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Association of Long-Term Atorvastatin with Escalated Stroke-Induced Neuroinflammation in Rats

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Abstract Statins are widely used in high-risk patients to reduce the stroke incidence. However, little has been investigated about the impact of chronic pretreatment with statins on cerebral ischemic insult following defined arterial occlusion. To address this in experimental rats, in the present work, atorvastatin was orally dosed for 1 month to evaluate the outcomes of the subsequent occlusive stroke induced by middle cerebral artery occlusion (MCAO). Our data was suggestive of potential escalating impact of chronic atorvastatin (Atv; 10 mg/kg) on neurological function, but not infarct volume. According to our immunoblotting data, such escalations were consistent with the prominent rise in TNF-α and IL-6 which paralleled with augmented Bax/Bcl2 ratio and Caspase-9 activation; however, these were not enough to worsen acute neurodegeneration determined by Fluoro Jade B staining. Noteworthy, such deteriorating effects were also partly detected in non-ischemic animals. Conclusively, our data are indicative of cerebral proinflammatory effects of chronic Atv which might overwhelm the beneficial pliotropic of the drug and predispose animals' brain to ischemic insult. Further studies on different statins with discrete pharmacokinetic properties are highly suggested to precisely explore stroke outcomes following long term prophylactic treatment particularly in primates.

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Introduction

Statin therapy for protection against cerebral ischemia has been long admitted to medical practice as is supported by several meta-analyses on clinical (Reeves et al. 2008; Chróinín et al. 2013) and preclinical (Baryan et al. 2012; García-Bonilla et al. 2012) data. These strong evidences, indicative of up to 40–50 % reduction in stroke incidence (Amarenco et al. 2004; Unit 2005; Everett et al. 2010), have also brought statins to standard prophylaxis protocols in evidence-based databases (Hennekens 2015), even in normolipidemic non-stroke people conjunctive to antihypertensive and antithrombotic agents (Mozaffarian et al. 2016).

While stroke "incidence" as the probability of stroke development is feasibly measured in either clinical settings or preclinical testing in high-risk animal models like spontaneously hypertensive rats (Nagotani et al. 2005; Tanaka et al. 2007), stroke "outcomes" comparisons should be rigorously determined based on same arterial blockage in terms of size and duration which hardly could be addressed feasibly in clinical settings. In this regard, several empirical examined statins pretreatment in stroke animal models providing least variations in stroke occurrence and severity and suggested statins could also improve stroke outcomes at molecular and histological levels mainly through the so-called "pliotropic" effects eliciting vasoactive compounds like NO (Asahi et al. 2005; Ye et al. 2008), antioxidant effectors (Hong et al. 2006; Makabe et al. 2010), and anti-inflammatory signaling (Gueler et al. 2007), independent of their lipid-lowering action (Li et al. 2014).

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Many of the statins' protective effectors in laboratory animals depend on HMG-CoA reductase inhibition which blocks the mevalonate cycle, and thus, isoprenoids and cholesterol production. Indeed, the two intermediate isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GPP) are essential elements for prenylation process, crucial for post-translational modifications of small GTPases (Wright and Philips 2006), enabling proteins for proper subcellular localization and trafficking (McTaggart 2006; van der Burgh et al. 2013). Importantly, given the very versatile potential effectors lie downstream to isoprenoids, statins could have legitimately widespread impacts not just encompassing protective ones. The fact would be clearly exemplified considering FPP is also the precursor for Coenzyme Q10 and possess antioxidant activities with substantial impact in defense against reperfusion injury (Jackson et al. 1997; Littarru and Langsjoen 2007). Therefore, it could be assumed the statins' net effects may remarkably vary depending duration of pretreatment as well as the existing physiological context.

Noteworthy, to our knowledge, the examinations of statin in animal models of stroke, as the unique precise tool to evaluate stroke outcomes, generally have not exceeded 2 weeks long. More importantly, there are empirical evidences indicating particular statins namely hydrophobic ones exert substantial cholesterol-lowering potential in the brain in which chronic administration may lead to meaningful pro-apoptotic and neurotoxic effects (März et al. 2007; Biondi 2011). Therefore, coupled with the controversial reports about statins safety as neuroprotective agents in stroke (Cappellari et al. 2011; Scheitz et al. 2014), in the present work, we sought to find whether chronic administration of atorvastatin as the most long half-lived brain permeable statin (Shitara and Sugiyama 2006), and above all, the most frequently used one (Golomb et al. 2004) may ameliorate cerebral insult following experimental stroke, particularly in the view of fine inflammatory and oxidative state of the brain.

Materials and Methods

Animals and Drug Administration

All experimental procedures were approved by the board of research ethics at the Neuroscience Research Center of Shahid Beheshti Medical University in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). Male Wistar rats (250–300 g) were housed at 25 ± 2 °C on a 12 h/12 h light/dark cycle with free access to food and water. Atorvastatin (Atv; Daroopakhsh Pharmaceutical Inc., Tehran-Iran) dosage was chosen according the lowest neuroprotective dosage identified elsewhere (García-Bonilla et al. 2012) and animals were randomly assigned to experimental groups receiving oral Atv (5 or 10 mg/kg/day; 30 days) prior to being subjected to 60 min MCAO. Functional assessments were conducted 24 h after reperfusion and animals, except those designated for Fluoro Jade B staining, were then euthanized with CO2 to harvest the brains. The corresponding parietal cortices and subcortical tissues (striatum) was separated and flash frozen in 80 °C to time of biochemical analysis.

Middle Cerebral Artery Occlusion Surgery

To induce experimental stroke, rats were anesthetized with chloral hydrate (10 % *v/v*, 0.36 ml/kg i.p.; Sigma, Germany) and the right middle cerebral artery (MCA) was occluded by the intraluminal suture technique as described by Longa with some modifications (Longa et al. 1989). Briefly, a 4-0 silicon-coated suture was introduced from the carotid bifurcation into the internal carotid artery and advanced until mild resistance was felt. Reperfusion was established by gently withdrawing the filament after 60 min of occlusion. Due to the common inter species variations in cerebral microvessels' anatomy (Ward et al. 1990), efficient occlusion was verified in model animals by Laser Doppler Flowmeter (Moor Instrument, England) showing cerebral blood flow (CBF) drop at least 80 % below the baseline in the MCA territory on parietal cortex. In the sham animals named as control group, all steps were included except the occlusion of the middle cerebral artery.

Neurological Deficit Assessment

Neurological deficits were evaluated 24 h after MCAO by a person blind to experimental groups using a five-point scoring system as described previously (Vakili et al. 2005). The scoring was as follows: 0 = normal motor function, 1 = flexion of contralateral torso or forelimb upon lifting by tail or failure to extend forepaw when suspended vertically, 2 = circling to the contralateral side but have normal posture at rest, 3 = loss of righting reflex, 4 = no spontaneous motor activity, and 5 = death, provided that postmortem brain sampling is indicative of large cerebral infarcts without intracranial hemorrhage.

Infarct Volume Measurement

To calculate the infarct volumes, the freshly harvested and PBS washed brains were sectioned into seven 2-mm-thick coronal slices using a brain matrix following 10 min dipping in ice cold saline. The slices were then immersed in Triphenyl-Tetrazolium Chloride 2 % solution (TTC; Sigma, Germany) at 37 °C for 10 min and photographed to get manually quantified using an Image Analyzer Software (Image J Analyzer).

Ischemic hemisphere edema was simply calculated by the hemispheres' volume subtraction and normalized to the corresponding differences in non-ischemic brains. The infarct volumes were expressed as *corrected infarct volume*, a percentage of the contralateral structure, to compensate for the effect of brain edema (Swanson et al. 1990).

Sample Preparation and Biochemical Analysis

To prepare total protein extractions, the cortical and subcortical samples were homogenized in protein extraction buffer containing protease inhibitor cocktail and centrifuged at 3000 rpm at 4 °C. Supernatants' protein contents were standardized with the Bradford's method.

Superoxide Dismutase Activity Assay

Superoxide dismutase (SOD) activity was performed based on the method of Kakkar et al. (1984). SOD present in mitochondria fractions were subjected to reaction with nicotinamide adenine dinucleotide (NADH) in assay mixture which was stopped by adding glacial acetic acid. The measured absorbance at 560 nm was considered as the color intensity of amino blue tetrazolium formazan (ABTF), the product of total nitroblue tetrazolium reduction by SOD.

Catalase Activity Assay

Catalase (CAT) activity was determined according to the method described by Aebi (1984). Briefly, H2O2 (0.01 M) was added to 60 μg of the isolated mitochondrial fractions. The rate of H2O2 breakdown was measured by the mixture's absorbance at 240 nm and considered as the CAT activity.

GSH Level Analysis

Total GSH levels were measured in mitochondrial fraction as described previously (Ellman 1959). The rate of colorimetric change of 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent) by reduced glutathione was determined in phosphate buffer by the spectrophotometric method determining absorbance at 412 nm and considered as GSH level (U/mg).

Lipid Peroxidation Evaluation

The extent of lipid peroxidation in CNS cells were estimated by measuring Malondialdehyde (MDA) production using double-heating method (Draper and Hadley 1990). Accordingly, cerebral homogenates were boiled in tricholoroacetic acid (TCA, 10 % *w*/*v*) solution to extract MDA and the aqueous part isolated at room temperature were subjected to boiling and react with thiobarbituric acid (TBA) to produce MDA/TBA adducts with purple color which as an index for MDA presence was detected spectrophotometrically.

DNA Peroxidation Evaluation

The amount of 8-hydroxy-2′-deoxyguanosine (8OHdG) as one of the major products of DNA oxidation was determined by the appropriate commercial ELISA kit (Abcam; Japan) to estimate DNA damage following stroke or Atv administration. DNA was first extracted using the YTA Genomic DNA Extraction Mini Kit for Tissue (Yekta tajhiz, Iran). Enzymatic digestions of DNA and further 8OHdG measurements were performed according to the kit manufacturer's instructions.

Western Blotting

Equal amounts of total proteins (60 μg) were loaded for each sample on SDS page and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). Subsequently, membranes were blocked by non-fat dry milk-TBST solution (2 %) and probed with specific primary antibodies against TNF-α (1/500), Interleukin-6 (IL-6; 1/500), Bax (1/1000), Bcl2 (1/1000), and Cleaved Caspase-9 (1/1000), all obtained from Cell Signaling Technology (Beverly, USA) except for TNF-α and IL-6 purchased from Abcam (Cambridge, UK). The membranes were then incubated with horseradish peroxidase-linked secondary antibody (1:10,000 *v*/*v*; Cell Signaling Technology, Beverly, USA), which could be directly detectable by chemiluminescence kit reagent (Amersham, Piscataway, USA). The corresponding scans were then analyzed semi-quantitatively by Image J software in proportion to β-actin band intensity as internal control.

Cerebral Fluoro Jade B Staining

For initial blood-free tissue preparation, anesthetized rats were transcardially perfused with ice cold PBS and 4 % phosphate-buffered paraformaldehyde to fix the cerebral parenchyma. The brain hemispheres were then removed and following postfixation in 4 % paraformaldehyde were paraffin-embedded with the aid of tissue processor. To appropriately detect acutely degenerating neurons, 5 μm coronal sections were cut and spread on microscope slides and were subjected to subsequent xylene and ethanol dipping with stained prior to 0.0004 % Fluoro Jade B (Histochem, CA) in 0.1 % acetic acid. The Entellan-mounted samples were then subjected to fluorescent microscopy (Olympus, TH4-200) and three 10× fields/animal were collected and Fluoro Jade B-positive cells were subsequently counted from each field to determine any changes in average degenerating cells/field across the different experimental groups.

Data Analysis

All data are represented as the mean ± SEM. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test to analyze the difference among groups. A p value less than 0.05 was considered statistically significant.

Results

Chronic Atorvastatin may Exacerbate Neurological Deficit but not Infarct Volume Following MCAO

Acute functional outcomes following 24 h reperfusion post 1-h MCAO were substantially abrogated by Atv at 10 mg/kg dosage as was eye-catching with enhanced stroke-induced mortality from 30 to 60 % (Fig. 1a). Unexpectedly, while the surviving animals yet demonstrated augmented neurological deficit, we could not detect any significant change in ischemic cell death determined by TTC staining (Fig. 1b, c). Notably, although in animals dosed at Atv 5 mg/kg, there was a discernible protection against I/R defined by reduced infarct volume, stroke-induced neurological deficit was not remarkably changed at 24 h reperfusion in such experimental group. Consistently, ischemic-edema in ipsilateral hemisphere in model animals (22,561.74 ± 13,026.03 mm^3) not receiving Atv was worsened in rats dosed at 10 mg/kg Atv (17,885.38 ± 10,326.13 mm^3; $p < 0.001$), but improved in those received Atv at 5 mg/kg (4078.444 ± 2354.691 mm^3; $p < 0.001$) over the last month.

Oxidative Stress Markers was Not Affected by Atorvastatin Pretreatment

Based on our ex vivo assays, induction of MCAO followed by 24 h reperfusion abolished the activity of oxidative stress defying agents like CAT and GSH which were in consistent with substantial elevation in lipids and DNA peroxidation as determined by MDA and 8-ohdG levels respectively (Table 1). Nevertheless, such alterations were not consistently detectable in examined cortical and subcortical samples which might be partly explained by different ratio of surviving cells in ischemic core and penumbra. However, the specific time point of cerebral sampling (24 h I/R) might also underlie the insufficiency of some significant changes namely SOD drop in cortical samples. According to these data, 30 days' administration of Atv did not induce any significant improvement in oxidative state of brains

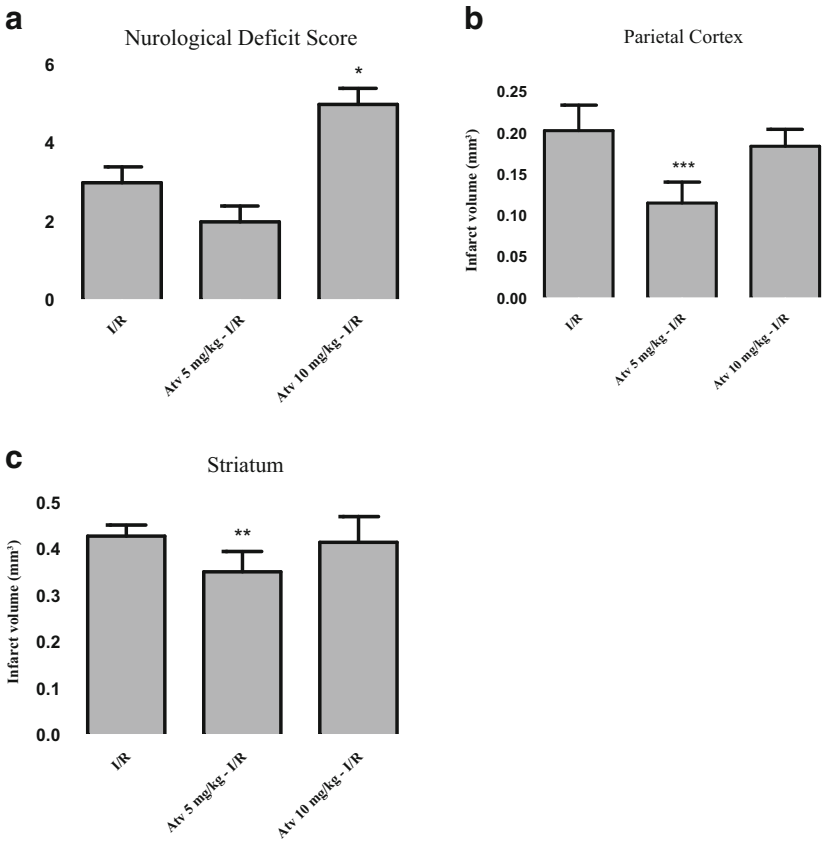

Fig. 1 Effect of chronic atorvastatin (*Atv*; 5 and 10 mg/kg; BO) pretreatment on neurological deficit scores (**a**) and the corresponding infarct volumes in cortex (**b**) and striatum (**c**) following 24 h cerebral ischemic/reperfusion (I/R) injury in rats. Values are expressed as mean ± SEM, each of which including at least seven replicates. *$p < 0.05$, *** $p < 0.001$ vs I/R (ischemia/reperfusion) group

Table 1 Effect of chronic atorvastatin (Atv; 5 and 10 mg/kg; BO) pretreatment on cerebral oxidative state following ischemia/reperfusion (I/R)

	SOD (U/mg)	CAT (nmol/mg)	GSH (U/mg)	MDA (nmol/mg)	8-OHdG (U/mg)
Cortex					
Control	0.337 ± 0.01	0.073 ± 0.005*	0.694 ± 0.02*	0.406 ± 0.007*	0.298 ± 0.01***
I/R	0.318 ± 0.001	0.043 ± 0.004	0.624 ± 0.01	0.522 ± 0.01	0.463 ± 0.01
Atv 5 mg/kg + I/R	0.325 ± 0.0004	0.029 ± 0.003	0.593 ± 0.005	0.515 ± 0.01	0.482 ± 0.006
Atv 10 mg/kg + I/R	0.323 ± 0.006	0.024 ± 0.005	0.570 ± 0.006	0.506 ± 0.01	0.516 ± 0.01
Striatum					
Control	0.370 ± 0.001***	0.036 ± 0.003	0.698 ± 0.02	0.349 ± 0.004*	0.298 ± 0.01***
I/R	0.318 ± 0.001	0.027 ± 0.003	0.777 ± 0.02	0.376 ± 0.01	0.474 ± 0.01
Atv 5 mg/kg + I/R	0.325 ± 0.0004	0.029 ± 0.003	0.755 ± 0.02	0.351 ± 0.009	0.474 ± 0.009
Atv 10 mg/kg + I/R	0.323 ± 0.006	0.024 ± 0.005	0.762 ± 0.01	0.367 ± 0.01	0.439 ± 0.03

Values are expressed as mean ± SEM, each of which including at least three replicates

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs I/R (ischemia/reperfusion) group

SOD superoxide dismutase activity, *CAT* catalase activity, *GSH* reduced glutathione level, *MDA* malondialdehyde content, *8-OHdG* 8-hydroxy-2′-deoxyguanosine content

following ischemic stroke despite the compelling evidences about anti-oxidant properties of subacute statins treatment (Hong et al. 2006).

Chronic Atorvastatin Elevated Cerebral TNF-α and IL-6 Levels

As represented in Fig. 3a., induction of MCAO significantly increased cortical TNF-α and IL-6 levels following 24 h reperfusion as compared to the due control groups. Such inflammatory responses were augmented by Atv (10 mg/kg) pretreatment in parietal cortices representing penumbral region in our stroke brains. To better understand if such finding is depends on I/R context, the low-dose Atv (5 and 10 mg/kg/day) was tested in separate groups of intact animal not underwent MCAO. To our surprise, there was an approximately dose-dependent rise in cerebral TNF-α which was also remarkable for Atv at 5 mg/kg dosage for both parietal cortex ($p < 0.01$) in and striatum ($p < 0.05$), indicating Atv may exert central pro-inflammatory effects in chronic oral administration. However, at least partly, this could explain the escalating impact of Atv on I/R reperfusion injury, the presumptive counteraction with other pliotropic effectors could not be ruled out. That is despite pro-inflammatory properties here, Atv at 5 mg/kg/day dosage could still provide partial protection against stroke-induced infarction, the pliotropic effects of Atv might have overweighed its mild pro-inflammatory impact in I/R context by a nominal decrease in Atv dosage (Fig. 2).

Atorvastatin Escalating Effects Were Associated with Apoptotic Molecules Overstimulation

According to immunoblots analysis for Bax/Bcl2 Ratio and also caspase-9 cleavage, Atv (10 mg/kg) accentuated cerebral apoptosis following stroke (Fig. 3a–f). Interestingly, in the separate experiments designed to evaluate Atv impact on intact brains, Atv (5 and 10 mg/kg) was found to exert pro-apoptotic properties on the brain as determined by significant rise in the mentioned pro-apoptotic molecules. Again, similar to the other long-lasting post-ischemic insults, i.e., inflammation, such effects were mostly detectable in parietal cortices ($p < 0.001$) representing penumbral regions in our experiments and thus prone to post-I/R apoptotic cascades. To certainly address the impact of exacerbated apoptotic signaling in Atv (10 mg/kg) treated I/R animals, the outcome of these alterations were evaluated in the view of virtually degenerating neurons. According to our Fluoro Jade B staining data, the remarkable number of degenerating neurons in the cortical regions which presented discernible inflammatory responses were not enhanced by Atv pre-treatment to a significant degree (Fig. 3g).

Discussion

Uncertainties about optimal statins therapy protocols (Moonis 2012; Scheitz et al. 2014) have been highlighted by recently provided sophisticated commentaries (Golomb 2015). In this regard, the present work apparently is the first experimental report providing preliminary evidences for potential neurotoxicity of chronic Atv in rodents. Indeed, while consistent with earlier evidences where we found that chronic low-dose Atv could also improve stroke outcomes with a minimal dosage increase to 10 mg/kg, we faced a conspicuous inversion in Atv impact on cerebral ischemic insult on which we focused for our further experiments. Noteworthy, it should be kept in mind such evidences could not be looked as a human warning since it strictly needs to be confirmed by at least non-human primate

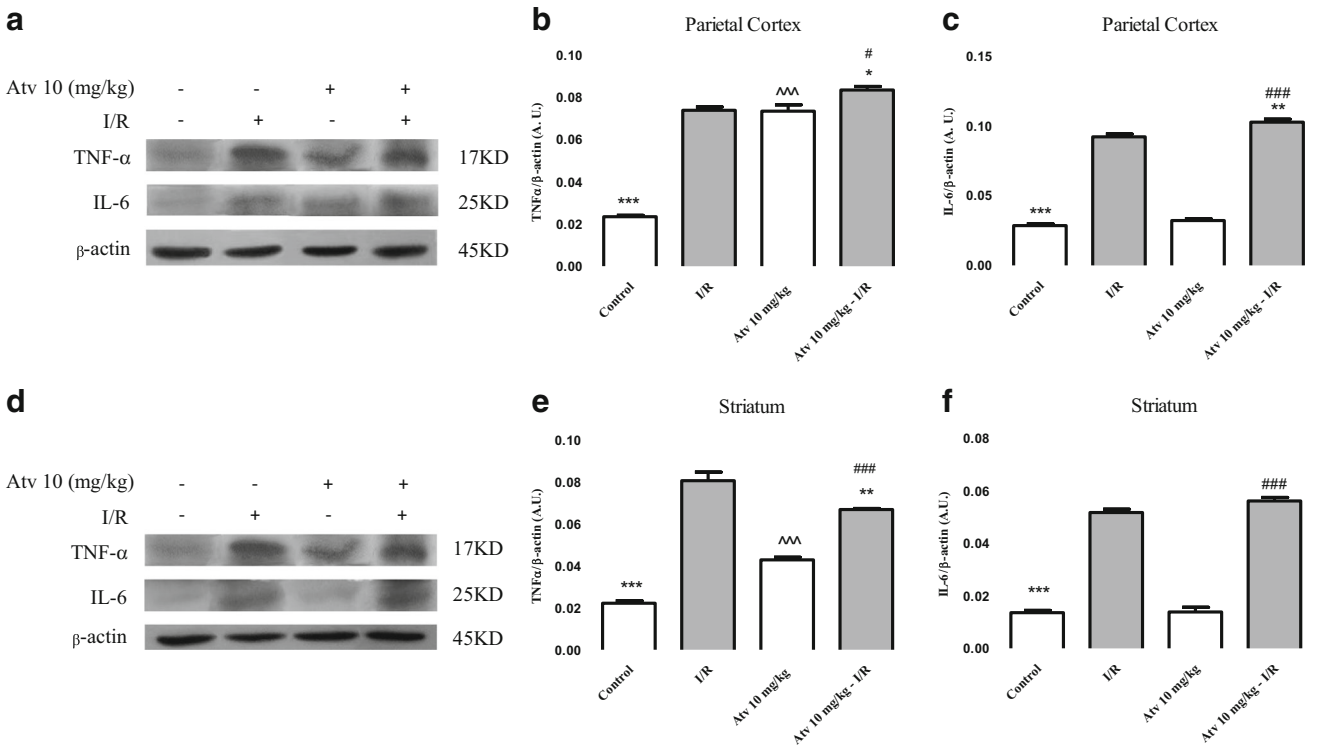

Fig. 2 Alterations in cerebral inflammatory cytokines in stroke animals pretreated with chronic atorvastatin (*Atv*; 10 mg/kg; BO) for 30 days. As shown in representative immunoblots of parietal cortices (**a**) and striatums (**d**), corresponding changes in TNF-α (**b**, **e**) and IL-6 (**c**, **f**) are implicative of pro-inflammatory impact of Atv pretreatment. Data represent mean ± SEM ($n = 3$–5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs I/R (ischemia/reperfusion) group; $^{\wedge\wedge\wedge}p < 0.001$ vs control, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs paired non-I/R group

examination. Rodent studies, despite several advantages, are not sufficiently relevant to be able to predict human responsiveness due to the lack of enough homology (Herodin et al. 2005).

According to solid evidences, while we may not rule out our data that is rodent-specific, rationally, it might be the matter of timing in our work that has been previously shown to bring significant alteration to also human effects. That is, nearly all subacute animal studies so far have considered a maximum of 14 days long treatment prior to experimental stroke which might not be long enough for all isoprenoid effectors being affected. This might be exemplified considering that statins serve antioxidant effects in as early as hours to days (Hong et al. 2006), while it takes weeks to significantly reduce systemic Coenzyme Q10 levels (Langsjoen and Langsjoen 2003) and about 10 months to ameliorate AD pathology (Kurata et al. 2015).

Although lack of earlier data about long-term atorvastatin in stroke animals does not permit for accurate in vivo comparison, our data seemingly is not supported by G. Hamann's team work (Trinkl et al. 2006) implying 4 weeks' pretreatment with pravastatin protects against transient ischemia which has been attributed to their effect on the cerebral vasculature (Asahi et al. 2005; Hayashi et al. 2005). On the contrary, our results might be mostly supported by in vitro reports of neurotoxic effects of statins as well as their distinct pharmacokinetic. Indeed, there is a pile of concrete in vitro evidences demonstrating many of statins may substantially impair cholesterol biosynthesis in rodents primary neurons and thus Rho GTPases prenylation disturbing neuronal plasticity and protection (März et al. 2007; Murakoshi et al. 2011; Martino et al. 2013). In line with this, GPP and cholesterol reduction have been determined in neurons and to a less extend in glia for many statins namely lovastatin (Meske et al. 2003), atorvastatin (Schulz et al. 2004), and pravastatin (Tanaka et al. 2000).

Nonetheless, the ability of particular statins in penetrating blood–brain barrier (BBB) should be conservatively weighed while focusing on in vitro evidences. That is, atorvastatin's BBB penetration, while not as much as simvastatin, is still far higher than that of pravastatin or mevastatin based on higher hydrophobicity despite upper molecular weight (Eckert et al. 2004; Sierra et al. 2011). It is utterly consistent with previous studies implying statins substantially differ in their

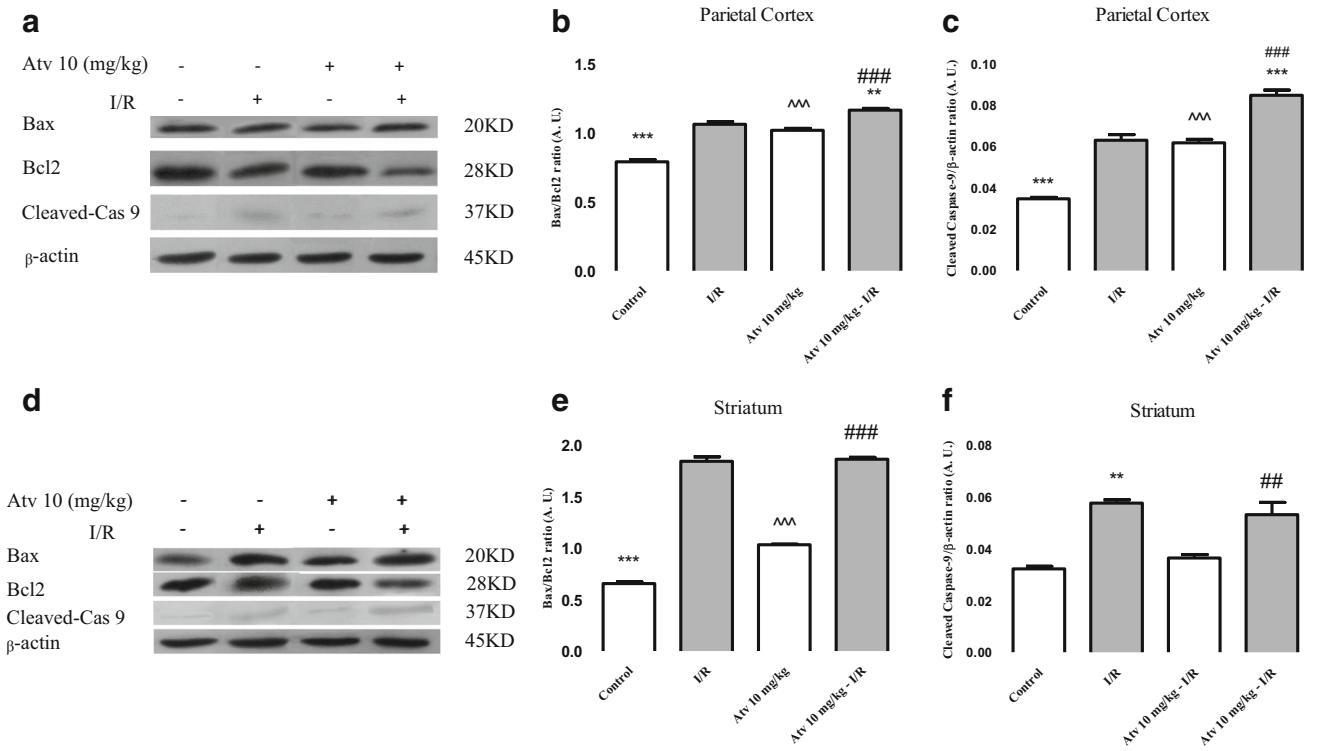

Fig. 3 Changes in molecular and histological markers of apoptosis in the brains of stroke animals pretreated with chronic atorvastatin (*Atv*; 10 mg/kg; BO) for 30 days. As represented in corresponding immunoblots due to parietal cortices (**a**) and striatums (**d**), changes in Bax/Bcl2 ratio (**b**, **e**) as well as cleaved Caspase 9 (**c**, **f**) were indicative of discernible pro-apoptotic effect of Atv (10 mg/kg) on cortical region. Such differences were not significant in the acute degenerative insult as determined by Fluoro Jade B staining in parietal cortical section images (**g**) obtained in fluorescent emission in gray scale (*lower row*) or in 515 nm > filter (*middle row*) in 40X magnitude in which arrows point to the representative degenerative cells. Data represent mean ± SEM (n = 3–5). $^{**}p < 0.01$, $^{***}p < 0.001$ vs I/R (ischemia/reperfusion) group; $^{\wedge\wedge\wedge}p < 0.001$ vs control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs paired non-I/R group

central effects following chronic in vivo administration as has been described by not surprisingly exceptional impact of simvastatin on cerebral gene expression namely anti/pro-apoptotic ones (Johnson-Anuna et al. 2005) and also reducing FPP, GPP, and cholesterol levels in mice brain (Eckert et al. 2009). Such evidences may also explain the proinflammatory and pro-apoptotic effects we detected on intact brains treated with Atv in both dosages of 5 and 10 mg/kg in our non-ischemic animals.

Conspicuously, cardiovascular investigations have shown HMG CoA-reductase inhibitors might worsen ischemia reperfusion injury (Ichihara et al. 1999; Satoh and Ichihara 2000), while administered chronically even in low dosage. This has been ascribed to substantial reperfusion injury as a consequence of coenzyme Q10 depletion leading to antioxidant defense loss (Pisarenko et al. 2001) as a side effect of mevalonate blockage leading to lack of selenocysteine and thus glutathione peroxidase and thioredoxin reductase (Moosmann and Behl 2004). This coupled with extensively cited evidences indicative of statins-induced protection against oxidative stress might be somehow implicative of antagonistic plieotropy at least in rodents.

As was mainly modulated by Atv in our work, inflammatory responses may be an important mediator of neurotoxic effects, especially considering the close cross talk with CNS. Significant cytokines elevation we detected in cortical samples following Atv 30 days' administration was not seemingly strong enough to exacerbate acute neurodegeneration as determined by our histological Fluoro Jade B staining. Nevertheless, it may somehow explain escalated neurological performance in stroke animals and also the augmented apoptotic cascade as determined by Bax/Bcl2 and Cas 9 activation in penumbral region which closely mirroring the long lasting parenchymal inflammation. In this regard, it should be noted that even nominal changes in cytokines could be of utmost potential to alter cerebral function (Mitchell et al. 2010) through the immense influence on gene expression, and thus, might obscure some of pleiropotent effects.

However, these data seem inconsistent to evidences suggesting anti-inflammatory effects for an already approved drug (Gauberti et al. 2013) is not contradictory to the

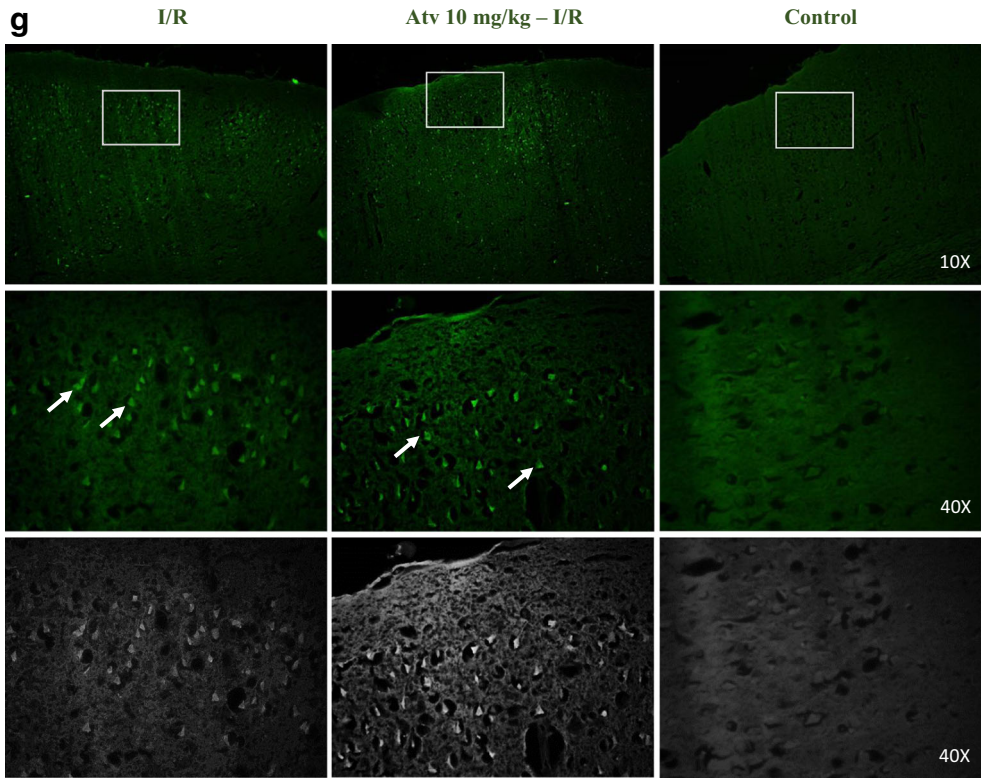

Fig. 3 (continued)

addressed controversies in previous professional remarks on chronic and acute statins in animal models (Schulz 2005). In this connection, it is of significant importance to note that human specific data are not yet devoid of such heterogenicity even in the view of its very distinctive immunity control (Hein and Griebel 2003) compared to rodents. In this regard, lots of evidences implying statins stimulate anti-inflammatory transcripts particularly in human vasculature (Dichtl et al. 2003; Gauberti et al. 2013) are contradicted by others concrete evidences indicative of pro-inflammatory attributes in human monocytes (Montero et al. 2000; Kiener et al. 2001) as it is clinically addressed by symptomatic inflammation in inherited deficiency of mevalonate kinase (Houten et al. 2003).

Certainly, our data considering general pathological outcomes in an animal model lets for specific interpretation neither about peripheral or central underlying mechanisms nor about human specific effects. But might be grossly explained by either Atv-induced cholesterol and GPP reduction in CNS cells leading to neuroinflammation and TNF-α upregulation (Bi et al. 2004; Churchward and Todd 2014) or have taken place as a consequence of statins peripheral actions namely elevated oxidized LDL as a chemotactic agent (Martínez-Castelao et al. 1999; Lankin et al. 2002) or the mentioned drop in antioxidants all predisposing the brain to later ischemic insult.

Conclusion

Our data, not undervaluing statin therapy in stroke, highlight the significance of accurate Atv which needs further confirmation in primates. In line with this, the debate of comparative weights of antagonistic plieotropy in long-term atorvastatin had better be more specifically investigated in terms of stroke incidence and outcomes. Intriguingly, dissecting peripheral and central effects of statins might provide new insights to optimal drug choice and/or dosage, particularly while the claimed statins neuroprotective effects apparently mainly rely on the peripheral impact namely cerebrovascular protection.

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