Effects of Different Atorvastatin Doses on Immunohistochemical Markers in the Hippocampus of Male Adult Rats

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Abstract
Statins, beyond their lipid lowering role, exert beneficial effect by acting as a neuroprotective agent in some clinical cases such as brain injury, stroke, ischemia, seizures and Alzheimer's disease. The aim of this study is toinvestigate the effects of different oral doses of atorvastatin on normal male rats, via studying immunohistochemical markers in the hippocampus. Twenty-four adult male Wistar rats were used in the experiment. They were obtained from the animal house (at Department of Pharmacology & Toxicology, College of Pharmacy/ Al-Mustansiriya University). Animals are divided into 4 groups randomly each group contains 6 animals as follow: Group 1: Animals are administered saline orally for 30 days and serve as (control group), Group 2: Animals are administered 5 mg/kg/day Atorvastatin in the form of oral suspension for 30 days, Group 3: Animals are administered 10mg/kg/day Atorvastatin for 30 days, Group 4: Animals are administered 20 mg/kg/day Atorvastatin for 30 days. All animals in this study were dissected under anesthesia; heads were decapitated for extraction of the brain. Immunohistochemical method was used to evaluate the expression of Glial Fibrillary Acidic Protein, Glutathione Reductase and neuronal Nitric Oxide Synthase markers in the rat's hippocampus. There was a significant reduction of GFAP expression in the group of rats treated with 20 mg/kg atorvastatin when compared with the control and 5 mg/kg atorvastatin. While, a significant increase in GR expression was noticed in the group treated with 20 mg/kg atorvastatin compared with other groups. Also, the results showed a significant reduction in nNOS expression in the hippocampus of rats treated with 20 mg/kg atorvastatin when compared with other groups. The present study demonstrates that atorvastatin exerts its neuroprotective effects in a 20 mg/kg dose, more than in 5 mg/kg or 10 mg/kg doses. The decreasing in astrocytes activity, promoting of anti-oxidant enzymes and reducing in nitrative stress were documented when a short term high atorvastatin dose used.
Keywords: hippocampus, atorvastatin, neuronal Nitric Oxide Synthase (nNOS), Glial Fibrillary Acidic Protein (GFAP), Glutathione Reductase (GR).

1. Introduction

The hippocampus is the part of the brain located beneath the cortex within the inner folds of the medial temporal lobes.The mammalian hippocampus is comprised of four main regions: the Dentate Gyrus (DG), the Cornuammonis (CA), the Presubiculum and the Subiculum. The CA regions are further subdivided into four regions called CA1, CA2, CA3, and CA4 (Amaral and Lavenex 2007) .The hippocampus has an important role in the formation of new memories about experienced events (Elzakker V *et al.*2008). Also, it plays a role in spatial memory and navigation. It is a one of the structures within the brain that makes up the limbic system, which is responsible for emotions, memories, motivation and other "preconscious" functions (Elzakker V *et al.*2008). It is one of the first regions affected by changes in the brain of Alzheimer's disease patients.

Statins are recommended as first-line therapy for hypercholesterolaemia (Pedersen T.R, *et al.*2004), since they have been shown to reduce the risk of cardiovascular morbidity and mortality in patient with or at risk of coronary heart disease in several clinical trails (Tziomalos K *et al.*2009 and Reiner Z.2013). Atorvastatin, which belongs to the second generation of statins, is a synthetic reversible inhibitor of HMG-CoA reductase. The dosage range used clinically is 10-80 mg/day (Plosker and Lyseng-Williamson 2007).They also act as a neuroprotective agent in some clinical cases such as brain injury, stroke, ischemia and seizures. Statins not only lower cholesterol but they also showed positive effects against Alzheimer-relevant amyloid beta-induced oxidative stress in mice models of AD (Kurinami H et al.2008 and Tong X.K et al.2009). It also serves as an anti-apoptotic by blocking Aβ 1-42, which is responsible for inducing neuronal death, as well as reducing caspase-3 activity (Sparks D.L et al.2008). It also serves as an anti-inflammatory by decreasing interleukin-1β, interleukin-6 and tumor-necrosis-factor-α (Zhang Y.Y et al.2013).Recent studies show that atorvastatin may act by reduction in the hippocampal astrogliosis, lipid peroxidation and COX-2 expression (Piermartiri T.C et al.2010), as well as an increase in glial glutamate transporters expression (Piermartiri T.C et al.2010 and Tavares R.G et al.2002). Therefore, the present study aims to investigate the effect of different oral doses of atorvastatin

on normal male rats via studying immunohistochemical markers in the hippocampus.

2. Material and method

Twenty-four adult male Wistar rats (weighing200–250g) were used in the experiment. They were obtained from the animal house (at Department of Pharmacology & Toxicology, College of Pharmacy/ Al-Mustansiriya University). Animals were kept under controlled conditions of temperature of $(22 \pm 1C^0)$ with light schedule of 12-12 hour's light/dark cycles. Tap water and foods in the form of pellets were accessible freely to theme. Atorvastatin tablets provided by HIKMA, Jordan. While streptozocin provided by Abcam, UK.

Animals are divided into 4 groups randomly each group contains 6 animals as follow: Group 1: Animals are administered saline orally for 30 days and serve as (control group), Group 2: Animals are administered 5 mg/kg/day Atorvastatin in the form of oral suspension by using oral gavage tube for 30 days, Group 3: Animals are administered 10mg/kg/day atorvastatin in the form of oral suspension by using oral gavage tube for 30 days, Group 4: Animals are administered 20 mg/kg/day atorvastatin in the form of oral suspension by using oral gavage tube for 30 days. All animals in this study were dissected under anesthesia using diethyl ether at day 31. With the animals under anesthesia and the heart still beating, heads were decapitated for extraction of the brain.

1-Samples Preparation for histological studies:

Immediately after separation, the specimens were fixed individually in 10% formalin buffer solution for 24 hours at room temperature, followed by a dehydration step, by immersing it in a gradually increasing concentration of alcohol. Then, the tissues were kept in xylene for one hour under a temperature of 60C, and then embedding it in paraffin wax.

2-Staining for general morphology:

5μm-thick sections were cut by a rotary microtome, sequentially mounted onto microscope slides, and stained with Hematoxylin and Eosin.

3-Immunohistochemistry for detection of neuronal Nitric Oxide Synthase (nNOS), Glial Fibrillary Acidic Protein (GFAP) and Glutathione reductase (GR) expression in paraffin-embedded sections:

Paraffin embedded blocks were sectioned at 5μm. The sections were processed for nNOS, GFAP and GR staining using a commercially available kit (provided by Abcam, UK). In brief, the sections were positioned on a positively charged slide. Subsequently, the sections were deparaffinized and rehydrated through a descending alcohol series followed by distilled water. The sections were incubated with sodium citrate buffer in humidity-heat chamber for antigen retrieval. Then, the endogenous peroxidase activity was inactivated with hydrogen peroxide. The non-specific bindings were blocked with a protein-blocking reagent. The sections were incubated with rabbit polyclonal anti nNOS antibody, Anti rabbit polyclonal anti GFAP antibody and anti rabbit polyclonal anti GR antibody. In sequence, the sections were incubated with horseradish peroxidase conjugate and then with complement solution. Finally, the reactions were revealed with 3-3'diaminobenzidine (provided by Abcam, UK) and the sections were counterstained with hematoxylin. Slides were examined with high magnification power to semi- quantitatively identify focally and completely stained cells that are defined as positive for the markers.

3.1-Neuronal Nitric Oxide Sunthase (Nnos):

The immunostaining was graded in five classes according to the percentage of stained tissue (Viaro F. *et al*.2010):0= when the staining was absent, 1=when the percentage of stained tissue varied from 1% -25%, 2= when the percentage of stained tissue varied from 26%- 50%, 3= when the percentage of stained tissue varied from 51%-75% and 4= when the percentage of stained tissue was superior to 75%.

3.2-Glutathione Reductase (GR):

An immunohistochemical based scoring system was utilized for analyses of GR as percentage ofpositive stained cells per field in a blind fashion and the scores calculated as following (Chen Y.T *et al*.2011) :0 = no stain %, 1 = <15%, 2 = 15-25%, 3 = 25-50%, 4 = 50-75% and 5 = >75-100%

3.3-Glial Fibrillary Acidic Protein (GFAP):

Scoring of GFAP expression was done for percentage of staining intensity per field as following (Jalal A.J.2010):0 = none, 1 =< 5%, 2 =5-25% , 3 = 25-75% and 4 =75-100%.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5. Data were tested for normality according to Shapiro test. Analysis of variance (ANOVA) was used and Kruskal-Wallise were performed. Comparisons among groups were submitted according to Dunn's Multiple Comparison Test. $P < 0.05$ was considered statistically significant.

3. Results

Effects of Atorvastatin on Glial Fibrillary Acidic Protein (GFAP)

Table (1) shows that there is a non-significant change (p>0.05) in the score of the GFAP expression of the control group and the group of rats who took 5mg/kg of atorvastatin. Moreover, there is another non-significant decrease in the group of rats who took 10mg/kg of atorvastatin when compared with the control group. There

were no signs of significancy (p>0.05) when the 10mg atorvastatin group was put against the 5mg/kg and 20mg/kg atorvastatin groups. On the other hand, significant decrease (p<0.05) in the atorvastatin 20mg/kg group was noticed when compared to the other two groups, the 5mg/kg atorvastatin and the control group. Figure (1) below testifies the effects of various atorvastatin doses on that marker.

Table 1. Effects of different doses of Atorvastatin on Glial Fibrillary Acidic Protein.

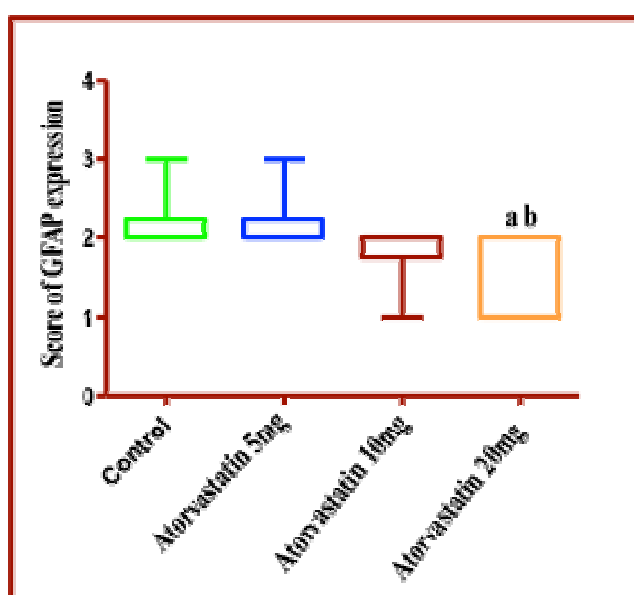| Dunn's Multiple Comparison Test	Difference in rank sum	Significancy
Control vsAtorvastatin.5mg	0.0000	NS
Control vsAtorvastatin 10mg	3.417	NS
Control vs Atorvastatin 20mg	8.917	S
Atorvastatin.5mg vs Atorvastatin 10mg	3.417	NS
Atorvastatin.5mg vs Atorvastatin 20mg	8.917	S
Atorvastatin 10mgvsAtorvastatin 20mg	5.500	NS

Figure 1. Box and whisker plot showing the effects of different doses of atorvastatin on the score of glial fibrillary acidic protein expression. The plots show the medians, 25th and 75th percentiles and extreme values. [a]: significantly different compared with the control. [b]: significantly different compared with the Atorvastatin 5mg/kg group.

Effects of Atorvastatin on Glutathione Reductase (GR)

Table (2) below shows that there is a non significant change (p>0.05) in the score of the GR expression between the control group and the group of rats who took 5mg/kg of atorvastatin. Also, there is another non significant increase in the group of rats who took 10mg/kg of atorvastatin when compared with the control. On the other hand, there is a significant increase (p<0.05) was shown in the atorvastatin 20mg/kg group when compared to the control group. All the 5mg, 10mg and 20mg atorvastatin groups showed a non significant change (p>0.05) when set side by side with each other. The figure (2) below demonstrates the Effects of different doses of atorvastatin on Glutathione Reductase.

Table 2. The Effects of various doses of Atorvastatin on Glutathione Reductase.

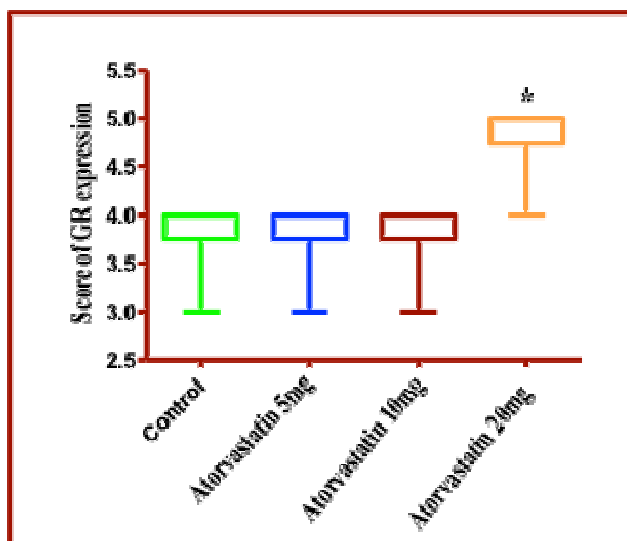| Dunn's Multiple Comparison Test	Difference in rank sum	Significancy
Control vs Atorvastatin.5mg	-3.333	NS
Control vs Atorvastatin.10mg	-3.333	NS
Control vs Atorvastatin.20	-11.00	S
Atorvastatin.5 vs Atorvastatin.10	0.000	NS
Atorvastatin.5 vs Atorvastatin.20	-7.667	NS
Atorvastatin.10 vs Atorvastatin.20	-7.667	NS

Figure 2. Box and whisker plot showing the effects of different doses of atorvastatin on glutathione reductase. The plots show the medians, 25th and 75th percentiles and extreme values.

*: significantly different compared with the control.

Effects of various doses of Atorvastatin on neuronal Nitric Oxide Synthase (nNOS)

Table (3) shows that there is a non significant decrease ($p > 0.05$) in the score of the nNOS expression of the group of rats who took 5mg/kg of atorvastatin when compared with the control. Moreover, there is another non-significant decrease in the group of rats who took 10mg/kg of atorvastatin when confronted with the control group. On the contrary, there is a significant decrease ($p < 0.05$) was shown in the atorvastatin 20mg/kg group when compared to the control. At the same time, all the 5mg, 10mg and 20mg atorvastatin groups showed a non-significant change ($p > 0.05$) when set side by side each other. The figure (3) below exhibits the effects of different doses of atorvastatin on neuronal Nitric Oxide Synthase (nNOS).

Table 3. Effects of various doses of Atorvastatin on neuronal Nitric Oxide Synthase (nNOS).

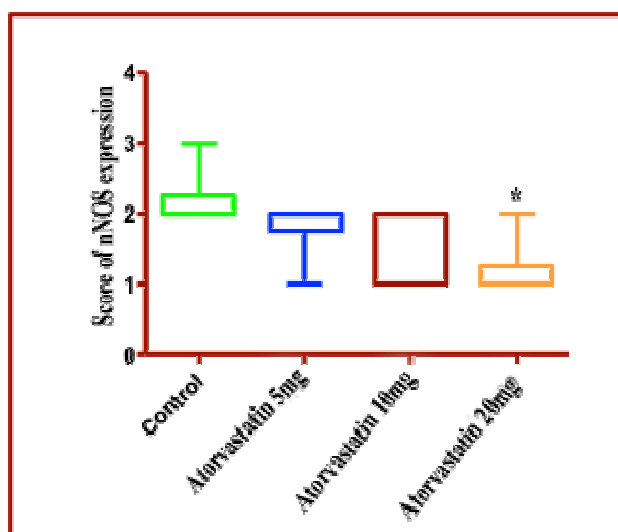| Dunn's Multiple Comparison Test	Difference in rank sum	Significancy
Control vs Atorvastatin.5mg	3.250	NS
Control vs Atorvastatin.10mg	5.167	NS
Control vs Atorvastatin.20mg	10.92	S
Atorvastatin.5 vs Atorvastatin.10mg	1.917	NS
Atorvastatin.5 vs Atorvastatin.20mg	7.667	NS
Atorvastatin.10 vs Atorvastatin.20mg	5.750	NS

Figure 3. Box and whisker plot showing the effects of different doses of atorvastatin on the score of the neuronal nitric oxide synthase expression. The plots show the medians, 25th and 75th percentiles and extreme values.

*: significantly different compared with the control.

4. Discussion

Normal brain aging is characterized by many macroscopic changes including decreased brain volume and weight, and microscopic alterations such as changes in blood–brain barrier permeability, modifications in the extracellular compartment, and both cellular and biochemical alterations in glia and neuronal cells (Castillo-Ruiz M.M *et al.*2007). Also, proliferation of astrocytes with the morphological changes associated with activation has been documented with age, that in turn lead to an increase in the expression of GFAP in the brain (Cowleya T.R *et al.*2012). Hayakawa et al. (2007) showed that increase GFAP immunoreactivity was observed in hippocampus of mice older than 50 weeks. The results collected in the present study showed a non significant change in the GFAP expression in the hippocampus of rats administered a dose of 5mg/kg or a 10mg/kg of atorvastatin orally. These findings come in agreement with the study of Piermartiri T.C.B *et al.* that they showed that there is no significant alteration in GFAP when 10 mg/ kg of atorvastatin treatment was administered. Meanwhile, this study show that there is a significant reduction in the GFAP expression when a dose of 20 mg/kg atorvastatin administered in compared with the control and 5 mg/kg atorvastatin groups. The possible explanation may be that the reactive astrocytes are a source of cytokines including IL-1β, TNF-α, and IL-6 (Gibson RM *et al.*2004). Reactive astrocytes may exacerbate inflammation by inducing the migration of other leukocytes into the brain, interrupting blood-brain-barrier function (Brambilla R *et al.*2009 and Vezzani A *et al.*2010), and producing reactive oxygen species (Swanson RA *et al.*2004 and Hamby ME *et al.*2006). The result showed that 20 mg/kg atorvastatin treatment reduced GFAP-positive astrocytes in the hippocampus. This atorvastatin-mediated suppression of astrocytosis may contribute to inhibition of neuroinflammation and neuronal loss, thus exert its neuroprotective effect.

Glutathione (γ-glutamylcysteinylglycine) is the most abundant non-protein thiol in cells (Dickinson and Forman 2002, and Zeevalk G.D *et al* 2008). Glutathione protects cells against exogenous and endogenous toxins, including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such radical species are removed via non-enzymatic reduction with GSH, whereas the removal of hydroperoxides requires enzymatic catalysis by glutathione peroxidase (Griffith O. W 1999, Dringen R. 2000, Dickinson and Forman 2002, and Zeevalk G.D *et al* 2008) . Both reactions lead to the generation of glutathione disulfide (GSSG, or oxidized glutathione), which is reduced back to GSH by glutathione reductase that uses NADPH from the pentose phosphate shunt (Dickinson and Forman 2002). The results of the this study showed that there is a significant increase in the expression of GR in the hippocampus of rats administered 20 mg/kg atorvastatin orally compared with control, 5mg/kg and 10 mg/kg atorvastatin groups in agreement with previous study (Kabel A.M *et al.* 2013). E. Barone et al. (2011) found that high doses of atorvastatin treatment reduced lipoperoxidation, protein oxidation and nitration, and increased GSH levels in parietal cortex of aged beagles. The most likely explanation for the increase of GSH by atorvastatin was attributed to the antioxidant effect of atorvastatin that results from inhibition of mevalonate pathway. This effect leads to a reduction in the synthesis of important intermediates including isoprenoids (geranylgeranyl pyrophosphate and farnesyl pyrophosphate). These isoprenoids serve as lipid attachments for intracellular signaling molecules (Rho, Rac, Ras and G proteins) which depend on isoprenylation for function and membrane localization. (Hadi N.R *et al.*2010). Rho cause a decrease in eNOS while, Rac leads to increase in NAD(P)H oxidase which cause increase production of ROS. (Mason J.C. 2003, and Liao J.K and Laufs U.2005). Atorvastatin cause inhibition of NAD(P)H oxidase activity and upregulation of eNOS via affect the Rac and Rho molecules respectively, that attenuate endothelial reactive oxygen species (ROS) formation, through attenuating endothelial superoxide anion production (Hadi N.R *et al.*2010).

Nitric oxide molecule is a little reactive radical produced by three enzymes: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and the inducible or inflammatory form expressed by macrophages (iNOS).The nitric oxide (NO) functions as a signaling molecule in the vascular system. Immediately after an ischemic insult or brain damage, eNOS is activated and exerts a protective vasodilation effects that enhance blood flow (van der Most P.J. *et al.*2009). Meanwhile, ischemic insults excessively activate the constitutively expressed nNOS and induce the expression and activation of iNOS, which lead to oxidative damage that caused by overproduction of NO by these tow enzymes. (Endres et al.2004). In this study, when neuronal nitric oxide synthase marker was used, the results show that that there is a non-significant decrease in the expression of this marker in the hippocampus of rats treated with a 10 mg/kg atorvastatin orally compared with the control and 5 mg/kg atorvastatin groups . this disagree with study of de Oliveira C.V. et al. (2013),that they found a decrease in NO content in the adult male rats cerebral cortex following 10 mg/kg for seven days of atorvastatin treatment or withdrawal at 24 hr. after the last atorvastatin administration. This discrepancy could be due to methodological differences, which Oliveira C.V. et al. measured total NO content that produced from all the NOS isoforms in the rats cerebral cortex while this study measure only the expression of nNOS enzyme in the rat's hippocampus. The present study displays there was a significant decrease in nNOS expression in the group administered 20 mg/kg atorvastatin when compared with control, 5 mg/kg and 10 mg/kg atorvastatin treated groups. The possible explanation comes from the fact that statins down-regulate activity of both neuronal NOS and inducible NOS isoforms (van der Most P.J. *et al.*2009), which are the predominant NOS isoforms in

the brain.

5. Conclusion

In conclusion, the present study demonstrates that atorvastatin exerts its neuroprotective effects in a 20 mg/kg dose. Decrease in astrocytes activity, promoting of anti-oxidant enzymes and reducing in nitrative stress were documented when a short term of high atorvastatin dose was used.

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