Effects of Atorvastatin and Streptozocin on Immunohistochemical Markers in Hippocampus of Male Adult Rats

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Abstract

In addition to the lipid lowering activity, statins act positively as a neuro-protective agent in some clinical cases such as Alzheimer’s disease, brain injury, ischemia and seizures. Alzheimer’s disease is a progressive neurodegenerative disorder representing the most common cause of dementia in the elderly population. Central STZ administration developed numerous behavioral, neurochemical and structural features that resembled those found in human Alzheimer’s disease. The results of the present study showed a significant increase in the expression of glial fibrillary acidic protein in the groups of rats administered intrathecal injection of streptozocin when put side by side with the control one. A reduction in this marker observed in the group administered 20 mg/kg atorvastatin combined with a single intrathecal injection of streptozocin when compared to group taken streptozocin alone. Anti-oxidant state represented by glutathione reductase showed a significant increase in the expression of this marker in the groups that administered 5, 10 and 20 mg/kg atorvastatin after intrathecal injection of streptozocin in compared with the group administered streptozocin alone. However, a significant reduction in this marker observed in the group administered streptozocin alone when compared with control group. A significant increase in the neuronal nitric oxide synthase enzyme in the hippocampus of rats administered intrathecal injection of streptozocin. Also, a reduction in this marker observed in the groups treated with 10 and 20 mg/kg atorvastatin combined with a single intrathecal injection of streptozocin when put side by side with group taken streptozocin alone. We can conclude from the results that administration of streptozocin intrathecally lead to a model of Alzheimer’s disease indicated by brain damage that may be improved by atorvastatin treatment but in a dose dependent manner.

1 Introduction

Hippocampus is one of the structures within the brain that makes up the limbic system, which is responsible for emotions, memories, motivation and other “preconscious” functions. It has an important role in the formation of new memories about experienced events. Also, it plays a role in spatial memory and navigation¹. Hippocampus is one of the first regions affected by changes in the brain of Alzheimer’s disease (AD) patients. Alzheimer’s disease causes two obvious changes in the hippocampus, neurofibrillary tangles and senile plaques². As the number of plaques and tangles increases, neurons function will be decrease³. Gradually, the neurons lose their ability to interact and eventually, die, leading to an overall shrinkage of brain tissue, particularly in the hippocampus, that leads to restrict the patient’s ability to form new memories⁴. Alzheimer’s disease is a progressive neurodegenerative disorder representing the most common cause of dementia in the elderly population. This disease was documented more than one hundred years ago by the German psychiatrist Dr. Alois Alzheimer ⁵. Streptozocin is used to generate experimental diabetic animal models by selectively causing beta-cell destruction, since it is...
transported through GLUT2, which is expressed relatively high in beta cells. Central STZ administration caused neither systemic metabolic changes nor diabetes mellitus, but developed numerous behavioral, neurochemical and structural features that resembled those found in human AD. Streptozocin has been administrated mostly in doses ranging from 1–3 mg/kg body weight, injected 1–3 times, either uni-or bi-laterally into the lateral cerebral ventricles. Treatment with very low to moderate Intracerebroventricular doses of STZ in short term experiments causes insulin resistance via a decrease in autophosphorylation and in total number of IRs. Also, reduced expression of genes encoding insulin, IRs, and insulin receptor-substrate 1 and reduced ligand binding to the insulin in CA3 region of hippocampus. Also, Intracerebroventricular streptozocin injection induced oxidative stress, neurotransmission deficits and induced behavioral alterations and structural changes. Although cholinesterase inhibitors (ChEIs) are the primary drug of choice for AD and related diseases, their effectiveness is frequently questioned. Recent studies reported that α7-neuronal acetylcholine nicotinic receptor (α7-nAChR), that mediated neurogenic vasodilatation of cerebral arteries, was blocked by ChEIs, and this blockade was prevented by statin pretreatment leading to activation of this receptor that is eventually result in nitric oxide release and vasodilatation by acting on presynaptic β2-adrenoceptors located on neighboring nitrergic nerve terminals. From a general point of view, the neuroprotective effects of statins include: (i) decrease of endothelial O2− formation by preventing the isoprenylation of p21 Rac; (ii) activate of SOD as well as endothelial progenitor cells; (iii) the increase of the expression of endothelial nitric oxide synthase (eNOS) and (iv) the activation of eNOS via activation of the PI3K/Akt pathway. Therefore, the present study aimed to investigate the effect of different oral doses of atorvastatin on male adult rats model of Alzheimer’s disease induced by 3 mg/kg streptozocin intracerebally via studying immunohistochemical markers in the hippocampus.

2 Materials and Methods

2.1 Animals and study design

Forty eight adult male Wistar rats (weighing 200–250 gm) were used in the experiment. They were obtained from the animal house (at Department of Pharmacology & Toxicology, College of Pharmacy/ The University of Mustansiriya). Animals were into 5 groups randomly each group contains 6 animals as follow: Group 1 were administered saline orally for 30 days and serve as a control group. Group 2 animals were administered intrathecal injection of 3 mg/kg Streptozocin as a single dose and saline orally for 30 days. Group 3 animals were administered intrathecal injection of 3mg/kg Streptozocin as a single dose. At the same day, 5 mg/kg/day Atorvastatin were administered in the form of oral suspension, by using oral gavage tube, and continued for 30 days. Group 4 animals were administered intrathecal injection of 3 mg/kg Streptozocin as a single dose. At the same day, 10 mg/kg/day Atorvastatin were administered in the form of oral suspension, by using oral gavage tube, and continued for 30 days. Group 5 animals were administered intrathecal injection of 3 mg/kg Streptozocin as a single dose. At the same day, 20 mg/kg/day Atorvastatin were administered in the form of oral suspension, by using oral gavage tube, and continued for 30 days. All animals kept under controlled conditions of temperature of (22 ± 10 °C) with light schedule of 12-12 hours light/dark cycles and the animal house was provided with an air vacuum. Tap water and foods in the form of pellets were accessible freely to the animals. The animals were kept for 2 weeks in the mentioned conditions before starting treatment to be adapted to the environment of the animal house. 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.

2.2 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.

2.3 Staining for general morphology

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

2.4 Immunohistochemistry for detection of neuronal Nitric Oxide Synthase, Gial Fibrillary Acidic Protein and Glutathione reductase 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. Then the sections were deparaffinized and rehydrated through a descending alcohol concentrations followed by distilled water. The sections were incubated with sodium citrate buffer in the 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 semiquantitatively identify focally and completely stained cells that are defined as positive for the markers.

2.4.1 Neuronal Nitric Oxide Synthase (Nnos)
The immunostaining was graded in five classes according to the percentage of stained tissue: 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%.

2.4.2 Glutathione reductase (GR)
An immunohistochemical based scoring system was utilized for analyses of GR as percentage of positive stained cells per field in a blind fashion, and the scores calculated as following : 0 = no stain %, 1 =<15%, 2 = 15-25%, 3 = 25-50%, 4 = 50-75% and 5 = >75-100%

2.4.3 Glial Fibrillary Acidic Protein (GFAP)
Scoring of GFAP expression was done for percentage of staining intensity per field as following: 0 = none, 1 = 5%, 2 =5-25%, 3 = 25-75% and 4 =75-100%

2.5 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-Wallis were performed. Comparisons among groups were submitted according to Dunn's Multiple Comparison Test. P < 0.05 was considered statistically significant.

3 Results

3.1 Effects of streptozocin alone and various doses of atorvastatin combined with streptozocin on glial fibrillary acidic protein (GFAP)
Our results make evident that there is a significant increase (p<0.05) in the score of the GFAP expression of the group of rats administered 3mg/kg of streptozocin intrathecally (Fig. 1A) when confronted with the control. On the contrary, there was no significant difference (P>0.05) when the control was compared with the groups treated with 3 mg/kg of streptozocin intrathecally combined with 5 mg/kg, 10 mg/kg and 20 mg/kg of atorvastatin orally. Non significant differences were noticed when streptozocin group wasopposed to both, the 5 mg/kg (Fig. 1B) and the 10 mg/kg (Fig. 1C) of the atorvastatin-streptozocin treated groups. Meanwhile, a significant decrease was observed (P<0.05) in the 20 mg/kg atorvastatin in addition to streptozocin group (fig. 1D) when set side by side against the streptozocin group. All the 5 mg/kg, 10 mg/kg and 20 mg/kg atorvastatin-streptozocin treated groups showed a non significant change (p>0.05) when compared with each other. The figure (2) authenticates the results given above on glial fibrillary acidic protein.

3.2 Effects of multiple doses of atorvastatin combined with streptozocin and streptozocin alone on glutathione reductase (GR)
Analysis of data makes certain that there is a significant decrease (p<0.05) in the score of the GR expression of the group administered 3 mg/kg of streptozocin intrathecally (fig.3A) when compared with the control. On the other side, there was no significant change (p>0.05) when the control was put against the groups who took 3 mg/kg of streptozocin intrathecally combined with 5 mg/kg, 10 mg/kg and 20 mg/kg of atorvastatin orally. Results demonstrated that a significant decrease (p<0.05) in the streptozocin group was minded when compared with both; the 5 mg/kg (Fig. 3B) and the 10 mg/kg (Fig. 3C) of the atorvastatin-streptozocin treated groups. At the same time, a significant decrease (p<0.05) in the same streptozocin group was seen when confronted with the 20 mg/kg atorvastatin-streptozocin treated group (fig. 3D).
All the 5 mg/kg, 10 mg/kg and 20 mg/kg atorvastatin-streptozocin treated groups showed a non-significant change (p>0.05) when set side by side with each other. The figure (4) indicates the results given above on glutathione reductase.

3.3 Effects of streptozocin alone and different doses of atorvastatin combined with streptozocin on neuronal Nitric Oxide Synthase (nNOS)
The result assures that there is a significant increase (P<0.05) in the score of the nNOS expression of the group administered 3 mg/kg of streptozocin intrathecally (Fig.5A) when compared with the control. On the contrary, there was no significant change (p>0.05) when the control was compared against the groups treated with 3 mg/kg of streptozocin intrathecally combined with 5 mg/kg, 10 mg/kg and 20 mg/kg of atorvastatin orally. Our findings demonstrated that a non-significant decrease (P>0.05) in the 5 mg/kg atorvastatin-streptozocin treated group (Fig. 5B) was minded when compared with the streptozocin group. Meanwhile, there is a significant decrease (P<0.05) in the 10 mg/kg atorvastatin-streptozocin treated group (Fig. 5C) when confronted with the streptozocin group. At the same time, significant decrease (P<0.05) in the 20 mg/kg atorvastatin-streptozocin treated group (Fig. 5D) was observed when compared with the streptozocin group. All the 5 mg/kg, 10 mg/kg and 20 mg/kg atorvastatin-streptozocin treated groups showed a non significant change (P>0.05) when compared with each other.

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Figure 1: Immunohistochemical staining for hippocampus expressions of GFAP marker in the (A) group administered 3 mg/kg streptozocin. (B) group treated with streptozocin and 5mg/kg atorvastatin. (C) group treated with streptozocin and 10mg/kg atorvastatin. (D) group treated with streptozocin and 20mg/kg atorvastatin. (Scale bar at left lower corner represents 15 μm (400 ×))

Figure 2: Box and whisker plot showing the effects of streptozocin alone and various doses of atorvastatin combined with streptozocin on the expression of glial fibrillary acidic protein marker

Figure 3: Immunohistochemical staining for hippocampus expressions of GR marker in the (A) group administered 3 mg/kg streptozocin. (B) group treated with streptozocin and 5mg/kg atorvastatin. (C) group treated with streptozocin and 10mg/kg atorvastatin. (D) group treated with streptozocin and 20mg/kg atorvastatin. (Scale bar at left lower corner represents 15 μm (400 ×))

Figure 4: Box and whisker plot showing the effects of streptozocin alone and various doses of atorvastatin combined with streptozocin on the expression of glutathione reductase marker

Figure 6 indicates the results given above on neuronal Nitric Oxide Synthase.
Figure 5: Immunohistochemical staining for hippocampus expressions of nNOS marker in the (A) group administered 3 mg/kg streptozocin. (B) group treated with streptozocin and 5mg/kg atorvastatin. (C) group treated with streptozocin and 10mg/kg atorvastatin. (D) group treated with streptozocin and 20mg/kg atorvastatin. (Scale bar at left lower corner represents 15 μm (400 ×)

4 Discussion

After cellular injury, the GFAP is synthesized plentifully, thus, it represents itself more conspicuously within the reactive gliosis process. Generalized astrogliosis, demonstrated by cellular hypertrophy and by an increase in expression of GFAP and astroglial S100B protein, was routinely observed in postmortem tissues from AD patients. Importantly, activated astrocytes are capable of accumulating large amounts of Aβ; the latter being taken up by astrocytes in association with neuronal debris. Beside, reactive astrocytes seem to accumulate large amounts of neuronal subtype of nicotinic cholinoreceptor (α-7nAChRs), which is known to have an exceptionally high affinity to β-amyloid. Processes of activated astrocytes were also reported to be a part in plaques formation. The present results show that there is a significant increase in the expression of GFAP in the group of rats administered 3 mg/kg of streptozocin intrathecally and this come in agreement with previous study. Although there are few studies regarding GFAP hippocampal alterations in STZ-treated rodents, Shoham S. et al. 2007 investigated that STZ increased the number of activated astrocytes in the hippocampus. Thus increasing in GFAP expression was seen after ICV STZ. In this study, the significant reduction in the GFAP expression was occurred in the rats administered 20 mg/kg atorvastatin orally after brain damage from 3 mg/kg intrathecal injection of streptozocin and this in agreement with the previous study indicated that the treatment with atorvastatin 30 mg/kg reduced GFAP staining density confront with the amyloid precursor protein transgenic mice group. The mechanism that the streptozocin causes an upregulation of GFAP may be related to the astrocytes. Astrocytes in the brain of normal persons usually do not express iNOS, but after brain damage, activation of NF-Kappa-B lead to increase expression of many inflammatory molecules in astrocytes [the expression of iNOS and proinflammatory cytokines (TNF-α, IL-1β, and IL-6)]. The expression of iNOS in the astrocytes causes production of excessive amount of NO which in turn led to upregulation of GFAP expression in reactive astrocytes by using the guanylate cyclase (GC)–cGMP-activated protein kinase (PK-G) signaling pathway to induce this expression. Statins exert anti-inflammatory effects by reducing the production of proinflammatory molecules such as tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) in astrocytes and that cause a decrease in GFAP expression indicating reduce in astrocytes activation that in turn cause a reduction in neuroinflammation response. This study shows that the effect of atorvastatin on expression of GFAP is happened on a high dose (20 mg/kg) that means the effect is dose dependent.
Under physiological conditions, a balance between pro-oxidant and anti-oxidant stimuli cell is regulated; while, certain stressors, damage, or diseases may affect this equilibrium and increase production of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which in turn may react with endogenous molecules including proteins, lipids, carbohydrates, DNA and RNA leading to cellular dysfunction resulting from their oxidative damage. Several lines of evidence have shown that the brains of individuals with Alzheimer’s disease demonstrated elevation in the oxidative stress levels.

This study indicates that the expression of glutathione reductase is significantly decreased in the group taken 3 mg/kg streptozocin intrathecally confronted with control group. These findings are nearly similar to the results of previous studies.

Ishrat T. et al. (2009) observed a significant decrease in reduced glutathione (GSH) and antioxidant enzymes (glutathione peroxidase GPx and glutathione reductase GR) in the hippocampus and cerebral cortex of ICV-STZ treated rats. The results of this study were in agreement with the study conducted by Javed H. et al. (2013) indicated that the activity of antioxidant enzymes (glutathione peroxidase, glutathione reductase, glutathione-S-transferase, catalase, and superoxide dismutase) was decreased in rats treated with 3 mg/kg STZ intracerebroventricular as compared with control group. Tota S. et al. (2011) also showed that streptozocin caused oxidative stress as evidenced by significant decrease in GSH level. Other studies reported that a streptozocin administration showed a significant reduced in the brain GSH level, indicating neuronal damage due to oxidative stress. The mechanisms by which STZ induces oxidative stress in the brain are not fully understood. It has been reported that treatment with ICV STZ shows reduced glucose consumption leading to hyperglycemia-like condition in brain. This may attribute to increase in non-enzymatic glycosylation of proteins and glucose auto-oxidation leading to oxidative stress and cellular damage by generation of free radicals. This free radical generation leads to decreased antioxidant enzymes like GSH which is indicated by a reduction in GR level. Also, ROS can be produced by microglia, which are activated by ICV STZ injection. This study shows that ICV STZ injection in rats can cause the progressive deterioration of brain functions due to oxidative stress, so, it has been postulated that it may provide a relevant model of sporadic AD.

In the present study, the expression of GR in the hippocampus significantly increases when the doses of 5 mg/kg, 10 mg/kg and 20 mg/kg atorvastatin orally are given after injection of 3 mg/kg streptozocin intrathecally compared with the group administered 3mg/kg intrathecal streptozocin only. These findings were in accordance with the results of previous studies that showed atorvastatin had potential antioxidant effect. Tramontina A. C. et al. (2011) reported that ICV-STZ injection reduced the total content of GSH in hippocampal slices and both simvastatin and pravastatin were capable of reversing this condition by preventing the effect of STZ on glutathione content. The possible explanation for upregulation of GSH system in atorvastatin treatment may be related to stimulation of the transcription factor Nrf2, Nuclear Factor (erythroid-derived 2)-like 2, by statins in several experimental systems, including cultured neurons. The Nrf2 mediated the expression of γ-glutamylcysteine synthetase, a key enzyme in GSH synthesis, which leads to increase its synthesis. The results of this study showed that atorvastatin increase the expression of GR after intrathecal STZ injection in a dose-dependent manner. As the doses increased from 5 to 10 to 20 mg/kg atorvastatin, the expression of GR in the hippocampus increased.

Nitric oxide molecule (NO) 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 functions as a signaling molecule in the vascular system. It is reported that NO molecules induce a vasodilation effect by improving blood flow locally. Immediately after an ischemic insult or brain damage, eNOS is activated and exerts a protective vasodilation effects that enhance blood flow. Meanwhile, ischemic insults cause activation of nNOS and iNOS, these enzymes turn lead to overproduction of (NO), which lead to oxidative damage. The findings of the present study show that a significant increase in the expression of nNOS in the hippocampus of rats administered 3 mg/kg streptozocin is agreed with previous study that found there was an increased in NO levels in the hippocampus of rats submitted to ICV- STZ injection. Rai S. et al. (2013) found that STZ (ICV) increased the nNOS mRNA and protein expression in hippocampus and cortex of adult male rats. Thus, increased free radical generation, nNOS and iNOS gene expression leading to formation of proinflammatory cytokines, by activation of microglia and astrocyte, which is an important pathophysiological component of Alzheimer’s disease. The possible mechanism explained this increase in nNOS may be related to the fact that STZ caused an increase in Ca$^{2+}$ level in cortex and hippocampus that mediate the binding of calmodulin to the nNOS, which in turn increase the activity of this enzyme that lead to energy depletion. In regard to atorvastatin, the present study findings are in accordance with the result of Moro M.A. et al. (2004) in which they documented that treatment with atorvastatin may lead to decrease overproduction of NO by nNOS and iNOS, after the onset of the ischaemic brain injury, through down-regulating both enzymes.

5 Conclusions

According to the results obtained from this study, one can conclude that administration of streptozocin intrathecally may yield a model of...
Alzheimer’s disease indicated by brain damage which in turn improved by increased doses of atorvastatin treatment.

6 Competing interests
No conflicts of interest are evident.

7 Authors’ contributions
MA and MI designed overall experiments. AA and SIS wrote the manuscript. AA carried out the in vivo experiments. MA and MI participated in discussion and manuscript writing. All authors read and approved the final manuscript.

8 References

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