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NEUROPROTECTIVE EFFECTS OF ATORVASTATIN THROUGH ANTI-OXIDANT, ANTI-INFLAMMATORY AND ANTI-APOPTOTIC MECHANISMS AGAINST TRANSIENT ISCHEMIC/REPERFUSION INJURY IN DIABETIC RATS

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ABSTRACT

Stroke and diabetes mellitus are two separate conditions which share multiple common threads. Atorvastatin plays an important role in the maintenance of hyperlipidemia especially in diabetic patients. Debate continues about the best strategies for management of diabetic stroke. Thus, interest was raised to investigate the neuroprotective effects of atorvastatin against transient ischemia/reperfusion (I/R) injury in diabetic rats targeting mainly the oxidative-inflammatory-apoptotic cascade which has been previously addressed as co-conspirators in this insult. Forebrain ischemia was induced in streptozotocin-diabetic rats by bicommon carotid occlusion for 15 min followed by 1h reperfusion. Atorvastatin (15 mg/kg; p.o) was administered daily for 2 weeks prior to I/R injury. The drug alleviated hippocampal injury inflicted by diabetes and/or I/R injury where it suppressed nuclear factor kappa NF-κB, and consequently the downstream inflammatory cytokines tumor necrosis factor-α and interleukin-6. In parallel, the anti-inflammatory cytokine interleukin-10 was elevated. Antioxidant potential of atorvastatin was depicted, where it reduced neutrophil infiltration, lipid peroxides, nitric oxide associated with replenished reduced glutathione. Decline of excitatory amino acid glutamate content is a main finding which is probably mediated by the NF-κB signaling pathway as well as improved oxidant status. Atorvastatin exerted an anti-apoptotic effect as reflected by the reduction of the cytosolic cytochrome c and the key downstream executioner caspase-3. In conclusion, atorvastatin is gifted with neuroprotective properties which are probably mediated by its antioxidant, anti-inflammatory, and anti-apoptotic mechanisms hence may provide a successful agent for the management of ischemic stroke.

INTRODUCTION

Diabetes mellitus (DM) is a widespread and severe metabolic disorder of multiple etiologies. It is extensively also a potent precipitator of ischemic cerebrovascular injuries and is considered one of the global etiological risk factors of stroke. Several studies showed a higher prevalence, recurrence rate and worse prognosis of stroke in diabetic subjects (Licata *et al*., 2002). DM, being a major cause of accelerated atherosclerosis (Bassi *et al*., 2012), may result in increased incidence of stroke (Araki *et al*., 2012). Hyperglycemia clearly plays a vital role in the development and progression of stroke as it activates secondary glucose metabolic pathways, such as the polyol and hexosamine pathways; protein kinase C isoforms and advanced glycation end products. Inevitably, diabetic patients are at least two folds more liable to have a stroke as compared to non-diabetics. Besides, they reveal poor functional outcomes and prognoses, and exhibit high rates of morbidity and mortality after stroke (Biller and Love, 1993). Oxidative stress, inflammation, and extensive programmed cell death are familiar culprits in the pathophysiology of both diabetes and ischemic brain injuries (Barone and Feuerstein, 1999; Aragno *et al*., 2000; Tsuruta *et al*., 2010). Therefore, modulation of these detrimental pathways may offer promising opportunities for the management of ischemic injuries in diabetics. In particular, in early stages cerebral ischemia activates the synthesis and release of inflammatory mediators, such as interleukin IL-1β, IL-6 and TNF-α, which promote the transformation of quiescent microglia into reactive microglia and the migration of granulocytes and macrophages into the ischemic brain parenchyma. Inflammation further precipitates

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the neuronal death after stroke (Dirnagl *et al*., 1999). Transcription factors like nuclear factor kappa B (NF-κB) are known to be induced during the acute phase after ischemia and promote postischemic inflammation, generation of reactive oxygen species (ROS), and neuronal damage (Stephenson *et al*., 2000). Like all statins, atorvastatin works by inhibiting hydroxymethylglutryl (HMG)-CoA reductase, an enzyme found in liver tissue that plays a key role in production of cholesterol in the body (Roth, 2002). Atorvastatin is primarily beneficial in dyslipidemia as well as the primary and secondary prevention of coronary heart disease. Indeed, statins have been shown to have pleiotropic effects including improving endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, and inhibiting the thrombogenic response, extrahepatic effects on the immune system, CNS, and bone.

In particular, inhibition of small GTP-binding proteins may play an important role in mediating the pleiotropic effects of statins (Liao and Laufs, 2005;Furie, 2012). A series of *in vitro* and *in vivo* experiments have confirmed that statins have the ability to suppress inflammation, which is independent of their potent function on blood lipid reduction (Jain and Ridker, 2005). Atorvastatin possesses antioxidant potential by which it maintains NO bioavailability by preventing its degradation by free radical molecules to peroxynitrite radicals thus it might protect diabetic vasculature against diabetes culprits (Wang *et al*., 2014). Based on the above considerations, this work was undertaken to investigate the cerebroprotective effects of atorvastatin against transient I/R injury in diabetic rats as well as the possible attenuation of inflammatory, oxidative, apoptotic, and excitotoxicity cascades involved in the generation of this effect.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ), atorvastatin and thiopental sodium were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity and analytical grade.

Animals

Male Wistar albino rats weighing 200-250 g were used in the present study. They were allowed an acclimatization period for at least one week prior to testing. Animals were kept under controlled environmental conditions; room temperature (24-27°C), constant humidity (60 ± 10%), with alternating 12 h light and dark cycles. Standard pellet diet and water were allowed ad libitum. All animals' procedures were performed in accordance with the ethical procedures and policies approved by the Ethics Committee of Faculty of Pharmacy, Cairo University and complies with the *Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).*

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of STZ (40 mg/kg), freshly prepared in 0.1 M citrate buffer, pH 4.5 (Muranyi *et al*., 2006). Rats were allowed to drink 5% glucose solution during the first 24 h of diabetes induction to overcome the drug-induced hypoglycemia (Matthaei *et al*., 1986). Two days later, blood samples were collected from rats' tails and hyperglycemia was confirmed by a blood glucose level reaching 300 mg/dl. Glucose was measured using an analyzer (Roche Diagnostic Accu-Check test strips, Germany).

Induction of transient cerebral ischemia

Rats were anaesthetized with thiopental (50 mg/kg, i.p.) and a midline ventral incision was made in the neck. Bilateral carotid artery occlusion using small artery clips was used to induce global cerebral ischemia for 15 min followed by 60 min reperfusion period (Aragno *et al*., 2000).

Experimental design

One hundred rats were randomly allocated into 5 groups (20 rats each). Group I and II are normal healthy rats received single intraperitoneal injection of citrate buffer and were kept for six weeks period. Group I served as normal sham operated control without induction of I/R, while in group II rats received 1% Tween 80 (10 ml/kg; p.o.) daily during the 5th and 6th week after which transient I/R was induced and served as I/R control group.

Groups III, IV and V are diabetic rats received single intraperitoneal injection of STZ dissolved in citrate buffer and were kept for six weeks period. Group III served as diabetic sham operated group without induction of I/R; group IV was subjected to I/R by the end of the 6th weeks period and served as diabetic I/R control group, the last group (Group V) received atorvastatin (15 mg/kg; p.o) daily (Elewa *et al*., 2009) dissolved in 1% Tween 80 during the 5th and 6th week after which transient I/R was induced and served as diabetic I/R atorvastatin treated group.

Immediately after reperfusion, animals in each were sacrificed by cervical dislocation and the both hippocampi of each rat were isolated. Each group was further subdivided into three sets. Those of the first set (n=7) were used for ELISA estimations; the second one (n=6) was employed for assessment of glutamate content by HPLC; the last set (n=7) served to determine the rest of biochemical parameters.

Biochemical determinations in hippocampus

Estimation of oxidative stress biomarkers

Lipid peroxides formation was determined in rat hippocampus homogenate (10% w/v normal saline) by estimating the content of thiobarbituric acid reactive substances (TBARS) using malondialdehyde (MDA) as a standard according to the method described by Mihara and Uchiyama (1978). Reduced glutathione (GSH) content was measured using Ellman's reagent as described by Beulter *et al*. (1963). Total nitrite and nitrate content was assessed according to the method of Miranda *et al*. (2001) based on the Griess reaction and employing vanadium trichloride as reducing agent.

Estimation of inflammatory and apoptotic mediators

The kinetic method described by Bradley *et al*. (1982) was employed to determine myeloperoxidase (MPO) activity.

Since the enzyme is located within the primary granules of neutrophils, its extraction necessitates the disruption of the granules to render MPO soluble in aqueous solution. This was achieved by sonication in potassium phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyl- trimethyl ammonium bromide (HTAB), a detergent that releases MPO from the primary granules of the neutrophil (Schultz and Kaminker, 1962). Hippocampal contents of cleaved caspase-3, interleukin-6 (IL-6), interleukin-10 (IL-10), as well as tumor necrosis factor alpha (TNF-α) were assessed using enzyme-linked immunosorbent assay (ELISA) kits supplied by R&D Systems, Inc., Minneapolis, USA. Likewise, the contents of NF-κB and cytosolic cytochrome-C were measured using ELISA kits supplied by EIAab Science Co., Wuhan, China.

Estimation of the excitatory amino acid glutamate

Glutamate content was estimated using a fully automated high-pressure liquid chromatography system (HPLC; Perkin-Elmer, MA, USA) according to the precolumn phenylisothiocyanate derivatization technique described by Heinrikson and Meredith (1984). Brain residues were reconstituted in 2:2:1 mixture (v) of methanol: 1 M sodium acetate trihydrate: triethylamine then re-dried under vacuum. The reaction of derivatization was performed for 20 min at room temperature using a 7:1:1:1 mixture (v) of methanol: triethylamine: double-distilled deionized water: phenylisothiocyanate, then subjected again to vacuum until dryness. Derivatized amino acids were reconstituted with sample diluent consisting of 5:95 mixture (v) of acetonitrile: 5 mM phosphate buffer (pH=7.2). After sonication, samples were filtered (0.45 µm; Millipore). A Pico-Tag physiological free amino acid analysis C18 (300 mm×3.9mm i.d.) column from Waters (MA, USA) and a binary gradient of Eluents 1 and 2 (Waters) were used. The column temperature was set at 46±1 °C and a constant flow rate of 1 ml/min was maintained throughout the experiment. Samples were injected in volumes of 20 µl, and the absorbance of the derivatized amino acids was measured at 254 nm. Glutamate standards were prepared in double-distilled deionized water.

Statistical analysis

Data were expressed as means ± S.E.M. Comparisons between means were carried out using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test using Prism (Version 5). The minimal level of significance was identified at $P<0.05$.

RESULTS

Effects of atorvastatin on hippocampal downstream inflammatory mediators after I/R in diabetic rats

Table 1 depicts that diabetic rats subjected to I/R exhibited a marked elevation of NF-κB downstream effectors namely MPO (47%), TNF-α (42%), and IL-6 (2.4 folds) versus their non-diabetic counter partners. Pretreatment with atorvastatin for 2 weeks prior to induction of I/R totally averted the modification in MPO enzyme while hampered the other aforementioned inflammatory mediators by 37 % for NF-κB, 47% for TNF-α, and 80% for IL-6 as compared to the diabetic ischemic group. On the other hand, diabetic rats subjected to I/R exhibited a prominent reduction in IL-10 by 81% versus the non diabetic ones, an effect which was reversed by atorvastatin pretreatment.

Effects of atorvastatin on hippocampal apoptotic biomarkers after I/R in diabetic rats

Ischemia reperfusion caused an eminent elevation of cytosolic cytochrome c in both normal and diabetic rats (approximately 5 folds), atorvastatin pretreatment reverted these levels back to its normal level (Figure 1a). Diabetic rats subjected to I/R showed an increment of cleaved caspase-3 by 68% as compared to non diabetic animals. Pretreatment with atorvastatin for 2 weeks prior to induction of I/R halted such elevation by 53% as related to the diabetic ischemic group (Figure 1b).

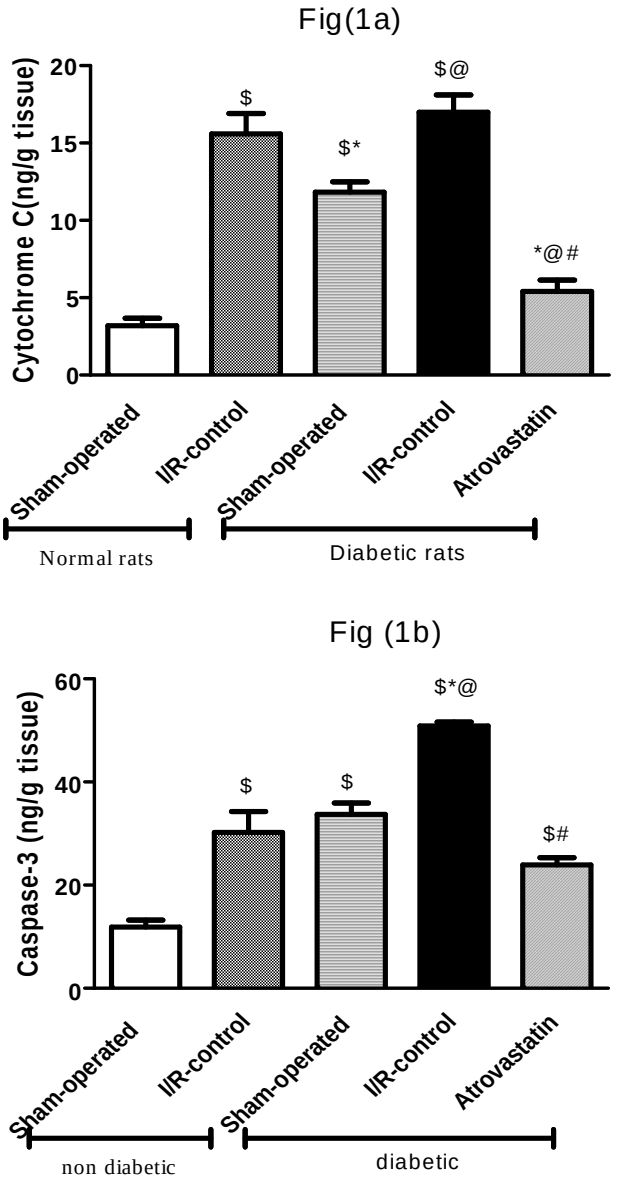

Figure 1. Effects of atorvastatin on hippocampal apoptotic biomarkers (cytochrome c, Fig. 1a; caspase 3, Fig. 1b) following ischemia reperfusion (I/R) injury in diabetic rats

Each bar with vertical line represents the mean ± S.E.M of 6-7 rats per group. $^{\$}$ *vs.* normal sham-operated, * *vs.* ischemic group, $^{@}$ *vs.* diabetic sham-operated, $^{\#}$ *vs.* diabetic ischemic (One-Way ANOVA followed by Tukey-Kramer multiple comparisons test; $p < 0.05$).

Table 1. Effects of atorvastatin on downstream pro-inflammatory and anti-inflammatory mediators in hippocampus following ischemia reperfusion (I/R) injury in diabetic rats

Parameters Groups	MPO (ng/g tissue))	TNF-α (pg/g tissue)	NF-κB (ng/g tissue)	IL-6 (pg/g tissue)	IL-10 (pg/g tissue)
Normal sham-operated	26.83 ± 4.24	3.01 ± 0.39	0.11 ± 0.01	27.11 ± 1.73	16.71± 2.34
Ischemia reperfusion	$40.20^{\$}$± 3.83	$14.85^{\$}$ ± 0.96	$0.35^{\$}$ ± 0.02	40.42 ± 5.37	$4.96^{\$}$ ± 1.22
Diabetic sham-operated	16.97^{*} ± 1.72	$11.74^{\$}$ ± 1.46	$0.27^{\$*}$ ± 0.01	51.69± 8.22	$6.23^{\$}$± 1.81
Diabetic ischemia	$59.17^{\$*@}$ ± 3.94	$21.15^{\$*@}$ ± 1.85	$0.38^{\$@}$ ± 0.02	$97.53^{\$*@}$ ± 11.38	$0.93^{\$}$ ± 0.46
Diabetic ischemia +Atorvastatin	$16.91^{*\#}$ ± 1.16	$11.23^{\$\#}$ ± 1.39	$0.24^{\$*\#}$ ± 0.01	$19.66^{@\#}$ ± 1.29	$8.44^{\$\#}$ ± 1.24

Values are expressed as mean ± SEM of 6-7 rats per group . $^{\$}$ *vs.* normal sham-operated, * *vs.* ischemic group, $^{@}$ *vs.* diabetic sham-operated, $^{\#}$ *vs.* diabetic ischemic (One-Way ANOVA followed by Tukey-Kramer multiple comparisons test; $p < 0.05$).

Table 2. Effects of atorvastatin on oxidative stress biomarkers in hippocampus of diabetic rats subjected to ischemia reperfusion (I/R) injury

Parameter Groups	GSH (µg/g tissue)	TBARS (nmol/g tissue)	NOx (µmol/g tissue)
Normal sham-operated	8.74 ± 0.81	0.51 ± 0.05	5.45 ± 0.39
Ischemia reperfusion	$2.51^{\$}$ ± 0.34	1.07 ± 0.07	$10.89^{\$}$ ± 0.66
Diabetic sham-operated	$5.06^{\$*}$ ± 0.37	1.32 ± 0.17	8.65 ± 1.18
Diabetic ischemia	$0.43^{\$*@}$ ± 0.15	$3.66^{\$*@}$ ± 0.32	$18.45^{\$*@}$ ± 2.01
Diabetic ischemia + Atorvastatin	$4.52^{\$*\#}$ ± 0.49	$1.25^{\#}$ ± 0.17	$9.81^{\$\#}$ ± 0.71

Values are expressed as mean ± S.E.M of 6-7 rats per group. $^{\$}$ *vs.* normal sham-operated, * *vs.* ischemic group, $^{@}$ *vs.* diabetic sham-operated, $^{\#}$ *vs.* diabetic ischemic (One-Way ANOVA followed by Turkey-Kramer multiple comparisons test; $p < 0.05$).

Effects of atorvastatin on hippocampal oxidant status after I/R in diabetic rats

A dramatic depletion of GSH, the key non enzymatic tissue antioxidant, was observed in diabetic ischemic rats as compared to non diabetic ischemic ones (83%). This metabolite was replenished by atorvastatin administration by 10 folds as compared to the untreated diabetic ischemic group. In parallel, a profound spike in hippocampal TBARS content (3.4 folds), a hallmark of lipid peroxidation, occurred in diabetic ischemic group as compared to non diabetic ischemic group. This was mitigated by atorvastatin (65%). Likewise, diabetic ischemic animals demonstrated a pronounced elevation in NOx content which reached 69% of the values of non diabetic ones subjected to the same insult. Atorvastatin administration for 2 weeks prior induction of ischemia reinstated normal NOx content in comparison with normal sham-operated animals (Table 2).

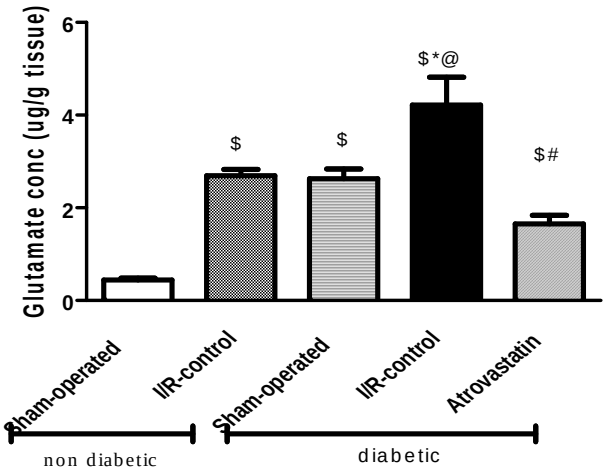

Figure 2. Effects of atorvastatin on hippocampal excitatory amino acid glutamate following ischemia reperfusion (I/R) injury in diabetic rats

Each bar with vertical line represents the mean ± S.E.M of 6-7 rats per group. $^{\$}$ *vs.* normal sham-operated, * *vs.* ischemic group, $^{@}$ *vs.* diabetic sham-operated, $^{\#}$ *vs.* diabetic ischemic (One-Way ANOVA followed by Tukey-Kramer multiple comparisons test; $p < 0.05$).

Effects of atorvastatin on hippocampal glutamate content after I/R in diabetic rats

A 1.5 folds increase in the content of glutamate was demonstrated in diabetic ischemic rats as compared to non diabetic ischemic group. The transmitter content was declined by 61% in the atorvastatin treated group as compared to the diabetic ischemic rats (Figure 2).

DISCUSSION

The present study was designed to determine whether atorvastatin could attenuate the cerebral ischemic injury due to transient forebrain ischemia in diabetic rats when administered prophylactically. To the best of our knowledge, this is the first study about the neuroprotective activity of atorvastatin against transient I/R injury in diabetic rats. NF-κB is well known as a stress-regulated transcription factor that has a crucial role in the control of inflammatory events. Several studies have confirmed that NF-κB is activated during development of brain injury (Nichols, 2004). Moreover, NF-κB enhances the expression of downstream cytokines as TNF-α and IL-6. in the current study, The results showed that I/R injury in diabetic rats causes elevation in NF-κB which when activated induces the expression of inflammatory cytokines including TNF-α and IL-6 which are produced and contribute to the neuronal damage (Luan *et al*., 2013; Stephenson *et al*., 2000).

However, atorvastatin treated rats exhibited marked decline in hippocampal NF-κB and pro-inflammatory cytokines including TNF-α and IL-6. In line with these results, several studies demonstrated the anti-inflammatory properties of atorvastatin via suppression of NF-κB and its downstream mediators such as TNF-α and IL-6 (Tu *et al*., 2014; Zhang *et al*., 2013; Saito *et al*., 2014). In the current study, the hippocampal content of the level of MPO, an index of neutrophil infiltration, and the anti-inflammatory cytokine IL-10 were estimated by ELISA kits. A significant increase in the levels of MPO and a significant decrease in the levels of IL-10 were induced by I/R in diabetic rats on non-diabetic counter partners. Pretreatment of diabetic rats with atorvastatin

for 2 weeks prior to I/R resulted in a significant reduction in the levels of MPO and a significant increase in the levels of IL-10 which extensively confirm the anti-inflammatory activity of atorvastatin. These results are consistent with those of other studies (Sakurai *et al*., 2011). MDA is an end product of lipid oxidation, the content of MDA directly reflects the free radical levels and is important indicator of oxidative neuronal injury (Boccuzzi *et al*., 1997). Additionally, reduced GSH is one of the chief endogenous antioxidant defense systems in the brain, which removes hydrogen peroxide and lipid peroxides. Depletion in GSH levels could either increase or reflect oxidative stress (Bains and Shaw, 1997). The results showed a significant increase in MDA levels and a significant decrease in reduced GSH levels in either non-diabetic or diabetic rats subjected to I/R injury when compared to their sham counter partners. There are several possible explanations for these results such as destruction of GSH to cysteine, shutting down the synthesis of GSH and consequently depleting the cellular stores of GSH (Bains and Shaw, 1997). Additionally, neuronal membrane is highly vulnerable to lipid peroxidation (Danbolt, 2001) that inhibits the function of membrane-bound receptors and enzymes (Coyle and Puttfarcken, 1993).

Here in, we demonstrated that diabetic animals show a higher level of oxidative stress and inflammation when compared with non-diabetic animals subjected to I/R injury (Collino *et al*., 2009). Nitric oxide (NOx) participates also in the pathogenesis of neuronal damage where the interaction of superoxide with NO results in the generation of peroxynitrite, one of the most detrimental ROS species, which causes neuronal tissue harm via lipid peroxidation, protein oxidation, nitration, and DNA breakage (Kidd *et al*., 2005;Virag *et al*., 2003). These results confirm that atorvastatin strengthened the oxidative defense mechanisms and reduced lipid peroxidation (Tu *et al*., 2014; Saito *et al*., 2014). Thus, these findings suggest that the cerebroprotective of atorvastatin may be attributed to inhibiting action of NADPH oxidase which is a predominant source of superoxide generation in the vasculature (Walder *et al*., 1997; Kusaka *et al*., 2004) diminished the cerebral expression of iNOS (Ouk *et al*., 2014) and its cellular anti-oxidative properties (Wassmann *et al*., 2002). Glutamate is an important excitatory neurotransmitter and has a crucial role in ischemic neuronal damage. Excess levels of glutamate result in ischemic neuronal death by increasing calcium influx via stimulation of NMDA receptor.

Astrocytic glutamate transporters remove synaptically released glutamate to maintain a low level of extra-neuronal glutamate (Danbolt, 2001). Disruption of glutamate transporters activity and expression can lead to excitotoxicity and is implicated in pathogenesis of cerebral injury. In global and focal ischemia, astrocytes established neuroprotection by maintaining the activity of glutamate transporters (Chen *et al*., 2005). In the present work, pretreatment with atorvastatin resulted in a significant decline in glutamate level. This effect seems to be related to its inhibitory effect on NF-κB signaling pathway. Apparently, NF-κB activation down regulates astrocytic glutamate transporters and suppresses glutamate uptake thereby contributes to ischemia via glutamate-induced excitotoxicity (Guan *et al*., 2011). Moreover, the excessive free radical production in diabetic ischemic animals can inhibit the conversion of glutamate to glutamine by glutamine synthetase and trigger glutamate synthesis as previously clarified by Oliver *et al*. (1990). Recent studies have reported that atorvastatin exerts specific anti-excitotoxic effect by reducing cortical neurons susceptibility to NMDA excitotoxicity (Bosel *et al*., 2005; Zacco *et al*., 2003). Moreover, a considerable amount of literature has been published on atorvastatin, these studies confirm the protective effect of atorvastatin on hippocampal neurons via reducing glutamate-mediated excitotoxicity (Posada-Duque *et al*., 2013; Lee *et al*., 2008).

In this investigation, the anti-apoptotic effects of atorvastatin were estimated by measuring the levels of cytochrome-c and cleaved caspase-3. These molecules are the known to be key players in the process of apoptosis (Zhang and Bhavnani, 2005). Following cerebral I/R injury in diabetic rats, a significant elevation of cytochrome-c and caspase-3 was observed (Ding *et al*., 2004; Muranyi *et al*., 2003). In agreement with our findings, several previous studies demonstrated the anti-apoptotic activity of statins through reduction of caspase-3 and cytosolic cytochrome-3 (Fonseca *et al*., 2009; Tanaka *et al*., 2004). In conclusion, the current study provides the first preclinical data that demonstrates the robust neuroprotective potential of atorvastatin against neuronal injury in diabetic rats submitted to transient forebrain I/R injury via inhibition of NF-κB signaling to enhance glutamate clearance and to lend support for the antioxidant, anti-inflammatory, and anti-apoptotic effects. Our results provide incentive to expand its use in diabetics.

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