31%, respectively, and that of the 24 h after treatment was 8% for the 500 mg/kg dose groups, and 22% to 24% in the 1000 mg/kg dose groups, when compared with the respective control value (Table 4-5). Twenty-four hours after statin treatments, plasma and brain ChE activities recovered from the 2 h values to gain more activity by 5%–32.9% and 5.7%–14.4%, respectively (Tables 4-3 and 4-5). Erythrocytes did not recover from the reduced values when determined 24 h later (Table 4-4).

Table (4-3): Plasma cholinesterase activity (U/ml) in mice dosed orally with statins by colorimetric method

Plasma cholinesterase activity			
Treatment group (mg/kg)	**2 h**	**24 h**	**% recovery from the 2-h values**
Distilled water-control	54.506±1.432	52.961± 0.941	0
Atorvastatin (500)	40.972±0.344*	43.035±0.435*†	5.0
Simvastatin (500)	38.802±0.567*	36.952±1.422*a	0
Rosuvastatin (500)	35.976±0.518*a	39.778±0.710*†ab	10.6
Atorvastatin (1000)	30.430±0.569*abc	40.430±0.491*†ab	32.9
Simvastatin (1000)	34.127±0.866*abd	38.693±0.614*†a	13.4
Rosuvastatin (1000)	26.515±0.958*abcde	34.780±0.677*†acde	31.2

Values are mean ± SE of 8 mice/group.

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

†Significantly different from the respective 2 h cholinesterase activity, $p < 0.05$.

a Significantly different from the respective atorvastatin 500 mg/kg dose group, $p < 0.05$.

b Significantly different from the respective simvastatin 500 mg/kg dose group, $p < 0.05$.

c Significantly different from the respective rosuvastatin 500 mg/kg dose group, $p < 0.05$.

d Significantly different from the respective atorvastatin 1000 mg/kg dose group, $p < 0.05$.

e Significantly different from the respective simvastatin 1000 mg/kg dose group, $p < 0.05$.

Table (4-4): Erythrocyte cholinesterase activity (U/ml) in mice dosed orally with statins by colorimetric method

Erythrocyte cholinesterase activity		
Treatment group (mg/kg)	**After 2 hours**	**After 24 hours**
Distilled water	77.835±0.888	76.222±0.941
Atorvastatin (500)	69.060±0.509*	65.468±0.790*†
Simvastatin (500)	68.615±0.776*	64.471±0.697*†
Rosuvastatin (500)	68.589±0.889*	63.936±0.951*†
Atorvastatin (1000)	58.725±0.561*abc	56.992±0.674*abc
Simvastatin (1000)	57.878±0.785*abc	53.518±0.689*†abc
Rosuvastatin (1000)	59.127±0.647*abc	53.215±0.745*†abcd

Values are mean ± SE of 8 mice/group.

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

†Significantly different from the respective 2 h cholinesterase activity, $p < 0.05$.

a Significantly different from the respective atorvastatin 500 mg/kg dose group, $p < 0.05$.

b Significantly different from the respective simvastatin 500 mg/kg dose group, $p < 0.05$.

c Significantly different from the respective rosuvastatin 500 mg/kg dose group, $p < 0.05$.

d Significantly different from the respective atorvastatin 1000 mg/kg dose group, $p < 0.05$.

Table (4-5): Brain cholinesterase activity (U/mg protein) in mice dosed orally with statins by colorimetric method

Brain cholinesterase activity			
Treatment group (mg/kg)	**after2 hours**	**after24 hours**	**% recovery from the 2-h values**
Distilled water	75.418±0.958	75.672±0.712	0.3
Atorvastatin (500)	65.797±0.964*	69.887±0.628*	6.2
Simvastatin (500)	62.671±0.651*a	69.812±0.619*†	11.4
Rosuvastatin (500)	64.020±0.767*	69.453±0.593*†	8.5
Atorvastatin (1000)	55.277±0.706*abc	58.725±0.561*†abc	6.2
Simvastatin (1000)	54.777±0.879*abc	57.878±0.785*†abc	5.7
Rosuvastatin (1000)	51.668±0.379*abcd	59.127±0.647*†abc	14.4

Values are mean ± SE of 8 mice/group.

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

†Significantly different from the respective 2 h cholinesterase activity, $p < 0.05$.

a Significantly different from the respective atorvastatin 500 mg/kg dose group, $p < 0.05$.

b Significantly different from the respective simvastatin 500 mg/kg dose group, $p < 0.05$.

c Significantly different from the respective rosuvastatin 500 mg/kg dose group, $p < 0.05$.

d Significantly different from the respective atorvastatin 1000 mg/kg dose group, $p < 0.05$.

Section Two

4-3: Single-dose statin treatments

Single dose treatments with the statins atorvastatin, simvastatin and rosuvastatin at 500 and 1000 mg/kg of body weight significantly ($p < 0.05$), and dose-dependently reduced GSH level in the plasma and the whole brain when compared with respective control values (Table 4-6). The most effective statin in causing significant reductions in plasma and brain GSH levels was rosuvastatin at 1000 mg/kg (mean ± SE, 36.533 ± 1.33 mg/L and 0.742 ± 0.03 mg/g protein, respectively) when compared with respective control values (69.7 ± 1.43 and 1.411 ± 0.02), and in comparison with the reductions caused by atorvastatin (56.49 ± 1.17 and 1.137 ± 0.03) and simvastatin (50.303 ± 1.07 and 1.087 ± 0.03). Atorvastatin was the least effective statin, since only the high dose achieved a significant reduction in brain GHS level (1.137 ± 0.03) in comparison with the respective control value (1.411 ± 0.02).

Table (4-6): Plasma and brain glutathione levels in mice 2 hours after a single dose oral treatment with statins

Statin groups	Single statin dose			
	500 mg/kg	p-value	1000 mg/kg	p-value
Plasma GSH (mg/L)				
Distilled water-control	65.707 ±1.66	-	69.700 ±1.43	0.03†
Atorvastatin	54.131 ±1.48	0.0001*	56.490 ±1.17	0.0001*,0.192†
Simvastatin	48.930 ±1.00	0.0001* 0.005a	50.303 ±1.07	0.0001*,0.001a 0.445†
Rosuvastatin	43.496 ±0.70	0.0001*a 0.0004b	36.533 ±1.33	0.0001*ab†
Brain GSH (mg/g protein)				
Distilled water- water	1.405 ± 0.03	-	1.411 ±0.02	0.874†
Atorvastatin	1.327 ± 0.02	0.055*	1.137 ±0.03	0.0001*†
Simvastatin	1.182 ± 0.02	0.0001*a	1.087 ±0.03	0.0001*,0.208a 0.019†
Rosuvastatin	1.127 ± 0.03	0.0001*a 0.166b	0.742 ±0.03	0.0001*ab†

Values are mean ± SE of 8 mice/group.

GSH= Glutathione.

*p-value for the statistical difference from the respective control group.

[a]p-value for the statistical difference from the respective atorvastatin dose group.

[b]p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 500 mg/kg dose group of the same statin.

Analysis of variance: plasma GSH data, F= 75.038; brain GSH data, F= 61.319, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

The two single doses of each statin (500 and 1000 mg/kg) significantly increased the level of the oxidative stress biomarker MDA in the plasma and brain of mice in a dose-dependent manner in comparison with respective control values (Table 4-7). The statin that caused the highest significant increase in MDA level in the plasma (983.4 ± 8.46 ng/ml) and whole brain (948.225 ± 5.17 ng/mg) was rosuvastatin at 1000 mg/kg vs. respective control values (75.916 ± 2.0 and 76.012 ± 2.09, respectively). These plasma and brain MDA levels in rosuvastatin-treated mice were even significantly higher than those of the respective atorvastatin (180.842 ± 3.69 and 172.875 ± 2.35) and simvastatin (581.310 ± 3.63 and 536.737 ± 5.19) groups. Single doses of atorvastatin (500 and 1000 mg/kg) were the least effective statin treatments in increasing the MDA level in the plasma (159.250 ± 3.17 and 180.842 ± 3.69) and brain (133.0 ± 1.81 and 172.875 ± 2.35) of mice vs. respective plasma (72.075 ± 2.40 and 75.916 ± 2.0) and brain (69.167 ± 2.64 and (76.012 ± 2.09) control values. Concomitantly with single dose changes in GSH and MDA levels, the liver enzyme ALT in the plasma of mice treated with single doses of the satins (500 and 1000 mg/kg) were significantly above the respective control values (Table 4-8). On the other hand, at 500 mg/kg, only rosuvastatin significantly increased plasma AST activity in comparison with the respective control value, whereas atorvastatin and simvastatin effects did not attain statistical significance differences ($p > 0.05$). However, the three statins showed a dose-dependent effect, as the 1000 mg/kg dose level significantly increased plasma AST activity in comparison with the control value (Table 4-8). Additional statistical analysis revealed that the most prominent and significant adverse effects on ALT and AST activities were due to rosuvastatin treatments at 500 mg/kg (27.157 ± 0.51 and 37.446 ± 1.11 IU/L, respectively) and 1000 mg/kg (30.842 ± 0.62 and 53.661 ± 1.08,

respectively) when compared to respective plasma ALT (4.568 ± 0.21 and 4.802 ± 0.23) and AST (10.815 ± 0.56 and 10.701 ± 0.47) control values.

Table (4-7): Plasma and brain malondialdehyde levels in mice 2 hours after a single dose oral treatment with statins.

Statin groups	Single statin dose			
	500 mg/kg	p-value	1000 mg/kg	p-value
Plasma MDA (ng/ml)				
Distilled water-control	72.075 ± 2.40	-	75.916 ± 2.00	0.548†
Atorvastatin	159.250 ± 3.17	0.0001*	180.842 ± 3.69	0.0001*†
Simvastatin	226.312 ± 2.63	0.0001*a	581.310 ± 3.65	0.0001*a†
Rosuvastatin	655.087 ± 4.80	0.0001*ab	983.400 ± 8.46	0.0001*ab†
Brain MDA (ng/mg)				
Distilled water- control	69.167 ± 2.64	-	76.012 ± 2.09	0.184†
Atorvastatin	133.00 ± 1.81	0.0001*	172.875 ± 2.35	0.0001*†
Simvastatin	222.812 ± 1.86	0.0001*a	536.737 ± 5.19	0.0001*a†
Rosuvastatin	652.875 ± 5.15	0.0001*ab	948.225 ± 5.17	0.0001*ab†

Values are mean ± SE of 8 mice/group.

MDA= Malondialdehyde.

*p-value for the statistical difference from the respective control group.

[a]p-value for the statistical difference from the respective atorvastatin dose group.

[b]p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 500 mg/kg dose group of the same statin.

Analysis of variance: plasma MDA data, F= 5368.996; brain MDA data, F= 8076.438, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

Table (4-8): Plasma alanine aminotransferase and aspartate aminotransferase activities in mice 2 hours after a single dose oral treatment with statins

Statin groups	Single statin dose			
	500 mg/kg	p-value	1000 mg/kg	p-value
Plasma ALT (IU/L)				
Distilled water-control	4.568 ± 0.21	-	4.802 ± 0.23	0.233†
Atorvastatin	12.187 ± 1.33	0.0001*	12.846 ± 0.41	0.0001*
Simvastatin	11.516 ± 0.42	0.0001* 0.439a	16.003 ± 0.32	0.0001*a†
Rosuvastatin	27.157 ± 0.51	0.0001*ab	30.842 ± 0.62	0.0001*†ab
Plasma AST (IU/L)				
Distilled water-Control	10.815 ± 0.56	-	10.701 ± 0.47	0.978†
Atorvastatin	16.702 ± 0.48	0.153*	33.123 ± 0.90	0.0001*†
Simvastatin	17.096 ± 0.59	0.128* 0.923a	25.240 ± 0.64	0.001* 0.058 a 0.05†
Rosuvastatin	37.446 ± 1.11	0.0001*ab	53.661 ± 1.08	0.0001*†ab

Values are mean ± SE of 8 mice/group.

ALT= Alanine aminotransferase; AST= aspartate aminotransferase.

*p-value for the statistical difference from the respective control group.

[a]p-value for the statistical difference from the respective atorvastatin dose group.

[b]p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 500 mg/kg dose group of the same statin.

Analysis of variance: ALT data, F= 245.973; AST data, F= 364.383, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

4-4: Repeated statin treatments

Repeated dosing of mice with each of the three statins at 200 mg/kg/day for 14- and 28 consecutive days significantly and time-dependently reduced plasma and brain GSH levels in comparison with respective control values (Table 4-9). Within these time frame treatment regimens, rosuvastatin was the most effective statin among the statin treatment groups in reducing plasma (27.402 ± 0.96 and 19.908 ± 0.85) and brain (0.747 ± 0.03 and 0.408 ± 0.03) GSH levels, respectively, when compared with concurrent control values in the plasma (67.333 ± 1.04 and 68.070 ± 1.10) and the brain (1.417 ± 0.02 and 1.442 ± 0.03).

Similar to single-dose treatment regimens, the three statins after repetitive treatments for 14- and 28 consecutive days significantly and time-dependently increased plasma and brain MDA levels in comparison with respective control values (Table 4-10). The effects of rosuvastatin treatments for 14- and 28 days on MDA levels in the plasma (1134.153 ± 8.08 and 1234.221) and brain (566.712 ± 5.95 and 1082.00 ± 3.35) of mice were the most prominent and significant ones among the statins when compared with respective control values in the plasma (75.195 ± 1.64 and 81.812 ± 1.73) and the brain (64.190 ± 1.27 and 67.912 ± 2.22).

Repetitive administration of the three statins at 200 mg/kg/day for 14, or 28 consecutive days caused liver injury which was similar to that of single dose statin treatments mentioned above. This was evident by significant and time-dependent elevations in plasma ALT and AST activities in comparison with respective control values (Table 4-11). The most injurious statin was rosuvastatin repetitive dosing for 14- and 28 consecutive days, as the ALT (44.065 ± 1.15 and 55.317 ± 1.22) and AST (56.036 ± 1.10 and 71.065 ± 1.35) activities were significantly elevated compared to those of respective control ALT (4.720 ± 0.20 and 4.638 ± 0.25) and AST (11.522 ± 0.49 and 13.037 ± 0.50) values.

Table (4-9): Plasma and brain glutathione levels in mice dosed orally with statins at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Statin groups	Duration of statin treatment			
	14 days	p-value	28 days	p-value
Plasma GSH (mg/L)				
Distilled water-control	67.333 ± 1.04	-	68.070 ± 1.10	0.626†
Atorvastatin	45.572 ± 1.18	0.0001*	51.756 ± 1.10	0.0001*†
Simvastatin	44.773 ± 1.21	0.0001* 0.597^{a}	43.837 ± 1.00	0.0001^{*a} 0.536†
Rosuvastatin	27.402 ± 0.96	0.0001^{*ab}	19.908 ± 0.85	0.0001$^{*\dagger ab}$
Brain GSH (mg/g protein)				
Distilled water-control	1.417 ± 0.02	-	1.442 ± 0.03	0.731†
Atorvastatin	1.122 ± 0.03	0.0001*	0.857 ± 0.03	0.0001*,0.001†
Simvastatin	0.843 ± 0.12	0.0001^{*a}	0.678 ± 0.03$^{*\dagger a}$	0.0001*,0.017^{a} 0.0260†
Rosuvastatin	0.747 ± 0.03	0.0001^{*a}, 0.189^{b}	0.408 ± 0.03	0.0001$^{*\dagger ab}$

Values are mean ± SE of 8 mice/group. The mice were sacrificed 24 hours after the last 14-or 28-day consecutive treatments.

GSH= Glutathione.

*p-value for the statistical difference from the respective control group.

^{a}p-value for the statistical difference from the respective atorvastatin dose group.

^{b}p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 14-day value of the same statin.

Analysis of variance: plasma GSH data, F= 254.242; brain GSH, F= 50.059, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

Table (4-10): Plasma and brain malondialdehyde levels in mice dosed orally with statins at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Statin groups	Duration of statin treatment			
	14 days	p-value	28 days	p-value
Plasma MDA (ng/ml)				
Distilled water-control	75.195 ± 1.64	-	81.812 ± 1.73	$0.378^{\dagger}$
Atorvastatin	187.953 ± 4.25	0.0001^{*}	187.568 ± 2.46	0.0001^{*} $0.959^{\dagger}$
Simvastatin	268.850 ± 3.74	0.0001^{*a}	620.487 ± 7.59	$0.0001^{*a\dagger}$
Rosuvastatin	1134.153 ± 8.08	0.0001^{*ab}	1234.221±7.42	$0.0001^{*\dagger ab}$
Brain MDA (ng/mg)				
Distilled water-control	64.190 ± 1.27	0.747	67.912 ± 2.22	$0.747^{\dagger}$
Atorvastatin	134.00 ± 1.77	0.0001^{*}	226.037 ± 4.37	$0.0001^{*\dagger}$
Simvastatin	185.475 ± 2.20	0.0001^{*a}	332.887 ± 3.51	$0.0001^{*a\dagger}$
Rosuvastatin	566.712 ± 5.95	0.0001^{*ab}	1082.00 ± 3.35	$0.0001^{*ab\dagger}$

Values are mean ± SE of 8 mice/group. The mice were sacrificed 24 hours after the last 14- or 28-day consecutive treatments.

MDA= Malondialdehyde.

*p-value for the statistical difference from the respective control group.

^{a}p-value for the statistical difference from the respective atorvastatin dose group.

^{b}p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 14-day value of the same statin.

Analysis of variance: plasma MDA data, F= 8004.814; brain MDA, F= 10270.293, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

Table (4-11): Plasma alanine aminotransferase and aspartate aminotransferase activities in mice dosed orally with statins at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Statin groups	Duration of statin treatment			
	14 days	p-value	28 days	p-value
Plasma ALT (IU/L)				
Distilled water-control	4.720 ± 0.20	-	4.638 ± 0.25	0.933†
Atorvastatin	12.682 ± 0.40	0.0001*	16.685 ± 0.38	0.0001*†
Simvastatin	16.670 ± 0.59	0.0001^{*a}	21.311 ± 0.30	0.0001^{*a†}
Rosuvastatin	44.065 ± 1.15	0.0001^{*ab}	55.317 ± 1.22	0.0001^{*ab†}
Plasma AST (IU/L)				
Distilled water-control	11.522 ± 0.49	-	13.037 ± 0.50	0.225†
Atorvastatin	21.225 ± 0.68	0.001*	26.338 ± 0.75	0.0001*†
Simvastatin	25.581 ± 0.48	0.0001* 0.001^{a}	28.971 ± 1.14	0.0001* 0.037^{a} 0.008†
Rosuvastatin	56.036 ± 1.10	0.0001^{*ab}	71.065 ± 1.35	0.0001$^{*\dagger ab}$

Values are mean ± SE of 8 mice/group. The mice were sacrificed 24 hours after the last 14- or 28-day consecutive treatments.

ALT= Alanine amino transferase; AST= aspartate amino transferase.

*p-value for the statistical difference from the respective control group.

^{a}p-value for the statistical difference from the respective atorvastatin dose group.

^{b}p-value for the statistical difference from the respective simvastatin dose group.

†p-value for the statistical difference from the respective 14-day value of the same statin.

Analysis of variance: ALT data, F= 728.139; AST data, F= 577.321, $p < 0.0001$.

Statistically significant difference was set at $p < 0.05$.

Section Three

4-5: Propofol pharmacological challenge

Oral dosing of mice with the three statins, atorvastatin, simvastatin and rosuvastatin at 200 mg/kg/day for 28 days caused a significant ($p < 0.05$) decrease in the latency to onset of propofol sleep when compared with the respective control value (Table 4-12). Subsequently, the duration of propofol sleep was significantly reduced from respective control values in mice treated with atorvastatin and simvastatin by 46.6% and 31.6%, respectively, but not with rosuvastatin (7.3%) (Table 4-12).

Table (4-12): Propofol anesthesia (100 mg/kg, intraperitoneally) 24 h after the last repeated oral dosing of mice with statins (200 mg/kg/day for 28 consecutive days)

Statin treatment	Latency to onset of sleep (sec)	%decrease from control	Duration of sleep (min)	%decrease from control
Control	39.2 ± 0.66	-	11.80 ± 0.42	-
Atorvastatin	31.5 ± 0.70*	19.6	6.30 ± 0.30*	46.6
Control	40.2 ± 0.84	-	13.30 ± 0.62	-
Simvastatin	33.3 ± 1.90*	17.2	9.10 ± 0.38*	31.6
Control	37.2 ± 0.47	-	13.10 ± 0.43	-
Rosuvastatin	28.5 ± 0.54*	23.4	12.15 ± 0.33	7.3

Values are mean ± SE of 10 mice/statin group.

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

4-6: Dichlorvos toxicological challenge

Statin treatments significantly increased the latency to onset of signs of poisoning and delayed the latency to onset of death within 4 h after the dichlorvos oral dosing at 150 mg/kg (Table 4-13). As expected, dichlorvos dosing produced cholinergic signs of organophosphate poisoning in both control and statin treated mice. The toxidrome of dichlorvos poisoning in mice was characterized by excessive salivation, lacrimation, frequent defecation, tremors followed by death at varying percentages that ranged from 37.5% to 100% (Table 4-13). Statin treatments variably decreased these signs of poisoning and death (37.5%-87.5%) in comparison with the control group (62.5%-100%).

Considering the total toxicity score as calculated from the occurrence of signs of cholinergic poisoning and the 4- and 24 h lethality (grades of 1-4) in, the three statin treatments decreased dichlorvos toxicity score by 42%, 33% and 21%, respectively in comparison with that of the control group (Table 4-14).

Table (4-13): Dichlorvos (150 mg /kg, orally) poisoning 24 h after the last repeated oral dosing of mice with statins (200 mg/kg/day for 28 consecutive days)

Variable	Control	Atorvastatin	Simvastatin	Rosuvastatin
Latency to onset of signs of poisoning (min)	2.41 ± 0.11	7.36 ± 0.27*	5.64 ± 0.17*[a]	5.65 ± 0.22*[a]
Latency to onset of death in 4 h (min)	90.6 ±1.1	200.0 ± 3.5*	195.0 ± 4.3*	156.6 ± 3.1*[ab]
% occurrence of signs of poisoning				
Salivation	100	62.5	75	75
Lacrimation	87.5	37.5*	50	62.5
Frequent defecation	100	62.5	62.5	87.5
Tremor	87.5	37.5*	50	75
% Death				
4 h death	87.5	37.5*	50	62.5
24 h death	87.5	37.5*	62.5	75

Values are mean ± SE of 8 mice/statin group.

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

a Significantly different from the respective atorvastatin value, $p < 0.05$.

b Significantly different from the respective simvastatin value, $p < 0.05$.

Table (4-14): Dichlorvos (150 mg/kg, orally)-induced toxicity score in mice treated with each of the statins at 200 mg/kg/day for 28 consecutive days

Signs/ death	Grades allocated to % occurrence of signs of poisoning and death†			
	Control	Atorvastatin	Simvastatin	Rosuvastatin
Salivation	4	3	3	3
Lacrimation	4	2	2	3
Frequent defecation	4	3	3	4
Tremor	4	2	3	3
4 h death	4	2	2	3
24 h death	4	2	3	3
Toxicity score Statistics				
Median toxicity score	4	2	3	3
25 percentile	4	2	2	3
75 percentile	4	3	3	3.25
Mode	4	2	3	3
Total toxicity score (maximum 24)	24	14*	16*	19*

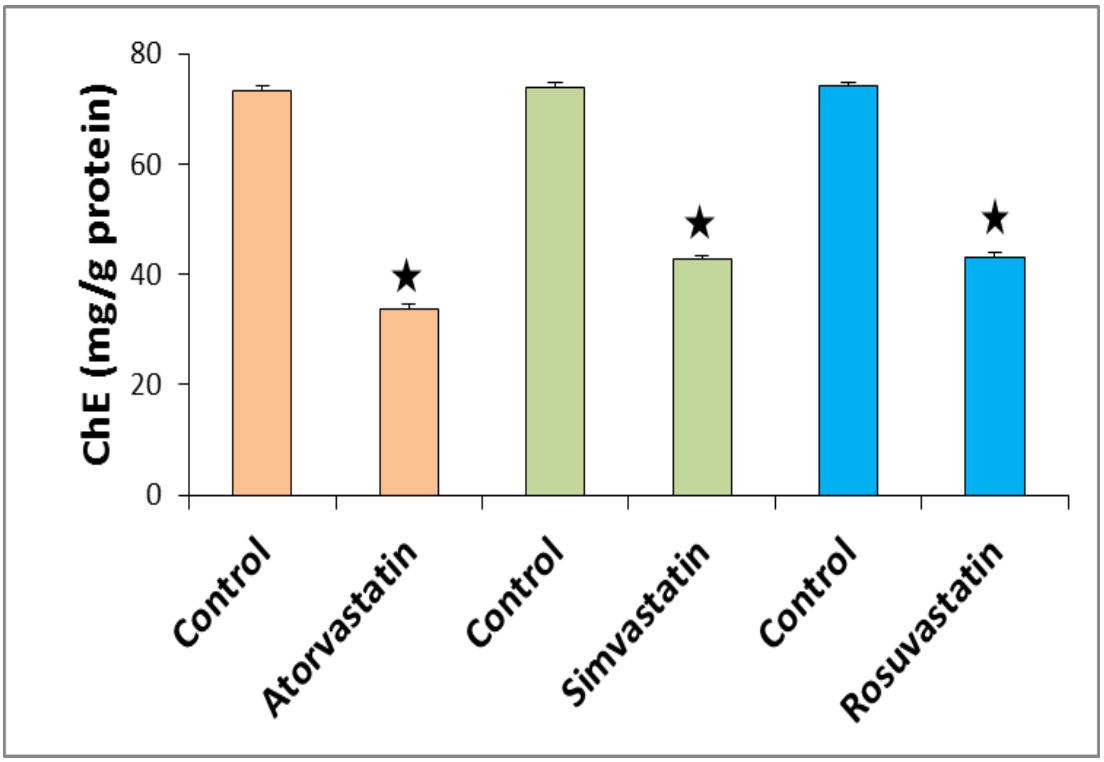
n= eight mice/statin group.

† Data of table 4-13 were used.

* Significantly different from the control value: Kruskal-Wallis test for equal medians, H (Chi^2) 14.69, p= 0.0007124; Dunn's test, p= 0.0001119, 0.001827, and 0.04821, respectively.

4-7: brain ChE activity

Repeated treatments with atorvastatin, simvastatin and rosuvastatin at 200 mg/kg/day for 28 consecutive days significantly reduced whole brain ChE activity by 54%, 42% and 42%, respectively, (table 4-15) compared to respective control values (Figure 4-1).



Values are mean ± SE of 10 mice/statin group.

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

Figure (4-1): Brain cholinesterase (ChE) activity (U/mg protein) in mice dosed orally with statins at 200 mg/kg/day for 28 consecutive days.

Table (4-15): Brain cholinesterase activity (U/mg protein) in mice dosed orally with statins at 200 mg/kg/day for 28 days

Statin treatment	ChE in Brain (U/mg protein)	% decrease
Control (distilled water)	73.28 ± 0.80	-
Atorvastatin	33.84 ± 0.80*	54
Control (distilled water)	74.02 ± 0.68	-
Simvastatin	42.79 ± 0.70*	42
Control (distilled water)	74.22 ± 0.62	-
Rosuvastatin	43.20 ± 0.81*	42

Values are mean ± SE of 10 mice/statin group.

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

Section Four

4-8:Neurobehavioral measurements

Repeated treatment of mice with each of the three statins (atorvastatin, simvastatin, and rosuvastatin) for 14 and 28 consecutive days significantly ($P < 0.05$, in comparison with respective control values) decreased open-field activities manifested as delays in the latency to onset of movement from the central square in the open-field arena, decreases in squares crossed for 5 min (general locomotion), and reductions in the frequency of 5-min rearing (Tables 4-16, 4-17, 4-18). In consonance with the depressed open-field behavioral performance, statin-treated mice suffered from significant reductions in head pocking activity behavior and slowed negative geotaxis performance at an angle of 45° in comparison with control mice (Tables 4-16, 4-17, 4-18). However, in contrast with the depressant actions of statins described above, the hypolipidemic drugs significantly increased the duration of forced

swimming behavior and decreased the duration of immobility in the swimming tank when compared with respective control values (Tables 4-16, 4-17, 4-18).

Table (4-16): Neurobehavioral performance in mice dosed orally with atorvastatin at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Behavioral measurement	**Duration of Atorvastatin treatment**			
	14 days		**28 days**	
Atorvastatin				
Open-field activity	Distilled water-control	Treated	Distilled water-control	Treated
Latency to move (sec)	0.19±0.03	0.59±0.03*	0.25±0.13	0.78±0.02*a
Squares crossed/5 min	128.40±0.73	67.40±1.45*	122.32±0.89	47.50±1.35*a
Rearing/5 min	24.60±0.56	2.70±0.37*	19.50±0.68	1.20±0.25*a
Head pocking/5 min	23.20±0.66	4.30±0.37*	18.40±0.78	2.00±0.26*a
Negative geotaxis (sec)	2.40±0.43	30.50±0.45*	4.60±0.62	48.80±1.33*a
Forced swimming (6min)				
Duration of initial swimming (min)	2.22±0.05	4.03±0.07*	2.10±0.09	4.28±0.06*
Duration of immobility(sec)	32.30±3.36	3.10±0.41*	26.20±1.54	1.700±0.21*

Values are mean ± SE of 10 mice/group.

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

[a]Significantly different from the respective value of the 14 days treatment group, $p < 0.05$.

Table (4-17): Neurobehavioral performance in mice dosed orally with simvastatin at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Behavioral measurement	Duration of simvastatin treatment			
	14 days		28 days	
Simvastatin				
Open-field activity	Distilled water-control	Treated	Distilled water-control	Treated
Latency to move (sec)	0.13±0.015	0.62±0.02*	0.27±0.23	0.68±0.02*a
Squares crossed/5 min	129.90±1.85	83.40±2.19*	130.76±1.62	53.20±1.37*a
Rearing/5 min	20.50±0.54	4.90±0.38*	21.30±0.61	1.90±0.28*a
Head pocking/5 min	24.20±0.76	24.20±0.76	27.34±0.87	3.30±0.37*a
Negative geotaxis (sec)	18.0±0.25	18.0±0.25	23.10±0.59	55.7±0.79*a
Forced swimming (6min)				
Duration of initial swimming (min)	2.34±0.08	4.31±0.09*	2.16±0.32	5.100±0.11*
Duration of immobility (sec)	34.20±0.61	4.90±0.50*	45.20±0.60	2.00±0.21*

Values are mean ± SE of 10 mice/group.

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

[a]Significantly different from the respective value of the 14 days treatment group, $p < 0.05$.

Table (4-18): Neurobehavioral performance in mice dosed orally with rosuvastatin at 200 mg/kg of body weight/day for 14- or 28 consecutive days

Behavioral measurement	Duration of Rosuvastatin treatment			
	14 days		28 days	
Rosuvastatin				
Open-field activity	Distilled water-control	Treated	Distilled water-control	Treated
Latency to move (sec)	0.14±0.02	0.62±0.02*	0.20±0.46	0.65±0.03*
Squares crossed/5 min	128.50±0.09	87.50±2.03*	120.40±0.80	38.60±1.57*a
Rearing/5 min	24.30±0.58	4.80±0.42*	20.50±0.75	3.70±0.34*
Head pocking /5 min	24.00±0.79	7.00±0.39*	21.72±1.20	3.6±1.15*a
Negative geotaxis (sec)	1.40±0.16	50.70±0.99*	3.22±0.25	49.30±0.92*
Forced swimming (6min)				
Duration of initial swimming (min)	2.35±0.06	4.32±0.07*	2.31±0.52	4.83±0.09*
Duration of immobility (sec)	34.70±0.62	11.70±0.76*	29.57±0.85	4.30±0.47*

Values are mean ± SE of 10 mice/group.

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

[a]Significantly different from the respective value of the 14 days treatment group, $p < 0.05$.

4-9: Blood and brain ChE activities

Plasma, erythrocyte and whole brain ChE activities were significantly reduced to various extents (24-53%, 13-48% and 24-49%, respectively) following 14- and 28-day statin treatments in comparison with respective control values (Table 4-19). Considering ChE inhibition values in the plasma, erythrocytes and the brain after the 28^{th} day statin treatments, the most prominent effect was seen with atorvastatin (53%, 48%, 49%), followed by simvastatin (41%, 40%, 44%) and then rosuvastatin (32%, 31%, 36%), respectively (Table 4-19). Regression analyses of brain ChE activity vs. plasma ChE and erythrocyte ChE activities, using the individual animal data of control and statin treatment groups, revealed high level of correlation with r^2 values of 0.92 and 0.93, respectively (Figure 4-2). Similarly erythrocyte ChE activity highly correlated with that of the plasma with an r^2 value of 0.91.

Table (4-19): Plasma, erythrocyte and brain cholinesterase (ChE) activities in mice dosed orally with statins at 200 mg/kg of body weight/day for 14 or 28consecutive days

Statin groups	14 days	% reduction from control	28 days	%reduction from control
Plasma ChE activity (U/mL)				
Distilled water-control	55.81±0.84	-	54.04±1.04	-
Atorvastatin	30.69±0.81*	44	25.18±0.65*†	53
Simvastatin	36.65±0.47*a	33	32.04±0.39*a†	41
Rosuvastatin	41.42±0.34*ab	24	36.51±0.42*ab†	32
Erythrocyte ChE activity (U/mL)				
Distilled water-control	77.57±0.76	-	77.70±0.54	-
Atorvastatin	52.36±0.53*	33	40.66±0.81*†	48
Simvastatin	59.70±0.62*a	23	46.71±0.44*a†	40
Rosuvastatin	64.65±0.95*ab	13	53.47±0.66*ab†	31
Brain ChE activity U/mg protein)				
Distilled water-control	75.52±0.71	-	77.33±0.67	-
Atorvastatin	45.96±0.78*	30	39.67±0.57*†	49
Simvastatin	53.97±0.63*a	40	43.12±0.80*a†	44
Rosuvastatin	57.26±0.57*ab	24	49.29±0.56*ab†	36

Values are mean ± SE of 8 mice/group. The mice were sacrificed 24 hours after the last 14- or

28-day consecutive statin treatments.

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

[a]Significantly different from the respective atorvastatin dose group, $p < 0.05$.

[b]Significantly different from the respective simvastatin dose group, $p < 0.05$.

†Significantly different from the respective 14-day value of the same statin, $p < 0.05$.

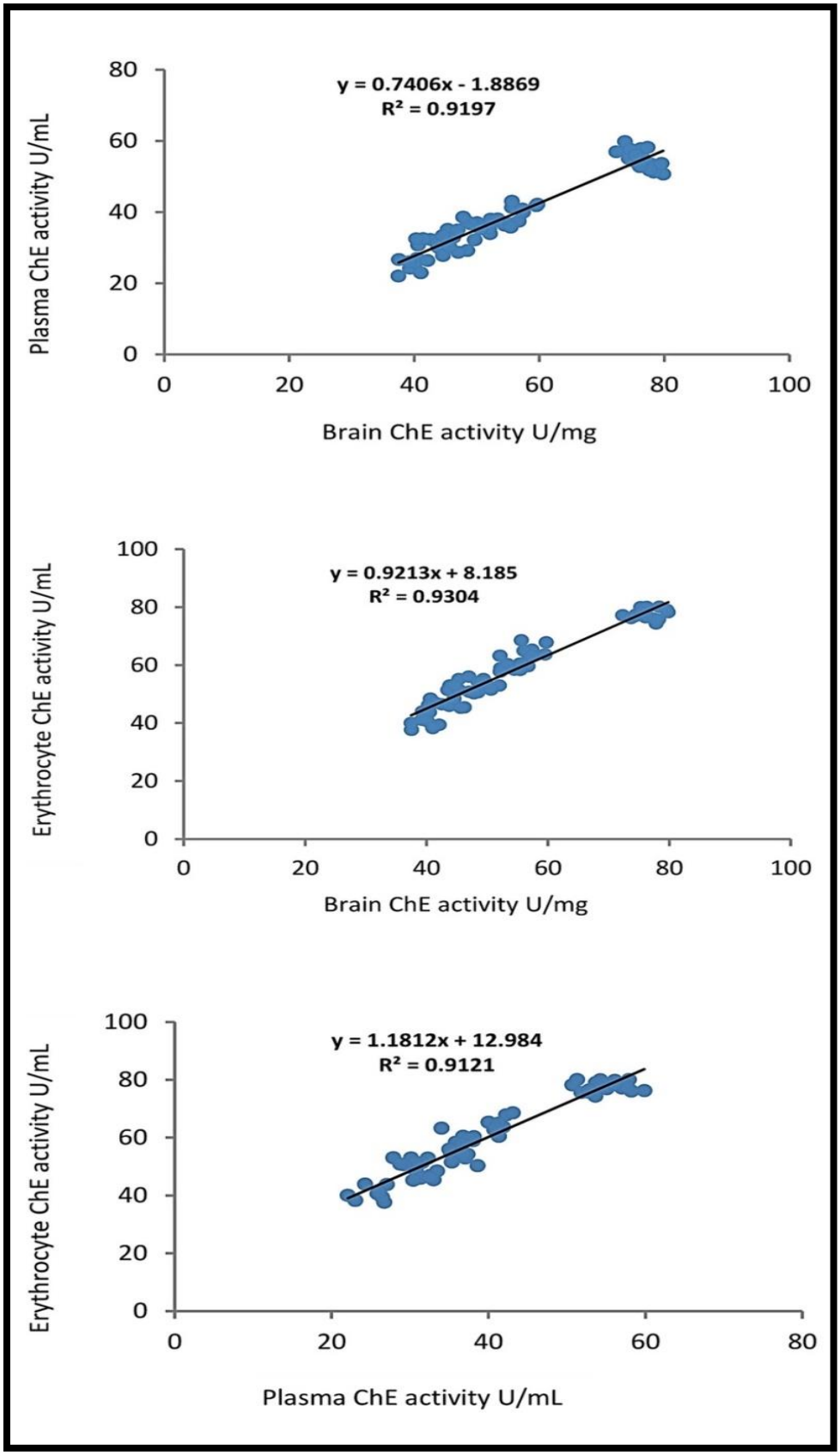


Figure (4-2): Regression and correlation analyses of brain and blood cholinesterase (ChE) activities.

4-10: cholesterol level

As a control measure of the pharmacological effects of the three statins, repetitive statin (atorvastatin, simvastatin, and rosuvastatin) treatments for 28 consecutive days significantly decreased plasma and brain cholesterol levels by 33% and 42%, 31% and 31%, and 12% and 14%, respectively in comparison with the respective control values (Table 4-20). Atorvastatin had the most prominent effect among the statin treatment groups in reducing plasma and brain cholesterol levels, and rosuvastatin had the least effect (Table 4-20).

Table (4-20): Plasma and brain cholesterol levels in mice dosed orally with Statins at 200 mg/kg of body weight/day for 28 consecutive days

Statin groups	Plasma cholesterol level (mg/100 mL)	Brain cholesterol level (mg/g)
Distilled water-control	116.375±1.37	33.375±0.68
Atorvastatin	78.500±0.87*	19.500±0.57*
Simvastatin	80.500±0.96*[a]	23.000±0.53*[a]
Rosuvastatin	102.25±1.25*[ab]	28.625±0.38*[ab]

Values are mean ± SE of 8 mice/group. The mice were sacrificed 24 hours after the last

28-day consecutive statin treatments.

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

[a]Significantly different from the respective atorvastatin dose group, $p < 0.05$.

[b]Significantly different from the respective simvastatin dose group, $p < 0.05$.

Section Five

4-11: In vitro statin ChE inhibition measured electrometrically

The three statins, atorvastatin, simvastatin and rosuvastatin at in vitro concentrations ranging from 10 to 100 µM significantly ($p < 0.05$) inhibited plasma ChE activity of mice in a concentration-dependent manner when compared with respective baseline-control (0 µM) values ((Tables 4-21, 4-22). Correspondingly, the percentages of reductions in plasma ChE by the three statins ranged from 3.5-41.4%, 0.3-24.1% and 0.8-21.3%, respectively, in comparison with respective control values (Table 4-21). The most effective statin in causing significant reductions in plasma ChE activity was atorvastatin by 41.4% at 100 µM (mean ± SE, 0.454±0.03 vs. control 0.775±0.03 Δ pH/30 min). Rosuvastatin had the least effect on plasma ChE (0.606±0.03) by 21.3% decrease when compared with the respective control value (0.770±0.03). Similarly, the percentages of reductions in brain ChE by the three statins ranged from 4.4-41.6%, 0.6-25.6% and 0.6-14.6%, respectively, in comparison with respective control values (Table 4-22). The most effective statin in causing significant reductions in brain ChE activity was atorvastatin by 41.4% at 100 µM (0.374±0.03 vs. control 0.640±0.03), and the least one was rosuvastatin by 14.6% (0.540±0.03 vs. control 0.632±0.02).

Table (4-21): In vitro effects of statins on plasma cholinesterase (ChE) activity of mice by electrometric method

Statin concentration (µM)	ChE activity (Δ pH/30 min)	% decrease from 0 (baseline) value
Atorvastatin		
0	0.775±0.03	-
10	0.748±0.02	3.5
25	0.736±0.02	5.0
50	0.580±0.02*[ab]	25.2
100	0.454±0.03*[abc]	41.4
Simvastatin		
0	0.772±0.03	-
10	0.770±0.03	0.3
25	0.742±0.03	3.9
50	0.654±0.02*[ab]	15.3
100	0.586±0.03*[ab]	24.1
Rosuvastatin		
0	0.770±0.03	-
10	0.764±0.03	0.8
25	0.756±0.02	1.8
50	0.652±0.02*[ab]	15.3
100	0.606±0.03*[ab]	21.3

Plasma samples of 25 mice were pooled before the experiment.

Values are mean ± SE of 5 determination/statin concentration.

*Significantly different from the respective baseline (0 µM), $p < 0.05$.

a Significantly different from the respective 10 µM concentration value, $p < 0.05$.

b Significantly different from the respective 25 µM concentration value, $p < 0.05$.

c Significantly different from the respective 50 µM concentration value, $p < 0.05$.

Table (4-22): In vitro effects of statins on brain cholinesterase (ChE) activity of mice

Statin concentration (µM)	ChE activity (Δ pH/30 min)	% decrease from 0 (baseline) value
Atorvastatin		
0	0.640±0.03	-
10	0.612±0.03	4.4
25	0.580±0.02	9.4
50	0.468±0.01*ab	26.9
100	0.374±0.03*ab	41.6
Simvastatin		
0	0.640±0.02	-
10	0.636±0.02	0.6
25	0.600±0.02	6.3
50	0.596±0.02	6.9
100	0.476±0.02*abc	25.6
Rosuvastatin		
0	0.632±0.02	-
10	0.628±0.01	0.6
25	0.604±0.02	4.4
50	0.578±0.02*a	8.5
100	0.540±0.03*ab	14.6

Whole brains of 25 mice were homogenized and pooled before the experiment.

Values are mean ± SE of 5 determination/statin concentration.

*Significantly different from the respective baseline (0 µM), $p < 0.05$.

a Significantly different from the respective 10 µM concentration value, $p < 0.05$.

b Significantly different from the respective 25 µM concentration value, $p < 0.05$.

c Significantly different from the respective 50 µM concentration value, $p < 0.05$.

4-12: In vitro pro-oxidant property of statins using whole brain homogenates and determination of brain ChE activity

In this experiment we used hydrogen peroxide at 500 µM as a positive control to induce in vitro generation of MDA, as a product of lipid peroxidation, in the brain homogenate. Expectedly, the hydrogen peroxide significantly increased brain MDA level by 593.7% to 663.6% relative to respective baseline-control (0 µM) values of the three statins (Table 4-23). Examining the generation of MDA, the three statins atorvastatin, simvastatin and rosuvastatin at in vitro concentrations ranging from 25 to 100 µM significantly ($p < 0.05$) increased MDA level in whole brain homogenates of the mice in a concentration-dependent manner, when compared with respective baseline-control (0 µM) values (Table 4-23). The recorded percentages of elevations in brain MDA by the three statins ranged from 5-41.1%, 22.2-130.1% and 33.2-297.6%, respectively, in comparison with respective control values (Table 4-23). The most effective statin in causing in vitro oxidative stress was rosuvastatin as seen by 297.6% increase in MDA production at 100 µM (mean ± SE, 330.722±5.72 vs. control 83.182±2.08 ng/mg), and the least one was atorvastatin by 41.1% (122.410±1.69 vs. control 86.772±3.90).

Table (4-23): In vitro effects of statins on brain malondialdehyde (MDA) level of mice

Treatments (µM)	MDA (ng/mg)	% increase from 0 (baseline) value
Atorvastatin		
0	86.772±3.90	-
25	91.090±2.67	5.0
50	108.628±1.76	25.2
100	122.410±1.69*[a]	41.1
H_2O_2 (500)†	601.978±19.91*[abc]	593.7
Simvastatin		
0	81.378±1.41	-
25	99.418±1.11*	22.2
50	150.840±4.98*[a]	85.4
100	187.264±4.451*[ab]	130.1
H_2O_2 (500) †	611.894±9.12*[abc]	651.9
Rosuvastatin		
0	83.182±2.08	-
25	110.782±2.92*	33.2
50	211.900±6.31*[a]	154.7
100	330.722±5.72*[ab]	297.6
H_2O_2 (500) †	635.210±9.33*[abc]	663.6

Whole brains of 25 mice were homogenized and pooled before the experiment.

† Hydrogen peroxide was the positive oxidant control.

Values are mean ± SE of 5 determination/statin or H_2O_2 concentration.

*Significantly different from the respective baseline (0 µM), $p < 0.05$.

a Significantly different from the respective 25 µM concentration value, $p < 0.05$.

b Significantly different from the respective 50 µM concentration value, $p < 0.05$.

c Significantly different from the respective 100 µM concentration value, $p < 0.05$.

Furthermore, determination of brain ChE activity using a spectrophotometric method revealed that the three statins atorvastatin, simvastatin and rosuvastatin at in vitro concentrations ranging from 25 to 100 µM significantly ($p < 0.05$) inhibited the enzyme activity in a concentration-dependent manner when compared with respective baseline-control (0 µM) values (Table 4-24). The percentages of reductions in brain ChE by the three statins ranged from 8.6-30.9%, 18.2-36.9% and 16.5-51.2%, respectively, in comparison with respective control values (Table 4-24). To this end, the maximum brain ChE inhibition induced by the three statins at 100 µM, as determined in the present experimental paradigm, was that of rosuvastatin (51.2%), followed by simvastatin (36.9%), and the least one was by atorvastatin (30.9%) in comparison with their respective control values (Table 4-24). Further, the brain ChE activity following in vitro exposure to hydrogen peroxide (500 µM) was also decreased (66.3%, 68.7%, and 69.8%) in comparison to respective control values used with each statin (Table 4-24). Additionally, the regression and correlation analyses of the in vitro inhibitory statin (atorvastatin, simvastatin and rosuvastatin) effects on brain ChE activity versus the corresponding increase in the MDA level showed strong correlations between the two variables within each statin exposure (0, 25. 50 and 100 µM), and the corresponding correlation coefficient (r) values were 0.761, 0.893 and 0.937, respectively

(Figure 4-3).

Table (4-24): In vitro effects of statins and hydrogen peroxide on brain cholinesterase (ChE) activity of mice by colorimetric method

Treatments (µM)	ChE activity (unit/mg)	% decrease from 0 (baseline) value
Atorvastatin		
0	79.362±1.15	-
25	72.548±1.31*	8.6
50	64.308±1.26*[a]	19.0
100	54.818±1.69*[a]	30.9
H_2O_2 (500)†	26.748±1.21*[abc]	66.3
Simvastatin		
0	79.656±1.58	-
25	65.126±1.40*	18.2
50	58.912±1.04*[a]	26.0
100	50.298±0.69*[a]	36.9
H_2O_2 (500) †	24.904±0.89*[abc]	68.7
Rosuvastatin		
0	79.256±0.89	-
25	66.166±2.58*	16.5
50	49.760±1.03*[a]	37.2
100	38.704±1.07*[a]	51.2
H_2O_2 (500) †	23.972±1.10*[abc]	69.8

Whole brains of 25 mice were homogenized and pooled before the experiment.

† Hydrogen peroxide was the positive oxidant control.

Values are mean ± SE of 5 determination/statin or H_2O_2 concentration.

*Significantly different from the respective baseline (0 µM), $p < 0.05$.

a Significantly different from the respective 25 µM concentration value, $p < 0.05$.

b Significantly different from the respective 50 µM concentration value, $p < 0.05$.

c Significantly different from the respective 100 µM concentration value, $p < 0.05$.

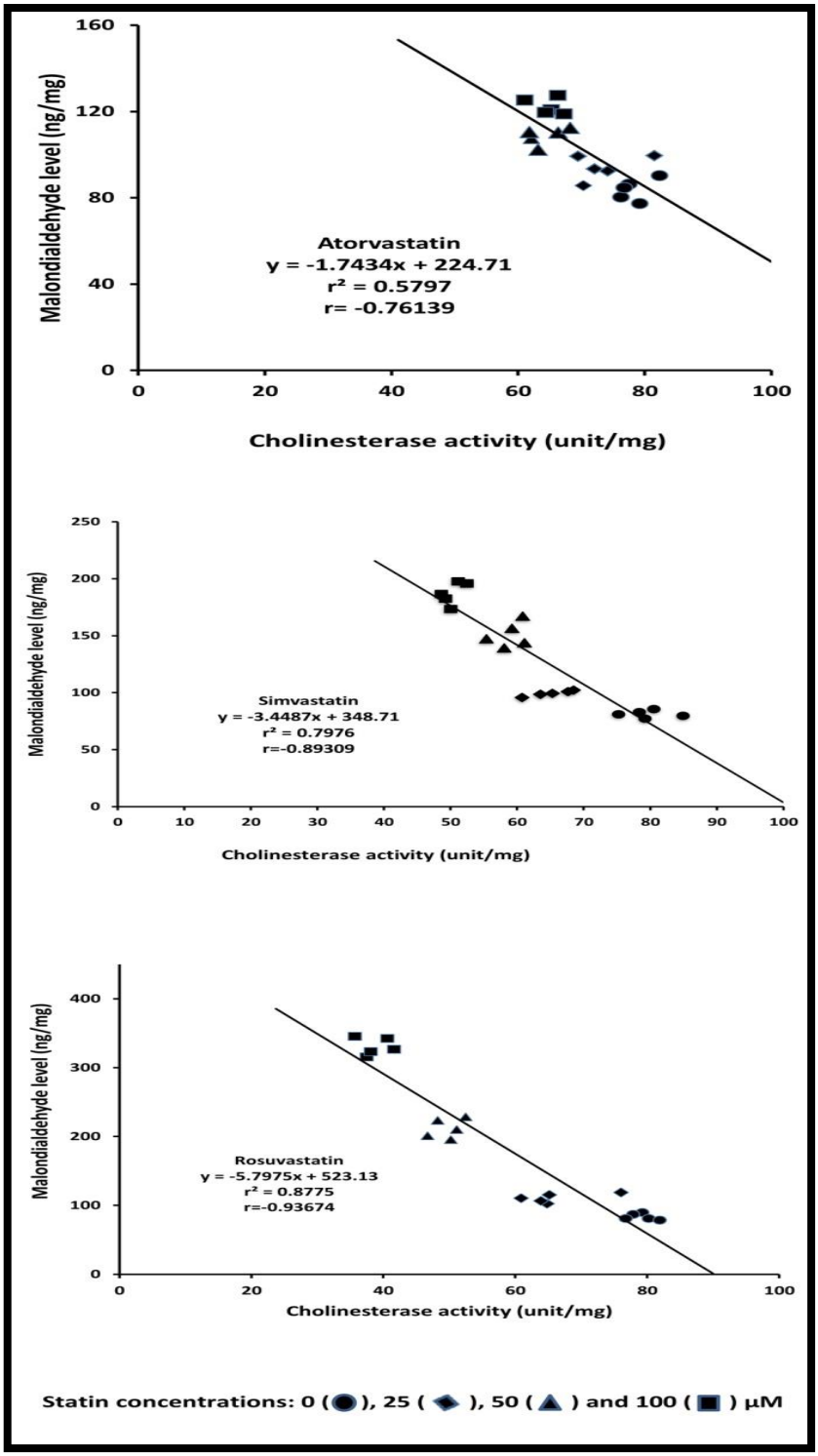


Figure (4-3): Regression and correlation analyses of individual assays of malondialdehyde level versus cholinesterase activity in the brain homogenates of mice following in vitro exposure to different concentrations of statins at 0, 25, 50 and 100 µM.

Chapter Five

Discussion

Chapter Five

Discussion

The present study demonstrated adverse behavioral effects characterized by reduced open-field activity measures, head pocking and negative geotaxis performance following single dose (2 h after dosing) or repeated statin (atorvastatin, simvastatin, and rosuvastatin) treatments for 14 or consecutive 28 days in mice. These effects could be an additional pleiotropic outcome of statins appended to the already known adverse behavioral changes not related to hypolipidemic actions (De Oliveira *et al*., 2018; Hirota *et al*., 2020; Attardo *et al*., 2022). The reported adverse behavioral effects of statins in humans include episodes of aggression, depression, loss of memory, or confusion (Rahola, 2012; Leppien *et al*., 2018). In laboratory mice or rats , they have been reported to cause memory alteration, changes in locomotor, social, and swimming activities, modulation of drug addiction, and antidepressant effects (Ghodke *et al*., 2012; Durankuş *et al*., 2023). In the present study, statins reduced general locomotor activity (open-field activity and negative geotaxis performance), produced possible anxiolytic effect (head pocking behavior) and antidepressant action (swimming endurance). These neurobehavioral changes collectively reported in the present study were dose-dependent and suggest a multifunctional aspects of statin-induced behavioral modulation in mice, which are in support of previous reports in laboratory animals (Ghodke *et al*., 2012; Durankuş *et al*., 2023). Furthermore, the present behavioral results, based on head pocking and swimming endurance as well as the current evidence in the literature (De Oliveira *et al*., 2018; Hai-Na *et al*., 2020; Durankuş *et al*., 2023), suggest that the depressive effects of statins on locomotor activity vary depending on their potential antidepressant properties. Interestingly, it was observed

in the mice of the present study that statin treatments in contrast to the exploratory depressed activities (open-field and head pocking) produced prolongation of swimming endurance and reduced immobility response. This effect has been found in animal models (mice) of antidepressant drugs (Bogdanova *et al*., 2013). The benefit of this effect of statins awaits further studies, specially, in the light of suggestions of potential psychotropic effects of statins (Cham *et al*., 2016; Durankuş *et al*., 2023). Further preclinical and possible therapeutic exploration of this concept in animal models should be explored.

The single doses of the three statins used in the present study were higher than those clinically used in humans (McGuinness *et al*., 2016; Hirota *et al*., 2020). However, the choice of statin doses in the present study was based on preliminary experiments in mice that did not show obvious signs of toxicosis. Emerging data indicate that statins are well tolerated even at high doses (Hu *et al*., 2012), which is an added advantage for any animal model that can tolerate high doses of statins in order to facilitate behavioral changes (Vukšić *et al*., 2019; Rashid and Mohammad, 2023b). In addition, species variation is expected in experimental animals in response to statin dosages, and they differ in pharmacological and toxicological profiles, especially when the end point effect is not the plasma cholesterol level; statin doses could be up to 10-80 times larger than those of humans (Kramer *et al*., 2011; Ghodke *et al*., 2012 ; Macan *et al*., 2015; Vukšić *et al*., 2019; Rashid and Mohammad, 2023a,b).

Reduced ChE activity in the plasma, erythrocytes, and brain represents another important finding of the present study in mice orally administered single doses of each of the three statins (500 and 100 mg/kg) or repeated doses at 200 mg/kg/day for 14 and 28 consecutive days. These results are consistent with previous studies on ChE activity in humans (Darvesh *et al*., 2004), rats (Cibicková *et al*., 2007; Cibickova *et al*., 2009;

Macan *et al*., 2015; Vukšić *et al*., 2019) and chicks (Rashid and Mohammad, 2023a) treated with single or repeated doses of statins. In contrast to the present study, we observed selective and differential effects of statins on ChE activity in the studies mentioned above. In the present study, high doses of statins were administered, and the results suggest generalized depressive effects of statins, in conjunction with behavioral alterations, on ChE activities in the plasma, erythrocytes, and brain. These results further support previous findings on reduced ChE activity as a result of statin therapy and highlight the fact that high doses of statins may modulate the enzyme activity toward depression, which remains to be determined in future clinical studies. Most statins reduce ChE activity; however, other statins may have no effect or even increase ChE activity (Cibicková *et al*., 2007; Husain *et al*., 2018).

The three tissue ChE activities (plasma, erythrocytes, and brain) examined in the present study represent pseudo-ChE in plasma (some in the brain) and true ChE in erythrocytes and brain (Mason, 2000; Mohammad *et al*., 2023). They are target subjects to various extents of pesticide inhibition (Mason *et al*., 2000; Mohammad *et al*., 2023). They also recover from inhibition in a differential manner, depending on the type of inhibitor and the enzyme source, with fast recovery observed in plasma ChE (Mason *et al*., 2000). Although it is too early to draw a cause and effect relationship from the present findings described above, several reports have indicated that inhibited brain ChE activity is associated with neurobehavioral changes such as increased anhedonia (preference to sucrose), depressed open-field activity, learning and memory impairment and forced swimming behavioral changes in rodents (Ribeiro *et al*., 2022). The pharmacokinetic, pharmacodynamic, and pleiotropic profiles of statins differ considerably (Hirota *et al*., 2020; Climent *et al*., 2021). However, the precise mechanisms of action of statins in inducing adverse effects described above and those in

the literature are not yet clear. Nevertheless, they might include targeting specific proteins such the ChE, cellular membranes or tissue redox oxidant/antioxidant systems (Ghodke *et al*., 2012; Liu *et al*., 2019; Vukšić *et al*., 2019).

Extensive in vitro and in vivo assessments of ChE activity in association with neurobehavioral outcomes are warranted to understand the neuronal actions of statins, taking into account their potential neurotoxicity versus beneficial effects in certain cases such as dementia (Tatley and Savage, 2007; McGuinness *et al*., 2016; Zhang *et al*., 2022; Attardo *et al*., 2022). Considering the latter effects and the present findings of neurobehavioral alterations in the mouse model in conjunction with reduced brain and blood ChE activities, it is plausible to deduce that relatively high doses of statins induce adverse effects in this animal model. As the changes are characterized by behavioral abnormalities coupled with neuronal changes at the cholinergic oxidant/antioxidant level, this could an initial step for developing an animal model (mice) for adverse statin intolerance.

In the light of pleiotropic effects of statins, there are suggestions of possible beneficial effects of statin in cases of dementia (Profumo *et al*., 2014; Sirtori, 2014; Sørensen *et al*., 2019). Indeed, the present results of reduced brain ChE activity in mice as well as the results of others in rats and mice (Cibicková *et al*., 2007; Husain *et al*., 2018; Vukšić *et al*., 2019; Karimani *et al*., 2021; Katić *et al*., 2021), chicks (Rashid and Mohammad, 2023a) support such a beneficial outcome. To this end, further in depth studies are needed to explore effects of statin on dementia animal models. An additional beneficial effect of reduced brain ChE activity of statins, which correlated well with those of the plasma and erythrocytes in the present study, was the reported reduction in the toxicity outcome of centrally and peripherally acting ChE inhibiting insecticide carbaryl in chicks (Rashid and Mohammad, 2023b).

The present study used the mouse model to assess the status of the oxidative stress induced by the three statins (atorvastatin, simvastatin, and rosuvastatin) that differ in their pharmacokinetic and pharmacodynamic effects (Sirtori , 2015; Hirota *et al*., 2020; Climent *et al*., 2021). The three statins treatment regimens applied in mice, consisting of single-day dosing and repetitive treatments for 14 or 28 days, produced unequivocal oxidative stress in the form of reduction in the level of the oxidative defense tripeptide GSH in the plasma and brain with a concomitant increase in the oxidative stress biomarker MDA. These antioxidant/oxidative changes induced by statins might have resulted from the metabolic production of reactive oxygen species that subsequently cause organ damage such as the liver at the cellular macromolecular level (Pal *et al*., 2015; Liu *et al*., 2019). While the oxidative stress of statins reported in the present study further supports and ascertains previous studies in which statin therapy was associated with stressful conditions at cellular and vital organ levels (Pal *et al*., 2015; Liu *et al*., 2019; Averbukh *et al*., 2024), caution should be practiced in interpreting such results. This is because many clinical studies have highlighted the documented beneficial antioxidant effects of statins in practice (Profumo *et al*., 2014; Sirtori, 2014). Nevertheless, in light of the present findings using relatively high doses of statins and other experimental findings about the adverse effects of statins reported herewith as well as earlier by others (Bouitbir *et al*., 2011; Rashid and Mohammad, 2023a,b), it can be deduced that oxidative stress is a marker associated with statin overdose and caution should be practiced clinically when treating patients with statins. This latter notion is especially important as statins can produce liver damage as reported in the current study in the form of increased plasma ALT and AST, and by others (Attardo *et al*., 2022), as well as the oxidative stress-related myopathy (Ahmadi *et al*., 2020). The present findings might be important to certain groups of patients such as those with liver failure and renal failure

since the kinetics of statins will be different in such patients predisposing them to higher serum concentrations of statins and a higher incidence of side effects like oxidative ones. Therefore, based on the current findings and those of others (Pal *et al*., 2015; Liu *et al*., 2019).

Additional significant specific organ insult caused by the statins could be the CNS since elevated oxidative stress biomarker MDA and the reduction of the antioxidant GSH content as well as reduced ChE activity were also found in the whole brain of the statin-treated mice in the present study. Albeit, these effects and the reported ChE activity reduction (Vukšić *et al*., 2019; Rashid and Mohammad, 2023a), myopathy (Sakaeda *et al*., 2011; Attardo *et al*., 2022), and the current evidence considering the brain as a non-therapeutic (hypolipidemic effect, as reduced brain cholesterol level) target for statins (Fracassi *et al*., 2019), could be related to neurobehavioral alterations seen in the present study and as reported in experimental animals by others. Keeping these adverse effects in mind, especially those of the single statin doses, and in light of the possibility of statin intolerance (Alonso *et al*., 2019; Bytyçi *et al*., 2022), reported clinically, further in-depth exploration of an animal model for single-dose statin intolerance in mice is warranted. It is, however, difficult to directly extrapolate experimental animal-based data to humans because of species variation, and the drug dosages used clinically could vary considerably from those of the experimental studies in animals, in which doses of statins could be up to 80 times higher than those applied in human beings (Kramer, 2011; Sirtori, 2014; Hirota *et al*., 2020; Climent *et al*., 2021). This is especially true when the endpoint effect is not the plasma cholesterol (Kramer, 2011; Fracassi *et al*., 2019).

The present study collectively demonstrated adverse behavioral effects characterized by reduced open-field activity measures, head pocking, negative geotaxic performance and changes in anesthesia response and

dichlorvos toxicity following single or repeated statin (atorvastatin, simvastatin, and rosuvastatin) treatments in mice. Hence, it should be stressed within the context of potential adverse neurobehavioral and biochemical effects of statins, that such effects could vary among different types of statins, since they have non-identical pharmacokinetic and pharmacodynamic properties (Sirtori, 2014; Hirota *et al*., 2020; Climent *et al*., 2021). In the present study, atorvastatin (single and repetitive treatments) had the least effect compared to simvastatin and rosuvastatin in producing MDA elevation or GSH reduction in the brain. This outcome could be associated with the reported intrinsic variations in pharmacokinetic properties, together with liver metabolism, neuronal effects, and tissue uptake of a particular statin (Wood *et al*., 2010; Kramer, 2011; Sirtori, 2014; Climent *et al*., 2021).

The pharmacological challenge with the short-acting anesthetic propofol (Sahinovic *et al.*, 2018) revealed that statin treatments for 28 days altered the response of mice to anesthesia. This result can be considered as an inadequate response to general anesthesia resulting from propofol. This became evident by the decrease of the time to onset of anaesthesia with the three statins and reduction of the duration of propofol anesthesia by atorvastatin and simvastatin, but not by rosuvastatin. This partial effect of rosuvastatin on propofol anesthesia in mice could be related to its low bioavailability into the central nervous system due to its hydrophilicity nature compared to more lipophilic statins atorvastatin and simvastatin (Ward *et al.*, 2019; Fong, 2020; Climent *et al.*, 2021). Propofol anesthesia is of short duration and results from potentiation of $GABA_A$-receptor-mediated inhibitory effect in the CNS as well as inhibition of the N-methyl-D-aspartate receptors (Kotani *et al.*, 2008; Sahinovic *et al.*, 2018). The nature of drug-drug interaction of propofol with the statins at the level of the CNS is not clear at present. However, it could be associated with the

reported neurotropic effects of high doses of statins in decreasing hippocampal neurotropins and irisin levels in conjunction with impaired cognitive function (Husain *et al.*, 2018; Okudan and Belviranli, 2020) and neurobehavioral alterations at the levels of locomotion, memory, anxiety, and depressive responses (Ghodke *et al.*, 2012; Husain *et al.*, 2018; Oliveira *et al.*, 2018; Hai-Na *et al.*, 2020). In accordance with these studies, locomotor activities and antidepressant modes of the statins have been also observed in the mice. Further, in accordance with the present results on propofol anesthesia, a single dose of simvastatin (100 mg/kg, orally) reduced xylazine-ketamine anesthesia when the latter was used as a pharmacological challenge in young chicks (Rashid and Mohammad, 2023b). Pharmacological challenges with centrally active drugs would be a suitable tool to further examine potential actions of statins on the brain and the associated behavioral outcomes, especially when considering exploration of additional pleiotropic effects of statins (Frankel *et al.*, 2007; Rashid and Mohammad, 2023b).

Dichlorvos is an organophosphate compound used as an insecticide that inhibits central and peripheral ChEs in the animal body (Okoroiwu and Iwara, 2018). This toxicant was used as a toxicological challenge in mice treated with statins. As expected (Mohammad *et al.*, 1989; Mohammad *et al.*, 2014; Okoroiwu and Iwara, 2018), dichlorvos induced a toxidrome of cholinergic poisoning (nicotinic, muscarinic and CNS effects) in control and statin-treated mice. The cholinergic toxidrome in the present study was characterized by excessive salivation, lacrimation, frequent defecation and tremors, followed by death at varying percentages. However, the three statin treatments reduced the dichlorvos-induced toxicosis in mice by prolonging the onset times of poisoning and death as well as by reducing the total toxicity score (21–42%) which is based on the occurrence of cholinergic toxidrome and death. These results are in agreement with those of a

previous study in which atorvastatin and fluvastatin reduced the toxicity of carbaryl, another anti-ChE, but reversible, insecticide, in young chicks (Rashid and Mohammad, 2023b). Furthermore, the results also highlight the diversity of statin effects, and suggest involvements of the cholinergic pathways in their protective action against ChE inhibitors.

To further elucidate the possible mechanism of action of statins on the cholinergic system in relation to the behavioral outcome to pharmacological and toxicological challenges of statins-treated mice, the present results clearly demonstrate reduced ChE activities in the blood and the whole brain. These effects are in accordance with reduced brain and blood ChE activities in rats (Vukšić *et al.*, 2019) and chicks (Rashid and Mohammad, 2023a). It is likely, therefore to deduce that the mechanism of statin-mediated protection against dichlorvos poisoning in mice is associated with prior inhibition of neuronal ChE activity in a manner that prevents additional ChE inhibition, thus reducing the cholinergic toxidrome and lethality. Such a protective mechanism of organophosphate poisoning has been reported earlier with the use of weak ChE inhibitors like physostigmine, pyridostigmine and metoclopramide to shield the animal against the ChE inhibitor poisoning (Al-Zubaidy and Mohammad, 2005; Hrvat and Kovarik, 2020). This is because the single most important mechanism of dichlorvos-induced toxicity, as is the case with other organophosphates, is irreversible inhibition of ChE activity at neuronal endings that leads to build up of acetylcholine, which in turn produces cholinergic toxidrome (Wilson 2014; Okoroiwu and Iwara, 2018).

Another possible reason for the interaction of statins with pharmacological and toxicological challenges is the induction of oxidative stress seen currently as undesirable effects of high doses of statins. This is probably as a result of reactive oxygen species buildup burst (Thomas *et al.*, 2022). In line with this consideration, oxidative stress was reported to

modulate the action of centrally acting drugs (Mousa and Mohammad, 2012) or toxicants (Al-Baggou *et al*., 2011). What complicates the matter more, organophosphates were reported to cause oxidative stress (Vanova *et al*., 2018). Therefore, outweighing such a beneficial effect of statin-induced ChE inhibition and its possible interaction with antiChE insecticides is needed, because of the oxidative stress-induced adverse effects of statins (Zubi *et al*., 2023). In addition, it was reported that oxidative stress produced by hydrogen peroxide might potentiate the toxicity of ChE inhibiting organophosphates in chicks (Al-Baggou *et al*., 2011). Considering the end effect on ChE activity after the 28th day statin treatment, the most effective statin in reducing blood and brain ChE activities was atorvastatin and the least effective one was rosuvastatin. This difference among the statins of the present study could be attributed to variations in pharmacokinetic, pharmacodynamics and pleiotropic profiles of statins (Sirtori, 2014; Hirota *et al*., 2020; Bytyçi *et al*., 2022). Further studies are also needed to explore this avenue of statin interactive, but differential effects. Furthermore, additional in depth exploration of statin actions on the oxidative stress inducing systems and cholinergic modulation are also needed. To this end also a word of caution is necessary herewith as the statin treatment regimens could cause liver injury, kidney damage, neuromuscular adverse effects, oxidative stress; as well as adverse psychiatric reactions (Sakaeda *et al*., 2011; Pal *et al*., 2015; Alonso *et al*., 2019; Attardo *et al*., 2022). Statin-induced adverse effects and involvements of many organ systems could quite possibly predispose statin treated subjects to additional burdens of drug interaction and/or toxicity (Bogdanova *et al*., 2013).

The present in vitro effects of three statins (atorvastatin, simvastatin and rosuvastatin) revealed inhibitory action of these drugs on pseudo ChE (plasma ChE) and true ChE (brain ChE) activities to various extents in a

mode depending on statin concentration. These in vitro effects were demonstrated by two independent methods (electrometric and spectrophotometric) of ChE determination that differ kinetically and temporally (Ataei *et al*., 2021; Ryan *et al*., 2024). This is an important finding as both methods are highly sensitive to in vivo and in vitro inhibitory effects of medications and toxicants on true (EC 3.1.1.7) and pseudo (EC 3.1.1.8) ChE activities (Mohammad *et al*., 2014; Sheridan *et al*., 2022; Granat *et al*., 2024). The in vitro inhibitory effects of the three statins coincide with other in vivo studies in mice treated with single (Wasim *et al*., 2022) or repeated doses (Sheridan *et al*., 2022) of the same statins as well as with other statins in chicks (Rashid and Mohammad, 2023a) and rats (Cibicková *et al*., 2007; Vukšić *et al*., 2019). Furthermore, the present results of reduced ChE activity in the plasma and the brain, which were statin concentration-dependent are in agreement with the reported using in vitro reductions of ChE activity by statins in various tissues (Roensch *et al*, 2007; Rashid and Mohammad, 2023a). However, in spite of the concordance of in vitro and in vivo studies, some discrepancies have emerged in the literature regarding changes in the ChE activity. This was in the form of increased ChE activity or no-change at all, albeit because of the evidences presented that such phenomena involving cholinergic pathways could be associated with differential effects of statins on this enzyme or the neuronal cholinergic pathways themselves (Kosowski *et al*, 2021; Rezakhani *et al*, 2023). However, in spite of the concordance of the in vitro and in vivo studies (Cibicková *et al*., 2007; Roensch *et al*., 2007; Vukšić *et al*., 2019; Rashid and Mohammad, 2023a), some discrepancies have been reported in the literature. This was in the form of increased ChE activity or no-change at all, albeit because of the evidences presented that such phenomena involving cholinergic pathways could be associated with differential effects of statins on this enzyme or the neuronal cholinergic

pathways themselves (Darvesh *et al*., 2004; Cibicková *et al*., 2007; Roensch *et al*., 2007; Macan *et al*., 2015; Vukšić *et al*., 2019; Rashid and Mohammad, 2023a). In the light of the in vitro evidence presented herewith and the associated in vivo studies demonstrating neurobehavioral actions of statins, it is reasonable to conclude involvement of the CNS in adverse/pleiotropic effects of statins in a manner related to the cholinergic associated pathway (Ludka *et al*., 2014; Husain *et al*., 2018; Okudan and Belviranli, 2020; Rashid and Mohammad, 2023a).

While statins modulate the cholinergic pathway as part of their adverse/pleiotropic effects that involve the CNS or even other systems, another aspect of statin action involves the induction of oxidative stress in vivo and vitro (Beltowski, 2005; Bouitbir *et al*., 2011; Ahmadi *et al*., 2020). In contrast, other studies have reported antioxidant effects of statins in a manner that could be attributed to different tissues examined and/or statin concentrations or doses applied (Beltowski, 2005; Liu *et al*., 2019; Mansouri *et al*., 2022). The present study has applied, a novel, but a simple in vitro approach using the whole brain homogenate to generate oxidative stress (increased MDA). This in vitro oxidative stress effect, which was statin concentration-dependent, is in accordance with the reported in vivo oxidative stress (increased plasma and brain MDA level and decreased GSH level) produced by the three statins in mice of the present study. Within this context, in vitro studies reported the oxidant properties of statins without involving the brain tissue per se (Acheampong *et al*., 2007; Schupp *et al*., 2008). The in vitro and in vivo pro-oxidant effects of the statins, could explain, in part, the adverse neurobehavioral, neuromuscular and cardiovascular effects reported earlier (Beltowski, 2005; Sodha *et al*., 2008; Kwak *et al*., 2012; Ahmadi *et al*., 2020; Liu *et al*., 2019). It is also possible that oxidative stress as generated by the three statins and by hydrogen peroxide (as a positive control) might have contributed to the decreased

ChE activity in the present study. The present study demonstrated that brain ChE in the brain homogenate exposed to hydrogen peroxide (500 µM) was reduced by 66.3% to 69.8%, compared to respective control values.

The present results also showed high level of correlation between anti-ChE and generation of MDA by the three statins. Indeed, it has been reported that in vivo exposure of chicks to the oxidative stress by the oral intake of hydrogen peroxide had resulted in reduced plasma, brain and liver ChE activities together with the potentiation of organophosphate intoxications (Al-Baggou *et al*., 2011). It is therefore, a useful approach to apply the in vitro experimental paradigms for the examination of statin effects on pseudo and true ChE activities without the possibility of metabolic and pharmacokinetic interferences (Koh *et al*., 2011; Ritiu *et al*., 2022; Sunami and Munakata, 2022). In the present study, the in vitro differences among the three statins in producing oxidative stress or ChE inhibition could be attributed to variations in the intrinsic activity, chemical-physical properties and structure of each statin (Koh *et al*., 2011; Ritiu *et al*., 2022; Sunami and Munakata, 2022). Furthermore, considering the clinical implication of reduced plasm and brain ChE activity and even that of the oxidative stress it is conceivable to conclude that these effects might influence responses to CNS active drugs, neuromuscular blocking agents and possibly anesthetics (Abdolmaleki *et al*., 2020; Ritiu *et al*., 2022; Sunami and Munakata, 2022). In support of this notion, statin pretreatments have been shown to alter the response of experimental animals to anesthetics and ChE inhibiting toxicants as seen in chicks (Rashid and Mohammad, 2023b) as well as in mice of the present study. To this end, based on the present in vitro findings which coincide with those of in vivo studies reported in the literature, it is perceivable to propose that at least two important mechanisms involving the cholinergic system together with oxidative stress that underscores the oxidant/antioxidant system,

might be involved in adverse or undesirable effects of statins. These effects, which included reductions in ChE activity in concordance with the induction of oxidative stress, were dependent on statin concentrations, indicating drug target efficacy in a consistent manner.

Limitations of the study

In spite of the new findings presented in the current study regarding the neurobehavioral outcome in mice treated with the three statins, the present study did not explore in depth the potential cognitive behavioral effects in mice treated with statins. The whole brain was used for measuring the ChE, GSH and MDA levels. However, statin treatments could produce varying changes involving their contents among different regions of the brain. In addition, as liver injury is deduced from the elevated ALT and AST activities, histopathological examinations were not performed, they were out of the scope of the current research. It is also possible that statin treatments might have affected other organ systems such as the kidney, as well as other biochemical variables such as the lipid profile of the mice. Albeit, future studies would address these potential research areas.

As the present results indicated potential pleiotropic effects of statins on the brain, different brain region should be examined and tested in future in vitro studies. The potential effect of statins on brain homocysteine level was not test. Homocyteine has been found to be affected by high levels of atorvastatin (10–100μM) using the in vitro experimental paradigm with peripheral blood mononuclear cells (Tajbakhsh *et al*., 2022). Further, in the present study, using the current in vitro system, the translation of the present findings and any clinical extrapolation should be extremely scrutinized first.

Chapter Six

Conclusions and Recommendations

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6-1: Conclusions

According to the results presented in the five sections of the present study, it is possible to deduce the following conclusions:

1. High single doses of the three statins differentially modulated neurobehavioral outcomes in mice in association with reduced ChE activity in the brain.
2. The data suggest and ascertain adverse behavioral effects of repeated statin treatments in mice.
3. Changes in ChE activity in plasma and erythrocytes indicate the potential of using them for biomonitoring statin adverse/therapeutic effects.
4. The results suggest and additionally ascertain differential alterations in neuronal functions in mice following statins treatments.
5. The present findings suggest an association of high doses of the three statins with the induction of oxidative stress, as adverse effects in the brain and plasma of mice, which also suffered from the additional burden of liver injury; though the hypocholesteremia effects of the statins were within the expected outcome.
6. This study also highlights the need to further investigate potential statin intolerance outcomes following single-dose treatments in mice.
7. The present results support the values of pharmacological and toxicological challenges of mice repeatedly treated with statins to uncover undesirable behavioral modifications of the anesthetic response to propofol and the desirable reductions of dichlorvos-induced cholinergic toxidrome.

8. The involvement of the cholinergic system in statin action has been suggested by the present results, however, a multidisciplinary approach is recommended to deeply explore the neurochemical bases of statin effects.
9. The in vitro results complemented the in vivo anti-ChE and oxidative stress effects of statins.

6-2: Recommendations

Based on the results of the present study and conclusions drawn from them, the following recommendations are suggested for future in depth exploration of statins' adverse effects in experimental laboratory animals:

1. Examining effects of other statins on blood and brain ChE activities.
2. Differentiation of neurotoxic effects of statins in mice.
3. Studying the in vitro and in vivo recovery of blood and brain ChE activities inhibited by statins.
4. Drawing a possible cause and effect relationship from inhibited brain ChE activity in association with neurobehavioral outcome under statin therapy.
5. Future studies should aim to examine and delineate any contradictions between the pro-oxidant and antioxidant impacts of statins in experimental animals.
6. Identifying a suitable animal model, possibly mice, for the induction of statin intolerance as part of the adverse effects of these drugs that specifically target the brain.
7. Further anti-depression studies on the value of statins as ChE inhibitors in the brain regarding the swimming behavioral outcome.
8. Screening other statins for in vitro and in vivo anti-ChE and oxidative stress effects, as they differ considerably in their pharmacologic properties.
9. Considering the in vitro findings of the present study, additional studies are needed to deeply explore the challenging adverse /pleiotropic brain-targeting effects of statins in animal models of intolerance and toxicosis.

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Appendix

Appendix 1

Cholinesterase (CHE) Activity Assay Kit

Catalog no: E-Bc-K052-S

Method: Colorimetric method

Specihication: 100assays(can detdct 96 samphes without duplication

Measuring instrument: Spectrohotometer

Sensitivity:1.17 U/mL

Detectionrange: 1.17-160 U/mL

General information

➢ Intended use

This kit can be used hor detection of cholinesterase (ChE) activity in whole Blood, serum, plasma and cell samples.

➢ Background

In the body the main type cholinesterase (ChE) is acetylcholinesterase (AchE) which is mainly found in the brain and red blood cell membranes. The other typs is butyrylcholinesterase (BchE), which is mainly hound in plasma both forms differ in genetics, structure and dynamic (ChE) is involved in the pathogenesis of some neurodegenerative and related diseases.

➢ Detection principle

Cholinesterase breaks down acetylcholine and acetic acid acetylcholine thet is not hydrolyzed by cholinesterase reacts with basic hydroxylamine to from actamidamine it reacts in an acidic solution to from a brown-red hydroxylamine iron complex the color depth is directly propprtional to the amount remaining acetylcholine can be

colorimertrically cholinesterase activily was cholinesterase activily was calculated.

➢ Kit components & storage

Item	component	Specification	storage
Reagent 1	Buffer Solution	60 mL × 2 vials	2-8 ^{O}C ,6 months
Reagent 2	Substrate	Powder× 2 vials	Shding hight , 6 months
Reagent 3	Diluent 1	10 mL × 1 vial	2-8 ^{O}C ,6 months
Reagent 4	Chormogenic Agent 1	Powder×1vial	2-8 ^{O}C ,6 months
Reagent 5	Alkali Reagent	60 mL ×1 vial	2-8 ^{O}C ,6 months
Reagent 6	Acid Aeagent	60 mL × 1 vial	2-8 ^{O}C ,6 months
Reagent 7	Protein Precipitator	40 mL × 1 vial	2-8 ^{O}C ,6 months
Reagent 8	Chromogenic Agent 2	Powder× 1 vial	Shding hight , 6 months
Reagent 9	Diluent2	2 mL × 1 vial	2-8 ^{O}C ,6 months
Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other.			

➢ Materials prepared by users

Instruments

Spectrophotometer (520 nm), Micropipettor, centrifuhe, Incubartoe, water bath, Votexer

Consumptive material

Tips (10 ɱL, 200 ɱL, 1000 ɱL), Ep tubes (1.5 Ml, 5mL)

Reagents

Double distilled water, Normal saline (0.9% Nacl)

➢ **Safety data**

Some of the reagents in the kit contain dangerous substances. It should be avoided to touch the skin and clothing. Wash immediately with plenty oh water if touhing it carelessly. All the samples and waste material should be treated according to the relevant rules of laboratorys biosafety.

➢ **Precautions**

Before the experiment, Please read the instructions carefully, and wear gooves and work cothes.

OD valuues must completed within 20 minutes

➢ **The key points of the assay**

1. The brown-red iron complex after reaction is unstable, and the measurement
2. Prevent the formulation of bubbles when measuring the OD value, otherwise the OD value will be affected.

Pre-assay Preparation

➢ **Reagent preparation**

1. Preparation of 80 μmol/mL reagent 2 stock solution

Dissolve 1 vial of reagent 2 powder with 5 mL of reagent 3 and mix fully. Prepare the fresh solution before use and the prepared solution can be stored at 2-8°C for a week.

2. Preparation of reagent 2 application solution

Dilute the 80 μmol/mL reagent 2 stock solution with reagent 1 for 10 times Prepare the needed amount of fresh solution before use. The prepared solution can be stored at 2-8°C for 24 hours.

3. Preparation of reagent 4 stock solution

Dissolve 1 vial of reagent 4 powder with 60 mL of double distilled water and mix fully. The prepared solution can be stored at 2-8°C for 3 months.

4. Preparation of reagent 4 application solution

Dilute reagent 4 stock solution with reagent 5 at a ratio of 1:1. Prepare the needed amount of fresh solution before use. The prepared solution can be stored at 2-8°C for 24 hours.

5. Preparation of reagent 9 application solution

Dilute the reagent 9 with double distilled water at a ratio of 1:39 and mix fully. The prepared solution can be stored at 2-8°C for 6 months.

- **Sample preparation**

The samples should be prepared as conventional methods. Also please refer to appendix II.

- **Dilution of sample**

It is recommended to take 2-3 samples with expected large difference to do pre-experiment before formal experiment and dilute the sample according to the result of the pre-experiment and the detection range (1.17-160 U/mL).

The recommended dilution factor for different samples is as follows (for reference only):

Sample type	Dilution Factor
Human serum	2-3
Human plasma	2-3
Mouse serum	2-3
Mouse plasma	2-3
SH-SY5Y cells	1
10% Mouse brain tissue homogenate	1
10% Rat brain tissue homogenate	1
10% Rat kidney tissue homogenate	1
10% Rat spleen tissue homogenate	1
10% Mouse liver tissue homogenate	1

Note: The diluent is normal saline (0.9% NaCl).

Assay protocol	
Ambient temperature	25-30°C
Optimum detection wavelength	520 nm

Instructions for the use of transferpettor

1. Equilibrate the pipette tip in that reagent before pipetting each reagent.
2. Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Assay protocol

- Operating steps

1. For serum (plasma), tissue and cells samples

1. Blank tube: Take 0.3 mL of double distilled water to the 5 mL tube.
   Control tube: Take 0.05 mL of double distilled water and 0.25 mL of reagent 2 application solution to the 5 mL tube.
   Sample tube: Take 0.05 mL of sample and 0.25 mL of reagent 2 application solution to the 5 mL tube.
2. Add 0.5 mL of reagent 1 to each tube and mix fully.
3. Incubate at 37°C for 20 min.
4. Successively add 1 mL of reagent 4 application solution, 0.5 mL of reagent 6, 0.25 mL of reagent 7, 0.5 mL of reagent 8 application solution and mix fully.
5. Centrifuge at 2325 g for 10 min, then take the supernatant.
6. Set the spectrophotometer to zero with blank tube and measure the OD values of each tube at 520 nm with 1 cm optical path cuvette.

2. For whole blood samples

1. Blank tube: Take 0.35 mL of double distilled water to the 5 mL tube.
   Control tube: Take 0.25 mL of reagent 2 application solution to the 5 mL tube.
   Sample tube: Take 0.1 mL of sample and 0.25 mL of reagent 2 application solution to the 5 mL tube.
2. Add 0.5 mL of reagent 1 to each tube and mix fully.
3. Incubate at 37°C for 20 min.
4. Successively add 1 mL of reagent 4 application solution, 0.5 mL of reagent 6, 0.25 mL of reagent 7, 0.5 mL of reagent 8 application solution.
5. Add 0.1 mL of sample to control tube.
6. Mix fully and centrifuge at 2325 g for 10 min, then take the supernatant.

7. Set the spectrophotometer to zero with blank tube and measure the OD values of each tube at 520 nm with 1 cm optical path cuvette.

Summary operation table

1. For serum (plasma), tissue and cells samples

	Blank tube Sample tube	Control tube	
Sample (mL)			0.05
Double distilled water (mL)	0.3	0.05	
Reagent 1(mL)		0.25	0.25
Mix fully, incubate at 37°C for 20 min.			
Reagent 4 application solution (mL)	1	1	1
Reagent 6 (mL)	0.5	0.5	0.5
Reagent 7 (mL)	0.25	0.25	0.25
Reagent 8 application solution (mL)	0.5	0.5	0.5
Mix fully, then centrifuge at 2325 g for 10 min, then take the supernatant. Set the spectrophotometer to zero with blank tube and measure the OD values of each tube at 520 nm with 1 cm optical path cuvette.			

- **Calculation**

1. Serum (plasma) samples

Definition: The amount of ChE in 1 mL of serum or plasma that react with substrate in 20 minute at 37°C and decompose 1 μmol acetylcholine is defined as 1 unit.

ChE activity (U/mL) $= \frac{A_1 - A_2}{A\ \ 1} \times c \times \frac{V_1}{V_2} \times f$

2. Tissue and cell samples

Definition: The amount of ChE in 1 mL of serum or plasma that react with substrate in 20 minute at 37°C and decompose 1 μmol acetylcholine is defined as 1 unit.

ChE activity (U/mgprot) $= \frac{A_{1-A_2}}{A\ \ 1} \times c \times \frac{V_1}{V_2} \div C_{pr} \times f$

3. Whole blood samples

Definition: The amount of ChE in 1 mL of whole blood that react with substrate in 20 minute at 37°C and decompose 1 μmol acetylcholine is defined as 1 unit.

ChE activity (U/mL) $= \frac{A_{1-A_2}}{A\ \ 1} \times c \times \frac{V_1}{V_2} \times f$

Note:

A,: The OD value of control tube.

A2: The OD value of sample tube.

c: the concentration of control tube, 8 μmol/mL.

f: Dilution factor of sample before tested.

V_1: The volume of reagent 2 application solution (0.25 mL)

V2: The volume of serum and tissue added to the reaction (0.05 mL)

V3: The volume of whool blood added to the reaction (0.1 mL)

C_{pr}: Concentration of protein in sample, mgprot/mL

Notes

1. This kit is for research use only.
2. Instructions should be followed strictly, changes of operation may result in unreliable results.
3. The validity of kit is 6 months.
4. Do not use components from different batches of kit.

Appendix I Performance characteristics

Appendix I Performance characteristics			
Detection range	1.17-160 U/mL	Average intra-assay CV (%)	3.7
Sensitivity	1.17 U/mL	Average intra-assay CV (%)	9.4
Average inter-assay CV (%)	104	-	-

- **Example analysis**

Dilute mouse serum with normal saline (0.9% NaCl) for 2 times, take 0.05 mL of diluted sample and carry the assay according to the operation table. The results are as follows:

The average OD value of the sample is 0.208, the average OD value of the control is 0.725, and the calculation result is:

$$\text{ChE activity} = \frac{0.725-0.208}{0.725} \times 8 \times \frac{0.25}{0.05} \times 2 = 57.04\ U/mL$$

- **Note for sample**

1. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
2. If a lysis buffer is used to prepare tissue or cell homogenates, there is a possibility of causing a deviation due to the introduced chemical substance.

Appendix II Sample preparation

The following sample pretreatment methods are for reference only.

- **Whole blood**

Bottom up the heparin anticoagulated whole blood to make it mix fully, then take 0.1 mL of the whole blood and add 0.4 mL of pre-cooled double distilled water. Mix fully for 1 min and stand for 15 min until the prepared hemolysis is transparent when observing under light.

- **Serum**

Collect fresh blood and stand at 25°C for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4°C. Take the serum (which is the upper light yellow clarified liquid layer) to preserve it on ice for detection. If not detected on the same day, the serum can be stored at -80°C for a month.

- **Plasma**

Take fresh blood into the tube which has anticoagulant (Heparin is used as anticoagulant), centrifuge at 700-1000 g for 10 min at 4°C. Take the plasma (which is the upper light yellow clarified liquid layer, don't take white blood cells and platelets in the middle layer) to preserve it on ice for detection. If not detected on the same day, the plasma can be stored at -80°C for a month.

- **Tissue sample**

Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2– 8°C Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of homogenized medium (2 - 8 deg * C) (mL) : the weight of the tissue (g) = 9/1 then centrifuge the tissue homogenate for 10

min at 10000 g at 4 deg $4°c$ Take the supernatant to preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M). If not detected on the same day, the tissue sample (without homogenization) can be stored at - 80 deg $-80°c$ for a month.

- **Cells**

Collect the cells (don't use trypsin or EDTA) and wash the cells with PBS (0.01 M. pH 7.4) for 1-2 times. Centrifuge at 1000 g for 10 min and then discard the supernatant and keep the cell sediment. Add homogenization medium at a ratio of cell number (10^6) homogenization medium μL =1: 300-500. Sonicate or grind with hand-operated in ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M). If not detected on the same day, the cells sample (without homogenization) can be stored at $-\ 80°C$ for a month.

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(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !)

Reduced Glutathione (GSH) Colorimetric Assay Kit

Catalog No: E-BC-K030-S

Method: Colorimetric method

Specification: 100 assays **(**Can detect 96 samples without duplication)

Measuring instrument: Spectrophotometer

Sensitivity: 0.26 mg GSH/L

Detection range: 0.26-122.8 mgGSH/L

This manual must be read attentively and completely before using this product. If you have any problem, please contact our Technical Service Center for help. Phone: 240-252-7368(USA) Fax: 240-252-7376(USA) Email: techsupport@elabscience.com Website: www.elabscience.com

Application

This kit can measure GSH content in serum, plasma, cells, tissue and other samples.

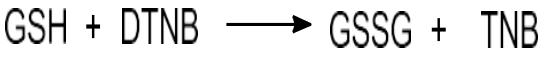
Detection significance

Reduced glutathione (GSH) is a tripeptide which composed of glutamic acid, glycine and cysteine. It is a kind of low molecular scavenger, which can remove O_2^-, H_2O_2, and LOOH. Beside as the main thiol compound of non-protein in the organization, GSH is the substrate of GSH-PX and GST which is indispensable to decomposing hydrogen peroxide for the two enzyme. What's more, it can stabilize the enzyme containing thiol and prevent hemoglobin and other auxiliary factors from the oxidative damage. Recently, it is proved that GSH is also involved in

the recovery of vitamin E to the reduction state. When lacking or depletion of GSH, it may cause producing toxic effects or increasing the toxic effects of many chemicals or environmental factors. It may be related to the increase of oxidative damage, so the amount of GSH is a vital factor to measure the body's antioxidant ability.

Detection principle

Reduced glutathione (GSH) can react with dithionitrobenzoic acid (DTNB) to produce thio-nitrobenzoic acid and glutathione disulfide. Nitromercaptobenzoic acid is a yellow compound which has the maximum absorption peak at 420 nm. The GSH content can be calculated by measuring the OD value at 420 nm.



Kit composition

Reagent	Specification	Storage
Reagent 1	Acid Reagent, 45 mL × 2 vials	2-8℃, 6 months, shading light.
Reagent 2	Phosphate, powder × 2 vial	2-8℃, 6 months.
Preparation of Reagent 2 application solution: dissolve Reagent 2 powder with 150 mL ddH2O completely. It can be stored at 4℃ for 6 months. The reagent is a saturated solution. If there is crystallization, please take the supernatant for experiment.		
Reagent 3	DTNB Solution, 30 mL × 1 vial	2-8℃, 6 months, shading light.
Reagent4	Salt Reagent, powder × 4 vials	2-8℃, 6 months, shading light.
Preparation of Reagent 4 application solution: dissolve a vial of Reagent 4 powder with 10 mL ddH2O completely. It can be stored at 4℃ for a month with shading light.		
Reagent 5	GSH Standard, 3.07 mg × 2	2-8℃, 6 months.

	vials	
Reagent 6	GSH Standard Stock Diluent, 6 mL × 1 vial	2-8℃, 6 months.
Preparation of GSH standard application solution: dilute GSH standard stock solution with ddH2O at a rate of 1: 9 and mix fully. Prepare the fresh solution before use.		
Preparation of 1 mmol/L GSH standard solution: molecular weight of GSH is 307, dissolve 3.07 mg GSH with 10 mL of GSH standard application solution fully. Prepare the fresh solution beforeuse.		

Preparation of 20 μmol/L standard solution: dilute 1 mmol/L GSH standard solution with GSH

Standard application solution at a rate of 1: 49 and mix fully	Prepare the fresh solution before use

Experimental instruments

Test tube, Micropipettor, Vortex mixer, Centrifuge, Spectrophotometer (420 nm) Preparation of sample

Serum (plasma):

Take 0.7 mL sample, add 0.7 mL reagent 1 and mix fully, then centrifuge at 4500 g for 10 min. Take the supernatant for test. (If the supernatant contains sediment, transfer the supernatant into a new EP tube and centrifuge again)

1. **5% tissue homogenate:** Weigh the tissue accurately and add PBS (0.01M, PH7.4) at a ratio of weight (g): volume (mL) =1: 19, homogenize the tissue in ice bath, centrifuge for at 10000 g 15 min.

Take 0.7 mL supernatant, add 0.7 mL reagent 1 and mix fully, then centrifuge at 4500 g for 10 min. Take the supernatant for test. Meanwhile, determine the concentration of supernatant (E-BC-K318-M, E-BC-K168-S, E-BC-K165-S). (**Note:** When absorbing supernatant, avoid a film on the surface and insert it into the supernatant to take 1 mL for determination.)

Operation steps

1. **Blank tube:** Add 1 mL of **Reagent 1** to the 5 mL EP tube.
   A- Standard tube: Add 1 mL of **20 μmol/L GSH standard solution** to the 5 mL EP tube.
   B- Sample tube: Add 1 mL of supernatant to the 5 mL EP tube.
2. Add 1.25 mL of **Reagent 2 application solution,** 0.25 mL of **Reagent 3,** 0.05 mL of **Reagent 4application solution** to each tube.
3. Mix fully and stand for 15 min at room temperature. Set spectrophotometer to zero with distilled water and measure the OD values of each tube at 420 nm wavelength with 1 cm optical path cuvette.

Note: It can be refer to the following operating table.

	Blank tube	**Standard tube**	**Sample tube**
Reagent 1 (mL)	1.0		
20 μmol/L GSH standard solution (mL)		1.0	
Supernatant (mL)			1.0
Reagent 2 application solution (mL)	1.25	1.25	1.25
Reagent 3 (mL)	0.25	0.25	0.25
Reagent 4 application solution (mL)	0.05	0.05	0.05
Mix fully and stand for 15 min at room temperature. Set spectrophotometer to zero with distilled water and measure the OD values of each tube at 420 nm wavelength with 1 cm optical path cuvette.			

Calculation of results

1. For serum(plasma) samples

GSH activity (mg GSH/L)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Concentration of standard } (20 \times 10^{-3} \text{ mmol}$$

× GSH molecular weight (307) × Dilution factor(2)× Dilution factor of sample before tested

Note: Concentration of standard: 20 μmol/L=20 × 10^{-3} *m*mol/L

Dilution factor = Dilution factor of Preparation of supernatant (2 times)

2. For tissue homogenate samples

GSH activity (mg GSH/gprot)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{Concentration of standard}(20 \times 10^{-3} \text{ mmol/L})$$

× GSH molecular weight (307) × Dilution factor(2)

× Dilution factor of sample before tested

÷ Concentration of protein in hemoglobin (gprot/L)

Note: Concentration of standard: 20 μmol/L=20 × 10^{-3} mmol/L

Dilution factor = Dilution factor of preparation of supernatant (2 times)

Technical parameter

1. The sensitivity of the kit is 0.26 mg GSH/L.
2. The intra-CV is 1.8% and the inter-CV is 2.4%.
3. The recovery of the kit is 102%.
4. The detection range of the kit is 0.26-122.8 mg GSH/L.

Appendix: Standard Curve

(This is for reference only)

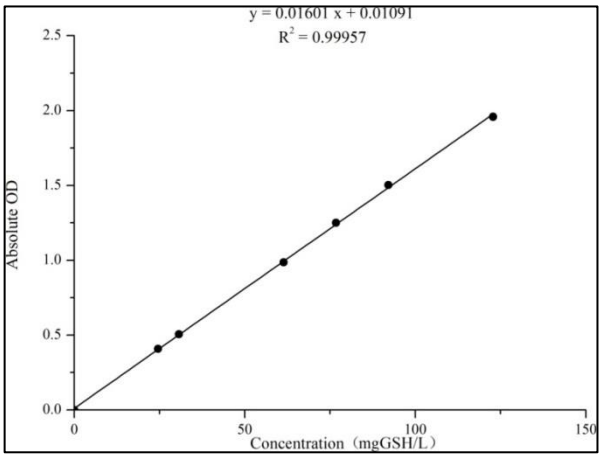
Dilution of standard

Dilute 1 mmol/L GSH (307 mgGSH/L) standard solution with GSH standard application solution to a serial concentration. The recommended dilution gradient is as follows: 122.8, 92.1, 76.75, 61.4, 30.7,24.56, 0 mgGSH/L.

Operation steps

	Standard tube
GSH standard solution with different concentration (mL)	1.0
Reagent 2 application solution (mL)	1.25
Reagent 3 (mL)	0.25
Reagent 4 application solution (mL)	0.05
Mix fully and stand for 15 min at room temperature. Set spectrophotometer to zero with distilled water and measure the OD values of each tube at 420 nm wavelength with 1 cm optical path cuvette.	

Standard curve



Catalog No : E-EL-0060

Product size: 96T/48T/24T/96T*5

References

1. Miester, A. and Anderson M.E.(1983).Glutathion.Annu Rev Biochem.52(6): 711-760.
2. Miester, A.(1982). Metabolism and function of glutathione: an overview. Biochemical Society Transactions.10(2):78-79.
3. Mannervik, B.(1985) Glutathion peroxidase. Methods Enzymol.77(5):490-495.
4. Miester, A. (1988).Glutathion metabolism and its selective modification. Journal of Biological Chemistry.263(33): 17205-17208.

Elabscience

MDA(Malondialdehyde) ELISA Kit

This manual must be read attentively and completely before using this product.

Intended use

This ELISA kit applies to the in vitro quantitative determination of MDA concentrations in serum, plasma and other biological fluids.

Character

Item	
Sensitivity	18.75 ng/mL
Detection Range	31.25-2000 ng/mL
Specificity	This kit recognizes MDA in samples. No significant cross-reactivity or interference between MDA and analogues was observed
Repeatability	Coefficient of variation is < 10%

Test principle

This ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with MDA. During the reaction, MDA in samples or Standard competes with a fixed amount of MDA on the solid phase supporter for sites on the Biotinylated Detection Ab specific to MDA. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450±2 nm. The

concentration of MDA in the samples is then determined by comparing the OD of the samples to the standard curve.

Kit components & Storage

An unopened kit can be stored at 2-8℃ for 1 week. If the kit is not supposed to be used within 1 week, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips 24T: 8 wells ×3 strips 96T*5: 5 plates, 96T	-20℃, 6 months
Reference Standard	96T: 2 vials 48T/24T: 1 vial 96T*5: 10 vials	
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 μL 48T/24T: 1 vial, 60 μL 96T*5: 5 vials, 120 μL	
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 μL 48T/24T: 1 vial, 60 μL 96T*5: 5 vials, 120 μL	-20°C(shading light), 6 months
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL 96T*5: 5 vials, 20 mL	2-8°C, 6 months
Biotinylated Detection Ab Diluent	96T/48T/24T: 1 vial, 14 mL 96T*5: 5 vials, 14 mL	
HRP Conjugate Diluent	96T/48T/24T: 1 vial, 14 mL 96T*5: 5 vials, 14 mL	
Concentrated Wash Buffer (25×)	96T/48T/24T: 1 vial, 30 mL 96T*5: 5 vials, 30 mL	
Substrate Reagent	96T/48T/24T: 1 vial, 10 mL 96T*5: 5 vials, 10 mL	2-8°C(shading light)

Item	Specifications	Storage
Stop Solution	96T/48T/24T: 1 vial, 10 mL 96T*5: 5 vials, 10 mL	2-8°C
Plate Sealer	96T/48T/24T: 5 pieces 96T*5: 25 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.

The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Microplate reader with 450 nm wavelength filter High-precision transfer pipette, EP tubes and disposable pipette tips Incubator capable of maintaining 37℃. Deionized or distilled water Absorbent paper Loading slot

Sample collection

Serum: Allow samples to clot for 1 hour at room temperature or overnight at 2-8℃ before centrifugation for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

Plasma: Collect plasma using EDTA-Na2 as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8℃ within 30 min of collection. Collect the supernatant to carry out the assay.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolyzed blood may affect the results, so the tissues should

be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5-10 min at 5000×g at 2-8℃ to get the supernatant.

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times or use an ultrasonic cell disrupter until the cells are fully lysed. Centrifuge for 10 min at 1500×g at 2-8℃. Remove the cell fragments, collect the supernatant to carry out the assay.

Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

Recommended reagents for sample preparation: 10×EDTA Anticoagulant (Cat No. E-EL-SR003), PMSF Protease Inhibitor (Cat No. E-EL-SR002), 0.25% Trypsin Solution (Cat No. EP-CM-L0043).

1) A freshly opened ELISA plate may appear a water-like substance, which is normal and will not have any impact on the experimental results. Return the unused wells to the foil pouch and store according to the conditions suggested in the above table.
2) Do not reuse the reconstituted standard, biotinylated detection Ab working solution, HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other

stock solutions should be stored according to the storage conditions in the above table.

3) The microplate reader should be able to be installed with a filter that can detect the wave length at 450±2 nm. The optical density should be within 0-3.5. Follow the Instructions of the Microplate Reader for set-up and preheat it for 15 min before OD measurement.
4) Do not mix or substitute reagents with those from other lots or sources.
5) Change pipette tips in between adding of each standard level, between sample adding and between reagent adding. Also, use separate reservoirs for each reagent.
6) The kit should not be used beyond the expiration date on the kit label.

■ Note for sample:

1) Tubes for blood collection should be disposable and be non-endotoxin. Samples with high hemolysis or much lipid are not suitable for ELISA assay.
2) Samples should be assayed within 7 days when stored at 2-8℃, otherwise samples must be divided up and stored at-20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen samples should be slowly thawed and centrifuged to remove precipitates.
3) Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
4) If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
5) If a lysis buffer is used to prepare tissue homogenates or cell lysates, there is a possibility of causing a deviation due to the introduced chemical substance.

6) Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

Dilution Method

If your test sample needs dilution, please refer to the dilution method as follows:

For 100 fold dilution: One-step dilution. Add 5 μL sample to 495 μL sample diluent to yield 100 fold dilution.

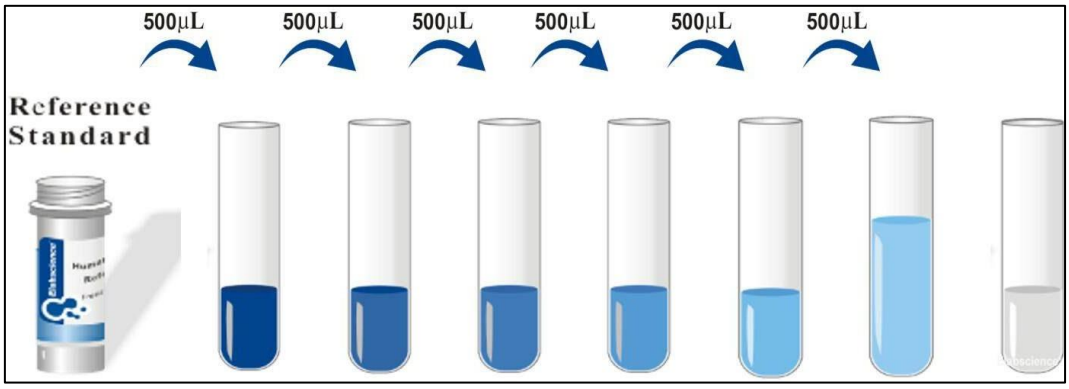
For 1000 fold dilution: Two-step dilution. Add 5 μL sample to 95 μL sample diluent to yield 20 fold dilution, then add 5 μL 20 fold diluted sample to 245 μL sample diluent, after this, the neat sample has been diluted at 1000 fold successfully.

For 100000 fold dilution: Three-step dilution. Add 5 μL sample to 195 μL sample diluent to yield 40 fold dilution, then add 5 μL 40 fold diluted sample to 245 μL sample diluent to yield 50 fold dilution, and finally add 5 μL 2000 fold diluted sample to 245 μL sample diluent, after this, the neat sample has been diluted at 100000 fold successfully.

Reagent preparation

1. Bring all reagents to room temperature (18-25℃) before use. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved.
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it

thoroughly with a pipette. This reconstitution produces a working solution of 2000 ng/mL(or add 1 mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then make serial dilutions as needed. The recommended dilution gradient is as follows: 2000, 1000, 500, 250, 125, 62.500, 31.25, 0 ng/mL. Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 2000 ng/mL working solution to the first tube and mix up to produce a 1000 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube. Gradient diluted standard working solution should be prepared just before use.



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (50 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent(Concentrated Biotinylated

Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).The working solution should be prepared just before use.

5. **Concentrated HRP Conjugate working solution:** HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).The working solution should be prepared just before use.

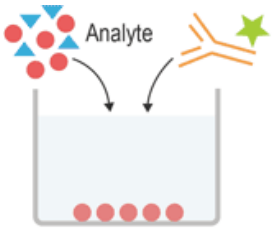
Assay procedure

1. Determine wells for **diluted standard, blank** and **sample**. Add 50 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations).Immediately add 50 μL of **Biotinylated Detection Ab** working solution to each well. Cover the plate with the sealer provided in the kit. Incubate for 45 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Decant the solution from each well, add 350 μL of **wash buffer** to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
3. Add 100 μL of **HRP Conjugate working solution** to each well. Cover

the plate with a new sealer. Incubate for 30 min at 37°C.

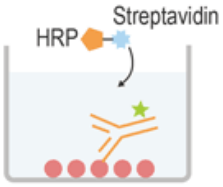
4. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 2.
5. Add 90 μL of **Substrate Reagent** to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
6. Add 50 μL of **Stop Solution** to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
7. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Assay Procedure Summary

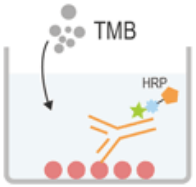


1. Add 50μL standard or sample to the wells, immediately add 50μL Biotinylated Detection Ab working solution to each well. Incubate for 45 min at 37°C

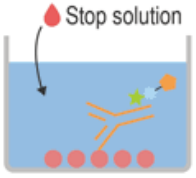
2. Aspirate and wash the plate for 3 times



3. Add 100μL HRP conjugate working solution. Incubate for 30 min at 37°C. Aspirate and wash the plate for 5 times



4. Add 90μL Substrate Reagent. Incubate for 15 min at 37°C



5. Add 50μL Stop Solution

6. Read the plate at 450nm immediately. Calculation of the results

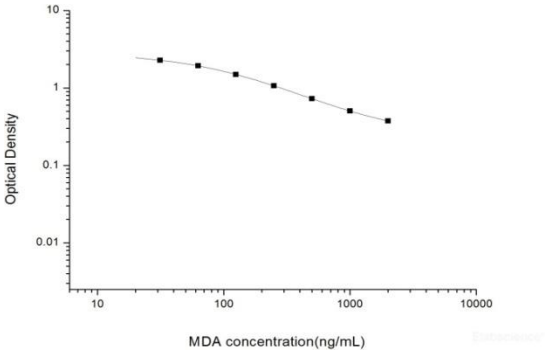
Calculation of results

Average the duplicate readings for each standard and samples. Plot a four parameter logistic curve on log-log axis, with standard concentration on the x-axis and OD values on the y-axis.

If the OD of the sample under the lowest limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

ng/mL	OD	Standard Curve
2000	0.375	
1000	0.503	
500	0.725	
250	1.063	
125	1.494	
62.500	1.931	
31.25	2.284	
0	2.811	

Performance

■ Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level MDA were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level MDA were tested on 3 different plates, 20 replicates in each plate, respectively.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
N	20	20	20	20	20	20
Mean	109.60	308.70	848.80	111.50	321.00	892.50
Standard deviation	6.10	17.30	37.30	7.00	17.00	46.40
C V (%)	5.57	5.60	4.39	6.28	5.30	5.20

■ Recovery

The recovery of MDA spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=8)	92-103	97
EDTA plasma (n=8)	94-107	99
Cell culture media (n=8)	93-110	100

■ Linearity

Samples were spiked with high concentrations of MDA and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)	Cell culture media(n=5)
1:2	Range (%)	92-107	84-99	89-101
	Average (%)	99	91	95
1:4	Range (%)	82-95	88-103	100-113
	Average (%)	89	95	105
1:8	Range (%)	86-97	87-102	99-111
	Average (%)	92	94	105
1:16	Range (%)	85-96	87-99	94-110
	Average (%)	91	92	100

Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.
3. The final experimental results will be closely related to the validity of products, operational skills of the operators, the experimental environments and so on. We are only responsible for the kit itself, but not for the samples consumed during the assay. The users should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
4. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions.
5. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings

of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.

6. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
7. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment, and so on. Intra-assay variance among kits from different batches might arise from the above reasons too.
8. Kits from different manufacturers or other methods for testing the same analyte could bring out inconsistent results, since we haven’t compared our products with those from other manufacturers.
9. The kit is designed for research use only, we will not be responsible for any issues if the kit is applied in clinical diagnosis or any other related procedures.

Appendix 2

ALT GPT Colorimetric Method

Reagent for quantitative determination of Alanine amino transferase [EC 2.6.1.2] in human serum and plasma

I INTENDED USE

This reagent is designated for professional use in laboratory (manual method). I It allows the quantification of global activity of the alanine amino transferase (ALT) enzyme in human serum and plasma to screen its level.

GENERALITIES (1) (2)

ALT is present in very high amounts in liver and kidney, and in smaller amounts in skeletal muscle and heart. Although serum levels of both AST and ALT become elevated whenever diseases process affecting liver cells integrity, ALT is the more liver-specific enzyme. A serum elevation of ALT activity is rarely observed in conditions other than parenchymal liver disease (cirrhosis, carcinoma, hepatitis, obstructive jaundice or liver stroke).

PRINCIPLE (4)

Colorimetric method developed by Tonhazy, White, and Umbreit and adapted for the determination of the activity in serum by Reitman and Frankel. Reaction scheme is as follows:

L-Alanine + 2- Oxoglutarate $\overset{ALT}{\rightleftharpoons}$ Pyruvate + L-Glutamate

Glutamate Then, Pyruvate reacts with 2, 4 DNPH to form 2, 4 Dinitrophenylhydrazones,

which absorbance at 505 nm in alkaline solution is proportional to AST or ALT activity in the reactional mixture.

I REAGENTS

R2 GPT / ALT Substrate

Phosphate Buffer pH 7.5 100 mmol/L

L-Alanine 200 mmol/L

2-oxoglutarate 2 mmol/L

Preservative

R3 GPT / ALT Dye

2,4-dinitrophenyl-hydrazine (DNPH) 1,7 mmol/L HCl 1 mol/L

EUH210: Safety datasheet on request (HCL 2.5 - < 10%)

R4 GPT / ALT Standard

Sodium Pyruvate	2	mmol/L
Sodium Mercurothiolate	0.1	%
Phosphate Buffer pH 7.5 Preservative	100	mmol/L

Preservative

According to 1272/2008 regulation, these reagents are not classified as dangerous

SAFETY CAUTIONS

Refer to current Material Safety Data Sheet available on request or on www.biolabo.fr

- Verify the integrity of the contents before use.
- Waste disposal: Respect legislation in force in the country.

All specimens or reagents of biological origin should be handled as potentially infectious. Respect legislation in force in the country.

I Any serious incident that has occurred in connection with the device is notified to the manufacturer and the competent authority of the Member State in which the user and/or patient is based.

REAGENTS PREPARATION

Ready for use.

STABILITY AND STORAGE

Stored away from light, well cap in the original vial at 2-8°C, reagents are stable when stored and used as described in the insert:

Unopened,

Until the expiry date stated on the label of the Kit. Once opened:

- Transfer requested quantity, well recap vials and store at 2-8°C,
- Separated reagents are stable at least 6 months without contamination Discard reagents if cloudy or if reagent blank at 505 nm is > 0.400.

SPECIMEN COLLECTION AND HANDLING (2)

Unhemolyzed serum.Do not use heparinized plasma.

ALT is stable in serum or plasma for:

· 24 hours at room temperature.

· 7 days at 2-8°C.

LIMITS (3)

For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S

MATERIAL REQUIRED BUT NOT PROVIDED

1. Medical analysis laboratory equipment
2. REF 92026: NaOH 0.4 N
3. Spectrophotometer

CALIBRATION

- REF 92027 (vial R4) or refer to the enclosed Standard Curve (batch specific) The value of the standard has been determined under

metrological control, by weighing on analytical balance. QUALITY CONTROL

- REF 95010 EXATROL-N Level I ·
- REF 95011 EXATROL-P Level II ·

External quality control program It is recommended to control in the following cases:

- At least once a run
- At least once within 24 hours
- When changing vial of reagent
- After maintenance operations on the instrument If control is out of range, apply following actions:

1. Prepare a fresh control serum and repeat the test
2. If control is still out of range, use a new vial of fresh calibrator
3. If control is still out of range, use a new vial of reagent and reassay If control is still out of range, please contact BIOLABO technical support or your local Agent.

REFRENCE INTERVAL (2)

ALT (IU/L)	at 37°C
New-born, Infants	13-45
Men	10-40
Women	7-35

Each laboratory should establish its own normal ranges for the population it serves.

PERFORMANCE

On Spectrophotometer 37°C, 505 nm

Measuring Range: within Standard Curve limits Detection limit: approximately 7.2 IU/L Precision .

.	Within-run N = 20		Between run N = 20	
	Normal level	High level	Normal level	High level
Mean IU/L	51.9	90.6	29.7	91.9
S.D. IU/L	2.2	2.5	1.7	8.2
C.V. %	4.2	2.8	5.8	8.9

Sensitivity for 100 IU/L:

approximately 0.200 Abs at 505nm.

Comparison study with commercially available reagent:

y = 1,0477 x - 2,3 r = 0,9737

Interferences:

Ascorbic acid	No interference up to 2500 mg/dL
Total bilirubin	Negative interference from 250 µmol/L
Hemoglobin	Positive interference from 30 µmol/L
Turbidity	Positive interference from 0.025 OD

Other substances may interfere (see § Limits)

Calibration frequency:

It is recommended to establish a new Standard Curve when using a new batch of reagent (§ CALCULATION) or to refer to the enclosed Standard Curve (batch specific).

MANUAL PROCEDURE

Let stand reagents and specimens at room temperature.

1- STANDARD CURVE ESTABLISHMENT:

Pipette into Test tubes (mL):						
Tube number:	1	2	3	4	5	6
Demineralized water	0.200	0.200	0.200	0.200	0.200	0.200
R2 (Substrate)	1	0.900	0.800	0.700	0.600	0.500
R4 (Standard)	--	0.100	0.200	0.300	0.400	0.500
R3 (Dye)	1	1	1	1	1	1
Mix. Let stand for 20 minutes at room temperature. Add:						
NAOH 0.4 N	10	10	10	10	10	10
Mix. Let stand 5 minutes and read absorbances at 505 nm against water.						
TGP (IU/L)	0	40	80	140	225	325
There's no need to plot a new curve at each determination. See §Calibration and Quality Control						

1- ASSAYS:

Pipette into test tubes:	
Reagent R2	1 mL
Incubate for 5 minutes at 37°C. Add:	
Serum	200 µL
Mix and incubate at 37°C during:	Exactly 30 minutes
Reagent R3	1 mL
Mix and let stand 20 minutes at room temperature. Add:	
NaOH 0.4 N	10 mL
Mix. Let stand 5 minutes and read absorbances at 505 nm against water.	

CALCULATION

Calculate the result as follows:

- Refer to enclosed Standard Curves (batch specific) or

- Plot Standard Curves on millimeter paper (Absorbances) handling as indicated in table 1. Abscissa: Units (IU/L)

Ordinate: Absorbances

Transfer “Assay” absorbances on Standard Curve and read activity (IU/L)

REFERENCES

(1) TIETZ N.W. Textbook of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders (1999) p. 652-.657

(2) Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2006) p. 64-67 et p.76-77.

(3) YOUNG D.S., Effect of Drugs on Clinical laboratory Tests, 4th Ed. (1995) p. 3-6 to 3-17 and p.3-68 to 3-79. (4) A colorimetric method for the determination of serum GOT and GPT, REITMAN S. and FRANKEL S., Amer. J. Clin. Path., 1957; 28, p.56-63

AST GOT Colorimetric Method

Reagent for quantitative determination of Aspartate amino transferase [EC 2.6.1.1] in human serum and plasma REF 92025 R1 1 x 100 mL R3 1 x 100 mL R4 1 x 10 mL

I INTENDED USE

This reagent is designated for professional use in laboratory (manual or automated method). It allows the quantification of global activity of the aspartate amino transferase (AST) enzyme in human serum and plasma to screen its level.

GENERALITIES (1) (2)

AST is distributed in all body tissues, but greatest activity occurs in liver, heart, skeletal muscle and in erythrocytes. Minimal activity occurs in skin, kidney and pancreas. Although serum levels of both AST and ALT become elevated whenever diseases processes affecting liver cells integrity (viral hepatitis, liver necrosis and cirrhosis), an increased AST activity in serum or plasma appears in more than 97% of cases of myocardial infarction. AST levels (and occasionally ALT) are also elevated in progressive muscular dystrophy, pulmonary emboli, acute pancreatitis…

PRINCIPLE (4)

Colorimetric method developed by Tonhazy, White, and Umbreit and adapted for the determination of the activity in serum by Reitman and Frankel. Reaction scheme is as follows:

L-Aspartate + 2-Oxoglutarate $\xleftrightarrow{\text{AST}}$ Oxaloacetate + L-Glutamate

Then, Oxalate reacts with 2, 4 DNPH to form 2, 4 Dinitrophenylhydrazones, which absorbance at 505 nm in alkaline solution is proportional to AST or ALT activity in the reactional mixture.

I REAGENTS

R1 GOT / AST Substrate

Phosphate Buffer pH 7.5	85	mmol/L
L-Aspartate	200	mmol/L
2-oxoglutarate	2	mmol/L
Preservative		

R3 GOT / AST Dye

2,4-dinitrophenyl-hydrazine (DNPH)	1,7	mmol/L	HCl 1 mol/L

EUH210: Safety datasheet on request (HCL 2.5 - < 10%)

R4 GOT / AST Standard

Sodium Pyruvate	2	mmol/L
Sodium Mercurothiolate	0.1	%
Phosphate Buffer pH 7.5	100	mmol/L Preservative

According to 1272/2008 regulation, these reagents are not classified as dangerous

SAFETY CAUTIONS

- Refer to current Material Safety
- Data Sheet available on request or on www.biolabo.fr
- Verify the integrity of the contents before use.
- Waste disposal: Respect legislation in force in the country.
- All specimens or reagents of biological origin should be handled as potentially infectious. Respect legislation in force in the country. Any serious incident that has occurred in connection with the device is notified to the manufacturer and the competent authority of the Member State in which the user and/or patient is based.

REAGENTS PREPARATION

Ready for use.

STABILITY AND STORAGE Stored away from light, well cap in the original vial at 2-8°C, reagents are stable when stored and used as described in the insert:

Unopened, Until the expiry date stated on the label of the Kit. Once opened:

- Transfer requested quantity, well recap vials and store at 2-8°C.
- Separated reagents are stable at least 6 months without contamination Discard reagents if cloudy or if reagent blank at 505 nm is > 0.400.

SP-ECIMEN COLLECTION AND HANDLING (2)

Unhemolysed serum. Do not use heparinised plasma AST is stable in serum or plasma for:

- 24 hours at room temperature
- 28 days at 2-8°C ·
- At least for 1 year at –20°C.

Adding pyridoxal phosphate (0.1 mM) improves stability at room temperature to 7 days.

LIMITS (3)

For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S

MATERIAL REQUIRED BUT NOT PROVIDED

1. Medical analysis laboratory equipment
2. REF 92026: NaOH 0.4 N
3. Spectrophotometer

CALIBRATION

- REF 92025 (vial R4) or refer to the enclosed Standard Curve (batch specific) The value of the standard has been determined under

metrological control, by weighing on analytical balance. QUALITY CONTROL

- REF 95010 EXATROL-N Level I
- REF 95011 EXATROL-P Level II
- External quality control program It is recommended to control in the following cases: · At least once a run ·
- At least once within 24 hours
- When changing vial of reagent
- After maintenance operations on the instrument If control is out of range, apply following actions:

1. Prepare a fresh control serum and repeat the test
2. If control is still out of range, use a new vial of fresh calibrator
3. If control is still out of range, use a new vial of reagent and reassay If control is still out of range, please contact BIOLABO technical support or your local Agent.

REFERENCE INTERVAL (2)

AST (IU/L)	at 37°C
New-born, Infants	39-117
Men	23-94
Women	13-31

Each laboratory should establish its own normal ranges for the population it serves.

PERFORMANCE

On Spectrophotometer 37°C, 505 nm

Measuring Range: within Standard Curve limits

Detection limit: approximately 7.2 IU/L

Precision

	Intra-série N = 20		Inter-série N = 20	
	Taux normal	Taux élevé	Taux normal	Taux élevé
Moyenne UI/L	37,7	167	38	144,5
S.D. UI/L	1,1	9,4	3,75	13,5
C.V. %	2,9	5,6	9,9	9,3

Sensitivity for 100 IU/L: approximately 0.200 Abs at 505nm. Comparison study with commercially available reagent:

y = 0,8984 x + 3,6 r = 0,9729

Interferences:

Other substances may interfere (see § Limits)

Interferences:

Ascorbic acid	No interference up to 2500 mg/dL
Total bilirubin	Negative interference from 100 µmol/L
Haemoglobin	Positive interference from 90 µmol/L
Turbidity	Positive interference from 0.075 OD

Other substances may interfere (see § Limits)

Calibration frequency: It is recommended to establish a new Standard Curve when using a new batch of reagent (§ CALCULATION) or to refer to the enclosed Standard Curve (batch specific).

MANUAL PROCEDURE

Let stand reagents and specimens at room temperature.

1- STANDARD CURVE ESTABLISHMENT:

Pipette into Test tubes (mL):						
Tube number:	1	2	3	4	5	6
Demineralised water	0.200	0.200	0.200	0.200	0.200	0.200
R2 (Substrate)	1	0.900	0.800	0.700	0.600	0.500
R4 (Standard)	--	0.100	0.200	0.300	0.400	0.500
R3 (Dye)	1	1	1	1	1	1
Mix. Let stand for 20 minutes at room temperature. Add:						
NAOH 0.4 N	10	10	10	10	10	10
Mix. Let stand 5 minutes and read absorbances at 505 nm against water.						
AST (IU/L)	0	30	70	135	225	350
There's no need to plot a new curve at each determination. See §Calibration and Quality Control						

1- ASSAYS:

Pipette into test tubes:	
Reagent R2	1 mL
Incubate for 5 minutes at 37°C. Add:	
Serum	200 µL
Mix and incubate at 37°C during:	Exactly 1 hour
Reagent R3	1 mL
Mix and let stand 20 minutes at room temperature. Add:	
NaOH 0.4 N	10 mL
Mix. Let stand 5 minutes and read absorbances at 505 nm against water.	

Note: Volumes may be reduced proportionally without modifying results.

CALCULATION

Calculate the result as follows:

- Refer to enclosed Standard Curves (batch specific) or

- Plot Standard Curves on millimeter paper (Absorbances) handling as indicated in table 1. Abscissa: Units (IU/L)

Ordinate: Absorbances

Transfer “Assay” absorbances on Standard Curve. Read activity (IU/L)

REFERENCES

(1) Tietz, N.W. (1999). Textbook of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders. p. 652-.657

(2) Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2006) p. 64-67 et p.76-77.

(3) Young, D.S. (1995).Effect of Drugs on Clinical laboratory Tests, 4th Ed. p. 3-6 to 3-17 and p.3-68 to 3-79.

(4) Reitman,S. and Frankel, S., Amer. J. Clin. Path., (1957). A colorimetric method for the determination of serum GOT and GPT. 28:56-63

Cholestrol commercial spectrophotometric kit (Biolabo SA, Maizy, France)

INTENDED USE

This reagent is designated for professional use in laboratory. It may be used with manual procedure on spectrophotometer or with Biochemistry Clinical Analyzer. This quantitative test is to determine the concentration of Total Cholesterol in human serum or plasma.

GENERALITIES

(1) (2) I Hypercholesterolemia can be observed in case of dietary imbalance, in in hepatic and thyroid disorders, certain cases of diabetes, nephrotic syndrome, pancreatitis, myeloma or familial hypercholesterolemia. Total cholesterol increased levels may be isolated or associated to other increased lipids (hyperlipidemia). A decreased level of cholesterol may be due to deficiencies or malnutrition, cancer or hyperthyroidism.

PRINCIPLE

(4) Enzymatic method described by Allain and al., which reaction scheme is as follows:

Cholesterol esters $\longrightarrow$ CE Cholesterol + free fatty acids Cholesterol + O2 CO $\longrightarrow$ Cholesten 4 one 3 + H2O2

H_2O_2 + Phenol + PAP $\longrightarrow$ POD Quinoneimine (pink) + 4 H2O

REAGENTS

R1 CHO CHOLESTEROL Reagent

Phosphate buffer	100	mmol/L
Chloro-4-phenol	5	mmol/L
Sodium Cholate	2.3	mmol/L
Triton x 100	1.5	mmol/L

Preservative

Cholesterol oxidase (CO) ≥ 100 IU/L Cholesterol esterase (CE) ≥ 170 IU/L Peroxidase (POD) ≥ 1200 IU/L

4 - Amino – antipyrine (PAP) 0.25 mmol/L PEG 6000 167 µmol/L

According to 1272/2008 regulation, this reagent is not classified as dangerous.

SAFETY CAUTIONS

- Refer to current Material Safety Data Sheet available on request or on www.biolabo.fr · .Verify the integrity of the contents before use. · Waste disposal: Respect legislation in force in the country.
- All specimens or reagents of biological origin should be handled as potentially infectious.

Respect legislation in force in the country. Any serious incident that has occurred in connection with the device is notified to the manufacturer and the competent authority of the Member State in which the user and/or patient is based.

REAGENTS PREPARATION

Ready for use.

STABILITY AND STORAGE

Stored away from light, well cap in the original vial at 2-8°C, reagent is stable when stored and used as described in the insert: Unopened: · Until the expiry date stated on the label of the Kit. Once opened: · Reagent is stable at least 3 months when free from contamination. · Discard reagent if cloudy or if absorbance at 505 nm > 0.400.

SPECIMEN COLLECTION AND HANDLING

Serum or plasma (Heparin or EDTA). Do not use oxalate, fluoride or citrate. Collect on fasting patient. Separate serum from cells within 2 h. Cholesterol is stable in the specimen for: · 5-7 days at 2-8°C · 3 months at –20°C · Many years at –70°C. · Avoid repeated freezing and thawing.

LIMITATIONS

Enzymatic methods increase analytic specificity. Cholesterol oxidase also reacts with 3b-hydroxycholesterols (insignificant quantity in human serum – i.e. DHEA, pregnenolone). For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S or N. W. Tietz.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Basic medical analysis laboratory equipment.
2. Biochemistry Clinical Analyzer Kenza One, Kenza 240TX/ISE or Kenza 450TX/ISE

QUALITY CONTROL

REF 95010 EXATROL-N Level

I. REF 95011 EXATROL-P Level

II. External quality control program. It is recommended to control in the following cases: · At least once a run. · At least once within 24 hours.

III. When changing vial of reagent.

After maintenance operations on the instrument. If control is out of range, apply following actions:

1. Prepare a fresh control serum and repeat the test.
2. If control is still out of range, use a new vial of calibrator or a fresh calibrator and repeat the test.

3. If control is still out of range, repeat with a new vial of reagent. If control is still out of range, please contact BIOLABO technical support or your local Agent.

REFERENCE INTERVAL (2)

Values for adults, in term of risk for atherosclerotic diseases:

Total cholesterol	**mg/dL**	**[mmol/L]**
Recommended values	< 200	[< 5.18]
Low risk	200-239	[5.18-6.19]
High risk	≥ 240	[≥ 6.22]

Each laboratory should establish its own normal ranges for the population that it serves.

PERFORMANCES

On KENZA ONE, 37°C, 505 nm

Linearity Range: between 4 and 400 mg/dL

Detection limit: approx. 0.1 mg/dL

Precision:

Within-run N = 20	***Low level***	***Normal level***	***High level***
Mean (mg/dL)	**107**	**199**	**270**
S.D. mg/dL	**1.6**	**1.8**	**1.5**
C.V. %	**1.5**	**0.9**	**0.6**

Analytical Sensitivity: approx. 0.3312 abs for 100 mg/dL Clinical comparison study with commercially available reagent, using serum specimens between 64 and 323 mg/dL (n=101)

y = 1.0292 x – 3.94 R= 0.9882

Interferences:

Turbidity	No interference up to 0.295 OD
Total bilirubin	Negative interference from 293 µmol/L
Direct bilirubin	Negative interference from 271 µmol/L
Ascorbic acid	Negative interference from 855 mg/dL
Glucose	No interference up to 1212 mg/dL
Haemoglobin	No interference up to 162 µmol/L

Other substances may interfere (see § Limits)

On the board stability: 2 months (*)

Calibration Stability: 2 months (*)

Make a new calibration when changing reagent batch, if quality control results are found out of the established range and after maintenance operations.

Performances and stability data on Kenza 450TX/ISE and (*) Kenza 240TX/ISE are available on request

CALIBRATION (6)

REF 95015 Multicalibrator traceable to SRM 1951 The calibration frequency depends on proper instrument functions and on the preservation of the reagent.

PROCEDURE

- Refer to validated application of the Kenza Analyzer used

CALCULATION

- The analyzer provides directly result in units (mg/dL).
- Refer to the instruction of use of Kenza analyzer.

REFERENCES

(1) Tietz, N.W. (1999).Textbook of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders p. 826-835.

(2) Tietz, N.W. (1995) Clinical Guide to Laboratory Test, 3rd Ed., p. 130-131.

(3) Young, D.S. (1995). Effect of Drugs on Clinical laboratory Tests, 4th Ed. p. 3-143 to 3-164 [mmol/L] [< 5.18] [5.18-6.19] [> 6.22] Between run N = 20 Low level Normal level Mean (mg/dL) 112 S.D. mg/dL 2.7 212 290 5.0 C.V. % 2.4 2.3 2.5 IVD

(4) Allain, C. C. et al. (1974). Clinical Chemistry., 20/4, p.470-475

(5) Allan C., Deacon et Peter J. G. Dawson, Clinical Chemistry (1979) 25/6, p.976-984 (6) High level 7.3 SRM: Standard Reference material ®

الخلاصة

إن أدوية الإستاتينات الخافضة للدهون لها آثار جانبية مختلفة، كما لها تأثيرات متعددة في سلوك البشر وحيوانات المختبر. بالإضافة الى تأثيراتها المؤكسدة/ الأكسدة الكولينية العصبية السلوكية في الفئران. الهدف من هذه الدراسة هو فحص التغيرات السلوكية العصبية ونشاط الكولين إستريز (ChE) والإجهاد التأكسدي في نموذج الفئران المعاملة بالأتورفاستاتين، والسيمفاستاتين، والروزوفاستاتين.

أُستخدمت ذكور فئران من أصل سويسري، وتم معاملتها عن طريق الفم بجرع مختلفة من الإستاتينات (أتورفاستاتين، سيمفاستاتين، وروزوفاستاتين). أُعطيت فئران السيطرة ماء مقطر.

القسم 1: بعد ساعتين من إعطاء جرعة الماء المقطر أو الإستاتينات بجرع 250 أو 500 أو 750 أو 1000 ملغم / كغم، أُخذ كل فأر لوحده لأداء السلوك العصبي والذي تمثل أدائه في الميدان المفتوح لمدة 5 دقائق، إختبار الإنتحاء الأرضي السالب بزاوية 45 درجة / لمدة60 ثانية، إختبار إدخال الرأس في الثقوب لمدة 5 دقائق ، و إختبار تحمل السباحة القسري.

تم تحديد نشاط إنزيم الكولين إستريز في البلازما وكريات الدم الحمراء والدماغ باستخدام جهاز المطياف الضوئي بعد 2 و 24 ساعة بعد أخذ جرع الإستاتينات 500 و 1000 ملغم / كغم عن طريق الفم.

القسم 2: أُعطيت الفئران جرع مفردة من الإستاتينات 500 أو 1000 ملغم / كغم. كما تم إعطاء الفئران جرع يومية متكررة من كل إستاتين بمقدار 200 ملغم / كغم / يوم لمدة 14 أو 28 يومًا. حددت مستويات الكلوتاثيون (GSH) والمالونديالديهايد (MDA) في الدماغ والبلازما، بالإضافة إلى قياس مستوى إنزيمات الكبد ألانين امينوترانسفيريز (ALT) وأسبارتات امينوترانسفيريز (AST) .

القسم3: عوملت الفئران بالإستاتينات (200 ملغم / كغم / يوم) لمدة 28 يومًا، وبعد 24 ساعة من آخر جرعة إستاتين أو الماء المقطر خضعت الفئران للتحدي الدوائي بعد حقنها بالبروبوفول بجرعة 100 ملغم / كغم في الخلب، أو للتحدي السمي باستخدام الدايكلوروفوس بجرعة 150 ملغم / 10 مل ماء مقطر / كغم، عن طريق الفم. تمت مراقبة تخدير البروبوفول والتسمم الكوليني ونشاط إنزيم الكولين استريز في الدماغ الناجم عن الدايكلوروفوس.

القسم 4: جرعت الفئران كل إستاتين بمقدار 200 ملغم / كغم /يوم لمدة 14 و28 يومًا، وبعد 24 ساعة من آخر جرعة تم اختبار كل فأر لوحده لأداء السلوك العصبي وقياس نشاط إنزيم الكولين استريز المذكور أعلاه.

القسم 5: أُستخدمت تراكيز مائية في المختبر لكل إستاتين على البلازما وجانسة الدماغ بتراكيز تراوحت 0 (السيطرة) أو 10 أو 25 أو 50 أو 100 مايكرومولار. حُدد تثبيط نشاط إنزيم الكولين استريز في البلازما والدماغ بالطريقة الكهرومترية بالإضافة إلى الطريقة اللونية بإستخدام جهاز المطياف الضوئي جنبًا إلى جنب ، فضلا عن معرفة أمكانية الإستاتينات في إحداث الإجهاد التأكسدي من خلال قياس تركيز المالونديالديهايد (MDA) في الدماغ.

النتائج:

القسم 1: أدت الإستاتينات اعتمادًا على الجرعة بشكل متفاوت ، إلى تأخير زمن الحركة في إختبار الميدان المفتوح، وقلة الحركة وعدد مرات الوقوف على القوائم الخلفية، وقلة عدد مرات إدخال الرأس في الثقوب ، والتأخير في أداء إختبار الإنتحاء الأرضي السالب . كما زادت الإستاتينات من مدة السباحة القسرية وقللت من مدة عدم الحركة في حوض السباحة. وبالإعتماد على الجرعة قللت الإستاتينات نشاط إنزيم الكولين إستريز في البلازما وكريات الدم الحمراء والدماغ للفئران بعد ساعتين و24 ساعة من تناول الجرعة.

القسم 2: أدت جرع الإستاتينات المفردة بجرعة 500 أو 1000 ملغم/كغم وبالإعتماد على الجرعة إلى تقليل تركيز الكلوتاثيون في البلازما والدماغ. أدى الإعطاء المتكرر لكل إستاتين بجرعة 200 ملغم/كغم / يوم لمدة 14 أو 28 يومًا وبالإعتماد على الوقت إلى تقليل مستويات GSH في البلازما والدماغ . بينما أدت إلى زيادة تركيز MDA بشكل كبير في البلازما والدماغ بعد جرعة واحدة أو متكررة من الإستاتينات، . كما أدت إلى زيادة في تركيز ALT و ASTفي البلازما.

القسم 3: عملت الإستاتينات على تقليل وقت بدء النوم الناتج عن تناول البروبوفول بشكل واضح وخفضت مدته، كما عملت الإستاتينات على زيادة ظهور علامات التسمم بشكل واضح، وأخرت ظهور الموت وقللت من علامات التسمم ودرجة السمية والموت. كما عملت الإستاتينات على تقليل نشاط إنزيم الكولين استريز في الدماغ .

القسم 4: أدت المعاملة المتكررة لكل إستاتين إلى قلة نشاط الفئران المعاملة في إختبار الميدان المفتوح و قلة عدد مرات إدخال الرأس في الثقوب ، والتأخير في وقت أداء إختبار الإنتحاء الأرضي السالب وزيادة المدة الزمنية لإختبار تحمل السباحة القسري مع التقليل في وقت عدم الحركة، كما أدت إلى نقصان في نشاط إنزيم الكولين إستريز في البلازما وكريات الدم الحمراء والدماغ .

القسم 5: أدت الإستاتينات بتركيز 10- 100 مايكرومول في المختبر بالإعتماد على التركيز إلى تثبيط نشاط إنزيم الكولين إستريز في البلازما والدماغ (الطريقة الكهرومترية). زاد بيروكسيد الهيدروجين بشكل ملحوظ من تركيز MDA في الدماغ. كما زادت الإستاتينات بتركيز 25 - 100 مايكرومول بالإعتماد على تركيز MDA في جانسات الدماغ، ثبط نشاط إنزيم الكولين إستريز ولوحظ زيادة كبيرة في تركيزMDA في الدماغ، باستخدام الإستاتينات عند التراكيز من 25 إلى 100 مايكرومول في المختبر وبالإعتماد على التركيز باستخدام جهازالمطياف الضوئي وتقنية الاليزا. كما قلل بيروكسيد الهيدروجين من نشاط إنزيم الكولين إستريز في الدماغ بالنسب (66.3٪و 68.7٪و 69.8٪) على التوالي.

نستنتج إن الجرع المفردة والمتكررة من الإستاتينات تعمل على تغيير نتائج السلوك العصبي بشكل مختلف في الفئران بالتزامن مع انخفاض نشاط إنزيم الكولين إستريز في البلازما وكريات الدم الحمراء والدماغ. ويمكن الإستفادة من نشاط إنزيم الكولين إستريز في البلازما أو كريات الدم الحمراء في المراقبة الحيوية للآثار الجانبية/العلاجية للإستاتينات. وقد تسببت الإستاتينات في إحداث إجهاد تأكسدي كآثار ضارة في الدماغ وبلازما الفئران، فضلا عن تأثيرها الضار على الكبد. وتدعم النتائج التحديات الدوائية والسمية في الفئران المعاملة بالإستاتينات للكشف عن التغيرات في الاستجابة للتخدير بالبروبوفول والتسمم بالدايكلوروفوس. وبشكل عام، تعتبر هذه التأثيرات أساسًا لاستكشافات واسعة للآثار الضارة للإستاتينات في الحيوانات التجريبية ولإيجاد نموذج حيواني، ربما الفئران، لتحريض الآثار الضارة للإستاتينات التي تستهدف الدماغ، فضلاً عن تسليط الضوء على نتائج إحتمالية عدم تحمل الإستاتينات المستخدمة في هذه الدراسة في هذا النوع من الحيوانات المختبرية. كما أن النتائج المختبرية تدعم تأثيرات الإستاتينات في الجسم الحي، فإن التأثيرات المضادة لإنزيم الكولين إستريز والإجهاد التأكسدي للإستاتينات قد تكون لها علاقة بتأثيراتها الضارة أوالسمية.

تقييم السلوك العصبي وحالة الكرب التأكسدي ونشاط إنزيم الكولين إستريز في ذكور الفئران المعاملة ببعض أدوية الإستاتين

أطروحة تقدمت بها

رونق فارس عبدالقادر حسن

إلى

مجلس كلية الطب البيطري في جامعة الموصل

وهي جزء من متطلبات نيل شهادة الدكتوراه فلسفة

في اختصاص الطب البيطري / الأدوية البيطرية

بإشراف

الأستاذ الدكتور

فؤاد قاسم محمد

جمادي الأول/ 1446هـ **تشرين الثاني/ 2024م**

جامعة الموصل

كلية الطب البيطري

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