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ORIGINAL PAPER

The Effects of Alpha Boswellic Acid on Reelin Expression and Tau Phosphorylation in Human Astrocytes

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Abstract Reelin is an extracellular glycoprotein which contributes to synaptic plasticity and function of memory in the adult brain. It has been indicated that the Reelin signaling cascade participates in Alzheimer's disease (AD). Besides the neurons, glial cells such as astrocytes also express Reelin protein. While functional loss of astrocytes has been reported to be associated with AD, dysfunction of astrocytic Reelin signaling pathway has not received much attention. Therefore, we investigated the effects of α-boswellic acid (ABA) as one of the major component of *Boswellia serrata* resin on primary fetal human astrocytes under a stress paradigm as a possible model for AD through study on Reelin cascade. For this aim, we used streptozotocin (STZ), in which from an outlook generates Alzheimer's hallmarks in astrocytes, and assayed Reelin expression, Tau and Akt phosphorylation as well as reactive oxygen species (ROS) generation and apoptosis in the presences of ABA. Our results indicated that while STZ (100 μM) down-regulated the expression of Reelin, ABA (25 μM) up-regulated its expression ($p < 0.01$) for 24 h. ABA efficiently reduced hyperphosphorylated Tau (Ser404) in STZ-treated astrocytes ($p < 0.01$). Furthermore, STZ-induced apoptosis by increasing cleaved caspase three ($p < 0.01$) and ROS generation ($p < 0.01$), a further pathological hallmark of Tauopathy. On the other hand, ABA decreased ROS generation and promoted proliferation of astrocytes through elevating Survivin expression ($p < 0.01$). These results showed that ABA could be considered as a potent therapeutic agent for prevention and decreasing the progression of Alzheimer's hallmarks in astrocytes; however, more in vivo studies would be needed.

Keywords Astrocytes · Alzheimer's disease · Alpha boswellic acid · Streptozotocin · Reelin · Tau

Abbreviations

ABA	α-Boswellic acid
BBA	β-Boswellic acid
AD	Alzheimer's disease
STZ	Streptozotocin
ROS	Reactive oxygen species

Introduction

Over the last few years, focus on glial cells has gained momentum as potential mediators of brain function (Araque et al. 1999; Bergles and Jahr 1998; Ullian et al. 2001). Among glial cells, astrocytes contribute to brain activities such as memory formation because of their metabolic function (Henneberger et al. 2010). Astrocyte dysfunction has been detected in many neurodegenerative diseases, such as Alzheimer's disease (AD) which is the most common form of dementia in the elderly characterized by progressive cognitive deficits, synaptic loss, severe neurodegeneration and specially two prominent hallmarks: Tau hyperphosphorylation and amyloid beta accumulation in the brain (Grilli et al. 2003; Sofroniew and Vinters 2010; Verkhratsky et al. 2010). One of the most important neuronal signaling cascades is Reelin cascade which its

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dysfunction involved in pathology of AD and neurodevelopmental diseases (Bothwell and Giniger 2000).

Reelin protein is an extracellular matrix protein expressed mainly by Cajal–Retzius (CR) cells, GABAergic interneurons, glutamatergic neurons as well as glial cells such as astrocytes (Pesold et al. 1998; Malone 2005; Hochstim et al. 2008; Siebert and Osterhout 2011). Reelin is required for the development and maturation of the brain by affecting neuronal migration, axonal branching and synaptogenesis through binding to lipoprotein receptors and phosphorylation of Dab 1 during brain development (Pesold et al. 1998; Roberts et al. 2005). Moreover, in the mature brain it modulates memory formation by increasing hippocampal neurogenesis and promoting synaptic plasticity (Folsom and Fatemi 2013; Pujadas et al. 2010). Relating to AD pathology, Reelin controls amyloid precursor protein (APP) processing (Hoe et al. 2006), counteracts Aβ-induced synaptic dysfunction (Durakoglugil et al. 2009) and reduces Tau phosphorylation by activating protein kinase B, also known as Akt, and inhibiting GSK3β (Ohkubo et al. 2003). Akt is a signaling kinase, which is involved in cell survival by negatively regulating GSK3β activity (Brazil and Hemmings 2001). Several studies have demonstrated that activated Akt attenuates neural death to protect neurons (Di Segni et al. 2005; Koh et al. 2003). In addition, more studies on AD brain samples implicate altered levels of Reelin (Botella-López et al. 2010; Chin et al. 2007). Finally, Reelin depletion has been reported to accelerate the onset of plaque formation and Tau hyperphosphorylation (Herring et al. 2012; Kocherhans et al. 2010). Hyperphosphorylation of Tau leads to destabilization of microtubules and the death of neurons (Spillantini and Goedert 2013).

Herbal medicine such as *Boswellia serrata* recommended by Avicenna, the ancient Persian physician, has been traditionally used for pregnant women and also elderly people to enhance the memory power of their infants and inhibit amnesia, respectively (Wynn and Fougere 2007). Boswellic acids, the major components of the *B. serrata* resin which have shown promising results in the treatment of AD and reinforcing memory performance, are consist of pentacyclic triterpenes such as α-boswellic acid (ABA), β-boswellic acid (BBA) and acetyl-11-keto-β-boswellic acid (Jalili et al. 2014; Yassin et al. 2013; Hosseini Sharifabad and Esfandiary 2007; Majeed and Badmaev 2006). Also, our laboratory earlier studies showed that BBA amplifies axonal outgrowth of embryonic hippocampal cells through enhancing microtubule polymerization dynamics (Karima et al. 2010). However, the effects of ABA have not received much attention.

According to above evidence, we tried to study two critical issues: (I) while Astrocyte dysfunction has been detected in AD as well as another neurodegenerative diseases (Sofroniew and Vinters 2010), there is lack of evidence about astroglial Reelin cascade. (II) Is ABA able to prevent and decrease pathological hallmarks of AD in astrocytes? For this aim, we investigated the effects of ABA on Reelin, Tau, Akt and Survivin expression in primary fetal human astrocytes. At first, we surveyed the effective dose of ABA (25 μM) on astrocytes viability by MTT assay at 24 h. Afterward, 100 μM streptozotocin (STZ) was utilized to induce AD condition in astrocytes according to the knowledge gained from Rajasekar et al. (2014) that have shown the mechanism of STZ (100 μM) through effecting on some factors such as GSK3α/β, Akt, plaque formation and oxidative stress which are relevant to AD pathology in astroglial cells. Secondly, we examined the effect of ABA (25 μM) and STZ (100 μM) in astrocytes viability and ROS production. Likewise, Survivin protein expression which is critical for cell proliferation was measured by western blot to confirm the MTT assay's results. Furthermore, the expression of Reelin, phosphorylation of Tau (Ser404 and Ser396) and p-Akt (Ser473) were measured in astrocytes by western blot. Moreover, acridine orange/ethidium bromide and Hoechst staining were performed to detect apoptosis.

Materials and Methods

Cell Culture and Treatment

Primary fetal human astrocytes, which were previously isolated from hypothalamus and cerebral cortex of two human fetuses on gestational weeks 9–12 (obtained from Bon Yakhteh Laboratory in Tehran) according to the protocol (Sharif and Prevot 2012), were cultured in complete DMEM (Gibco-BRL, Life technology, Paisley, Scotland) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine and 10 % heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Life technology, Paisley, Scotland) and were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % air. These primary fetal human astrocytes were used for all experiments which conducted here.

MTT Assay for Cell Viability Assessment

Primary fetal human astrocytes were seeded in 96-well plate (10,000 cells per well) in 100 μL complete DMEM and incubated for 24 h, then exposed to various concentrations (0, 10, 15, 20, 25, 30, 40, 50, 60, 80 and 100 μM) of ABA (Sigma-Aldrich, USA, solved in 0.05 % dimethyl sulfoxide (DMSO), Sigma-Aldrich, USA) for 24. After the treatment period, MTT salt (Sigma-Aldrich, USA) was added (5 mg/mL in PBS). The assay indicates the ability of the cells to convert the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide tetrazolium salt into a purple MTT formazan, by the mitochondrial dehydrogenase activity of live cells. DMSO was used to solubilize the colored crystals, and the absorbance was measured spectrophotometrically at 570 nm. The percentage of cell viability was determined as the percentage of reduced MTT, considering the absorbance of control cells as 100 % (Vistica et al. 1991). A total of 25 μM ABA was selected for all further experiments based on cell viability within 24 h.

Previous study by Rajasekar et al. (2014) on Rat astrocytoma cell line (C6) confirmed 100 μM STZ (Sigma-Aldrich, USA.) is the effective concentration for such studies as it could generate all required Alzheimer's hallmarks. To identify the effect of both ABA and STZ simultaneously, primary fetal human astrocytes divided into five groups: Group I: control group; Group II: cells treated only by 100 μM STZ (solved in DMEM) for 24 h; Group III: cells treated first with 100 μM STZ for 30 min and then incubated with 25 μM ABA for 24 h with considering this fact that biological half-life of STZ is 35–40 min (Adolphe et al. 1975); Group IV: cells treated first with 25 μM ABA for 30 min and then incubated with 100 μM STZ for 24 h and Group V: cells treated only with 25 μM ABA for 24 h. Then, MTT assay was employed to measure the cells viabilities.

Measurement of Oxidative Stress Induced by ABA and STZ Using DCF Assay

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are continually produced during metabolic processes and are the hallmark of astrocyte activation (Rajasekar et al. 2014). In this study, cells' ROS content was measured by 2′, 7′-dichlorofluorescein (DCF) assay (LeBel et al. 1992) using 2′, 7′-dichlorofluorescein diacetate (DCF-DA) dye (Sigma-Aldrich, USA).

As explained before, cells divided into the five groups and treated with ABA and STZ. Then, the five groups were incubated with DCF-DA at a final concentration of 10 mM for 30 min at 37 °C, washed twice in PBS, and their fluorescence intensity assessed by a Varian spectrofluorometer model Cary Eclipse with excitation and emission wavelengths of 485 and 530 nm, respectively. The data of treated group are presented as percent change in fluorescence as compared to control.

SDS-PAGE and Western Blotting

Astrocytes in the five groups were lysed in lysis buffer and prepared for immunoblotting (Heni et al. 2011). Western blotting analysis was carried out following the method of Fatemi et al. (2005). SDS polyacrylamide gels (6 % for Reelin and 10 % for β-Actin, Survivin, Tau and Akt) were prepared by standard Laemmli solutions of 25T/1C for Reelin and 29T/1C for β-Actin, Survivin, Tau and Akt (BioRad). A total of 60 μM of protein per lane were loaded onto the gel and electrophoresed for 15 min at 75 V followed by 75 min at 150 V at room temperature (RT). The proteins were electroblotted onto PVDF membrane for 2 h at 300 mAMP at 4 °C (β-Actin, Survivin, Tau and Akt) and 15 h at 150 mAMP (Reelin). Protein blots were blocked with 5 % BSA in Tris-buffered saline with 0.3 % Tween 20 (2 h at 4 °C for β-Actin, Survivin, Tau and Akt; 4 h at 4 °C for Reelin). The blots were then incubated with anti-Reelin antibody (Abcam, ab139691, rabbit, 1:200, 48 h at 4 °C), anti-β-Actin (Cell Signaling Technology, 1:5000, 2 h at RT), anti-Survivin (Sigma-Aldrich, 1:5000, 2 h at RT), anti-Tau Ser396 (Abcam, 1: 2000, 2 h at RT), anti-Tau Ser404 (Abcam, 1:1000, 2 h at RT), anti-Tau (Abcam, 1:1000, 2 h at RT), anti-Akt (Cell Signaling Technology, 1:1000, 2 h at RT), anti-Akt Ser473 (Cell Signaling Technology, 1:1000, 2 h at RT) and anti-caspase-3 (Santa Cruz Biotechnology Inc., 1:1000, 24 h at RT) washed with 0.3 % Tween phosphate-buffered saline (PBS) for 30 min and then incubated with the secondary antibody for 2 h at RT (Abcam, anti-rabbit IgG and anti-mouse 1:2000). Blots were washed for 2 × 15 min with 0.3 % Tween-PBS. Signals were detected using ECL (enhanced chemiluminescence) system provided by the Abcam, USA. Densitometry analyses of bands were accomplished by western blot imaging system (SABZ Biomedicals, Iran). Results obtained are based on three independent experiments.

Fluorescence Microscopy Evaluation of the Apoptotic Cells

Acridine Orange/Ethidium Bromide Double Staining Assay

The effects of ABA and STZ on astrocytes apoptosis were determined morphologically by fluorescent microscopy, after labeling with acridine orange and ethidium bromide (Kasibhatla et al. 2006a; Smith et al. 2012). Astrocytes were seeded in a 12-well plate (80,000 cells per well) in 500 μL DMEM containing 10 % fetal bovine serum and incubated for 24 h. After treatment with the 25 μM ABA and 100 μM STZ for 24 h, the cells were detached from the five groups of cells, washed by cold PBS and adjusted to a cell density of 1×10^4 cells/mL in phosphate solution (1:1 v/v); then, acridine orange/ethidium bromide solution containing 100 μg/mL acridine orange and 100 μg/mL ethidium bromide (acridine orange/ethidium bromide, Sigma-Aldrich, USA) were added to the cell suspension. The cellular morphology was evaluated by an Axoscope2 plus fluorescence microscope from Zeiss (Germany). Different types of cells detected which include living cells

(Uniform bright green nuclei, with organized structure), early apoptotic cells including membrane blebbing and chromatin condensation and fragmentation (bright green nucleus) and late apoptotic cells (orange to red nuclei with highly fragmented chromatin).

Hoechst Staining Assay

Hoechst 33,342 (Invitrogen, H3570) staining was conducted to confirm the detection of apoptotic cells (Allen et al. 2001; Kasibhatla et al. 2006b). Astrocytes were cultured in a 12-well plate (80,000 cells per well) in 500 µL DMEM containing 10 % fetal bovine serum and incubated for 24 h. Then, the cells were treated with the 25 µM ABA and 100 µM STZ for 24 h. Astrocytes in the five groups were harvested and washed with PBS and adjusted to a cell density of 1×10^4 cells/mL of phosphate solution (1:1 v/v), then incubated for 30 min at room temperature with Hoechst 33,342 (5 mg/mL) and nuclei were evaluated by an Axoscope2 plus fluorescence microscope from Zeiss (Germany). Apoptotic cells demonstrated nuclear condensation and DNA fragmentation.

Statistical Analysis

The results are expressed as mean ± S.E.M ($n = 3$). Statistical analyses were performed by applying one-way ANOVA or two-way ANOVA and Student's t test using Prism pad software. The criterion for statistical significance was $p < 0.05$ and $p < 0.01$ for highly significant.

Results

Effects of ABA on Astrocytes Viability

To determine the numbers of viable cells in response to ABA, we examined the effect of ABA on the cell viability of astrocytes using MTT assay. Cells were incubated with different concentrations of ABA (10–100 µM) for 24 h. The results demonstrated that exposure to 15, 20, 25 and 30 µM ABA for 24 h not only had any toxicity to the astrocytes, but also had significantly increased cell proliferation (Fig. 1): 15.7 % ($p < 0.05$), 19.15 % ($p < 0.05$), 21.54 % ($p < 0.01$) and 15.51 % ($p < 0.05$) increase versus control group. Therefore, we used the 25 µM concentration of ABA for further investigations.

Effects of ABA and STZ on the Cell Viability and Survivin Expression in Astrocytes

To identify the effect of both ABA and STZ simultaneously, (I) MTT assay and (II) Survivin protein expression were employed in the five groups to determine the viable cells. Survivin is an essential protein for cell proliferation and prevention of apoptosis (Colnaghi et al. 2006; Wheatley and McNeish 2005).

(I) The MTT result showed that presence of ABA before adding STZ reduced the effect of STZ (Fig. 2a, Group IV) and a 19.74 % increment was observed versus Group II ($p < 0.01$). The number of viable cells in the Group V (cells treated only by 25 µM of ABA) was 19.2 % more than the control group ($p < 0.01$). (II) Determination of 16 kDa Survivin by western blot analysis (Fig. 2b) showed pre- or post-treatment with 25 µM of ABA significantly up-regulated Survivin protein expression in astrocytes affected by 100 µM STZ (Groups III and IV) in comparison with Group II (16.38 and 23.02 % increase, respectively, $p < 0.01$). Furthermore, 25 µM concentration of ABA (Group V) significantly enhanced the Survivin protein expression in astrocytes compared to the Group II (43.34 %) and the control group (36.29 %; $p < 0.01$).

Effects of ABA and STZ on Apoptosis Through Caspase-3 Activation in Astrocytes

The proteolytic processing of caspase-3 was evaluated by western blot analysis to investigate the cytotoxic effects of ABA and STZ in the five groups of astrocytes. As presented in Fig. 2c, while 25 µM ABA (Group V) had not any significant toxic effect in astrocytes, 100 µM STZ increased 80.19, 77 and 51 % apoptosis through activation of caspase-3 in the Groups II, III and IV versus control group ($p < 0.01$), respectively. Pre-treatment of astrocytes with 25 µM ABA (Group IV) decreased 28.67 % induction of a caspase-dependent programmed cell death in comparison with Group II ($p < 0.01$).

Effects of ABA and STZ on ROS Generation in Astrocytes

To explore the effect of ABA and STZ on oxidative stress, the ROS (hydrogen peroxide and superoxide radical) generation in astrocytes was investigated using fluorescence spectrometry. ROS generation is one of the major characteristics of glial cell response in the AD pathophysiology (Bungart et al. 2014). To ascertain this, ROS level was measured in the five groups of astrocytes using DCF-DA reagent. Cell-permeate reagent DCF-DA, a fluorogenic dye measures H_2O_2 and other hydroxyl radical concentration within the cell. After diffusion into the cell, DCF-DA is deacetylated by cellular esterases to a non-fluorescent compound, which is later becomes fluorescent into DCF upon oxidation by ROS. DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy (LeBel et al. 1992). As shown in Fig. 2d, while

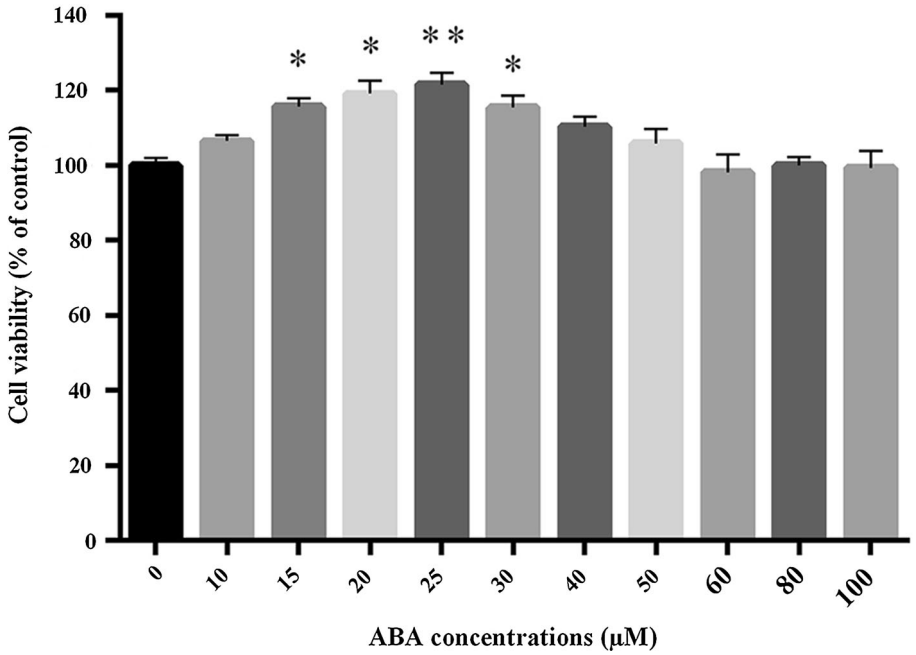

Fig. 1 Effect of ABA on astrocytes viability. Astrocytes are treated with different concentrations (0–100 μM) of ABA for 24 h. Their viabilities are assayed using MTT test. Concentrations of 10,15, 20, 25 and 30 μM of ABA increase the rate of growth in astrocytes for 24 h. Histograms show relative changes expressed as Mean ± SEM of three independent experiments ($n = 3$). Data values are expressed as % of control values. *Significant change ($p < 0.05$) in comparison with control group

100 μM STZ significantly ($p < 0.01$) increased ROS generation (13.76 %) in Group II compared to the control group, the presence of 25 μM ABA in Groups III and IV decreased the oxidative stress, respectively, 19.37 and 49.49 % compared to Group II ($p < 0.01$). Moreover, the result showed that ROS level in Group V (cells treated only with 25 μM ABA) noticeably reduced in comparison with Group II (61.76 %) and control group (48 %; $p < 0.01$).

Effects of ABA and STZ on Reelin Expression in Astrocytes

Although the anti-Reelin antibody [G10] is prevalent for Reelin evaluation, we used anti-Reelin antibody [EPR3330(2)] (ab139691) which is monospecific for human fetal brain lysate too. Reelin appears as several protein bands on SDS-PAGE: Reelin 410 (full-length Reelin), 330 and 180 kDa. Reelin protein expression in STZ- and ABA-treated astrocytes was assessed in the five groups of cells. Measurement of Reelin 180 and 410 kDa species in 100 μM STZ-treated astrocytes (Group II) showed, respectively, 26.98 and 26.41 % ($p < 0.01$) reduction when compared with control group (Fig. 3a). Pre-treatment with 25 μM ABA in Group IV up-regulated Reelin 180 (23.77 %), 330 (16.03 %) and 410 kDa (33.32 %) proteins expression, compared to Group II ($p < 0.01$). Also, western blot analysis revealed that treated astrocytes with 25 μM ABA (Group V) enhanced the Reelin 180 (23 %) and 410 kDa (13.67 %) proteins expressions in comparison with control group as well as a 49, 17.16 and 40.12 % increase, respectively, in expression of Reelin 180, 330 and 410 kDa compared to Group II ($p < 0.01$).

Effects of ABA and STZ on Inducing Phosphorylation of Tau and Akt in Astrocytes

In order to explore the intracellular mechanisms underlying the protective effect of ABA against STZ, phosphorylation of Akt and Tau was studied by immunoblotting. The result (Fig. 3b–d) showed that STZ and ABA did not have effect on total Akt and Tau expression. While p-Akt (Ser473)/Akt phosphorylation decreased 40.22 % in Group II versus control group ($p < 0.01$), the level of phosphorylated Tau (Ser396)/Tau increased by 22.96 ($p < 0.01$).

Pre-treatment and post-treatment with 25 μM ABA meaningfully up-regulated the level of phosphorylated Akt ($p < 0.01$) and down-regulated the level of phosphorylated Tau (Ser404) in Groups III and IV versus control group and Group II ($p < 0.05$ and $p < 0.01$).

Effects of ABA and STZ on Apoptosis in Astrocytes

Apoptosis Detection with Acridine Orange/Ethidium Bromide Double Staining Assay

To assess the type of cell death induced by STZ and ABA in astrocytes, the morphological changes after double staining cells with acridine orange and ethidium bromide

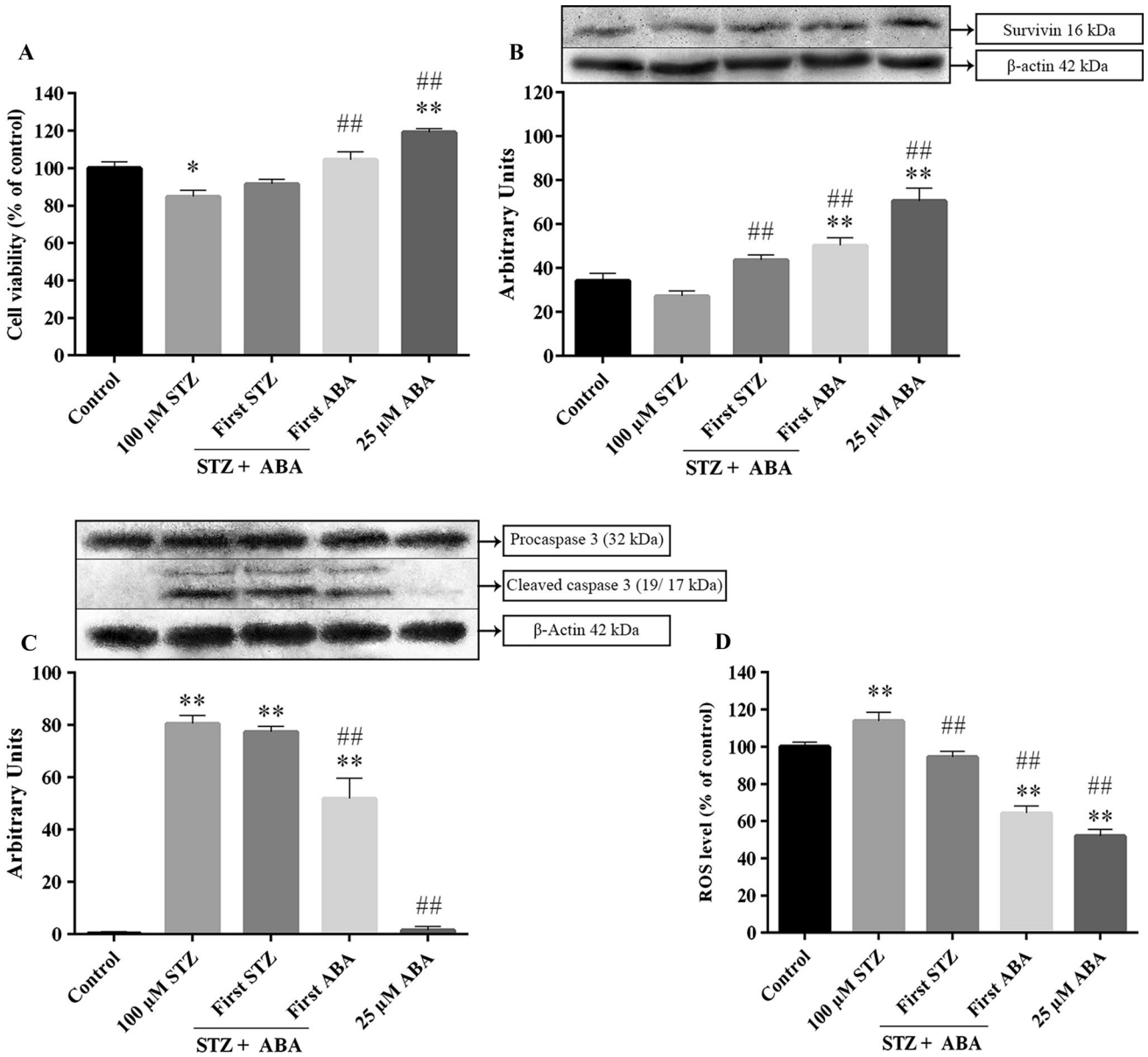

Fig. 2 Combined effect of ABA and STZ on **a** cell viability, **b** Survivin expression **c** caspase-3 activation and **d** induction of oxidative stress in astrocytes. **a** Astrocytes viabilities are assayed using MTT. The cells are treated with 25 μM ABA and 100 μM STZ for 24 h in five groups (Group I, no treatment as control; Group II, 100 μM STZ-treated cells; Group III, cells first treated with 100 μM STZ for 30 min and then incubated with 25 μM ABA for 24 h; Group IV, cells first treated with 25 μM ABA for 30 min and then incubated with 100 μM STZ for 24 h; Group V, cells treated only by 25 μM ABA for 24 h). A total of 100 μM STZ induces cytotoxicity and apoptosis in astrocytes, while the presence of 25 μM ABA inhibits its cytotoxicity effects in Group III. **b** The gel mobility of Survivin 16 kDa band of five groups. Mean Survivin/β-Actin are presented as *histograms bars*. Survivin/β-Actin is enhanced significantly in Groups IV and V ($p < 0.01$) versus control cells and Group II. **c** Activity of caspase-3/procaspase-3 is increased significantly in Groups II, III and IV compared with control ($p < 0.01$). **d** The production of oxidative stress in astrocyte is measured by DCF assay in the five groups of cells. The results reveal an increase in producing oxidative stress in Group II compared to control ($p < 0.01$), while the presence of 25 μM ABA reduces ROS generation in Groups III, IV and V in comparison with group II ($p < 0.01$). Results are expressed as Mean ± SEM of three independent experiments ($n = 3$). Data values are expressed as % of control values. *Asterisks* (*) indicate significant difference ($*p < 0.05$ and $**p < 0.01$) from control and *double pound sign* (##) represents substantial difference ($\#\#p < 0.01$) from the Group II

were investigated. Morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, alterations in the size and the shape of cells, as revealed by fluorescence microscopic analysis, were observed predominantly in the Groups II and III of cells as shown in Fig. 4a.

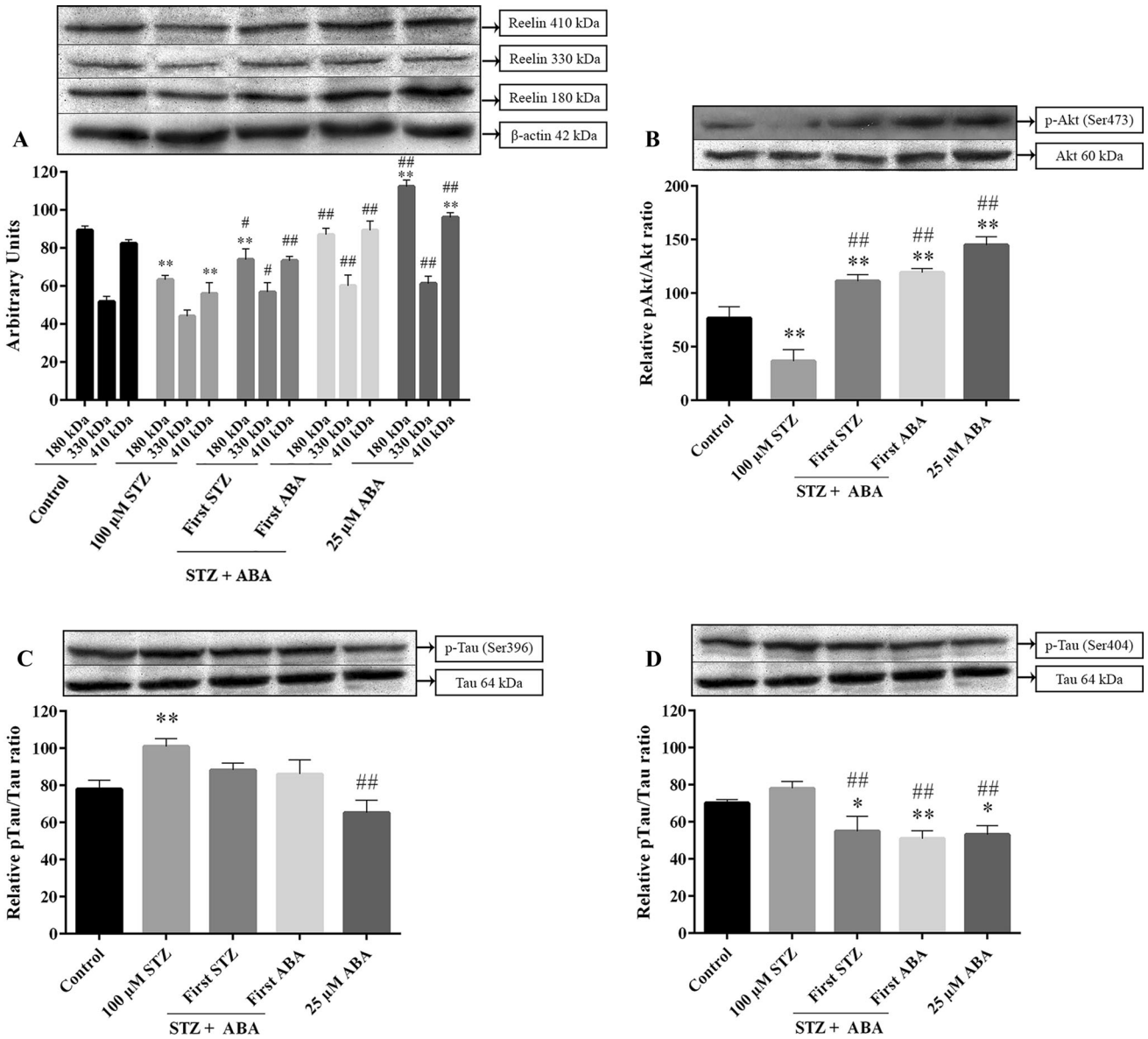

Fig. 3 Gel mobility of **a** Reelin 410, 330 and 180 kDa band, phosphorylated form of **b** p-Akt (Ser473) **c** Tau Ser396 and **d** Tau Ser404 of five groups are shown in the *upper panel*. **a** Mean Reelin 410, 330 and 180 kDa/β-Actin ratios for subjects are shown as *histograms bars*. Reelin 410 and 180 kDa/β-Actin are reduced significantly in 100 μM STZ-treated cells ($p < 0.05$ and $p < 0.01$) versus control subjects. However, Reelin 410, 330 and 180 kDa/β-Actin are improved considerably in Groups III, IV and V ($p < 0.05$ and $p < 0.01$) compared to Group II. **b** Activity of p-Akt (Ser473)/Akt is reduced in Group II cells ($p < 0.01$) versus control group, while activity of p-Akt (Ser473)/Akt are remarkably amplified in Groups III, IV and V ($p < 0.01$) versus Group II. **c** and **d** Phosphorylated form of Tau (Ser396)/Tau is increased meaningfully in Group II ($p < 0.01$) versus control group. However, phosphorylated form of Tau (Ser404)/Tau is reduced significantly in Groups III, IV and V ($p < 0.01$) versus Group II. *Histograms* represent Mean ± SEM of relative density of protein of three independent experiments ($n = 3$). (# and *) Significant differences (*$p < 0.05$ and **$p < 0.01$) versus control and (#$p < 0.05$ and ##$p < 0.01$) versus Group II cells, respectively

Apoptosis Detection with Hoechst Staining Assay

Hoechst staining was conducted for evaluation of cell death in each five groups. In the Hoechst staining, apoptotic cells (fragmented nuclei) were seen mostly in Groups II, III and IV (Fig. 4b).

Discussion

The present study investigated the effects of ABA on cell viability, Survivin protein expression as well as oxidative stress, apoptosis, phosphorylated Tau (Ser396 and Ser404)

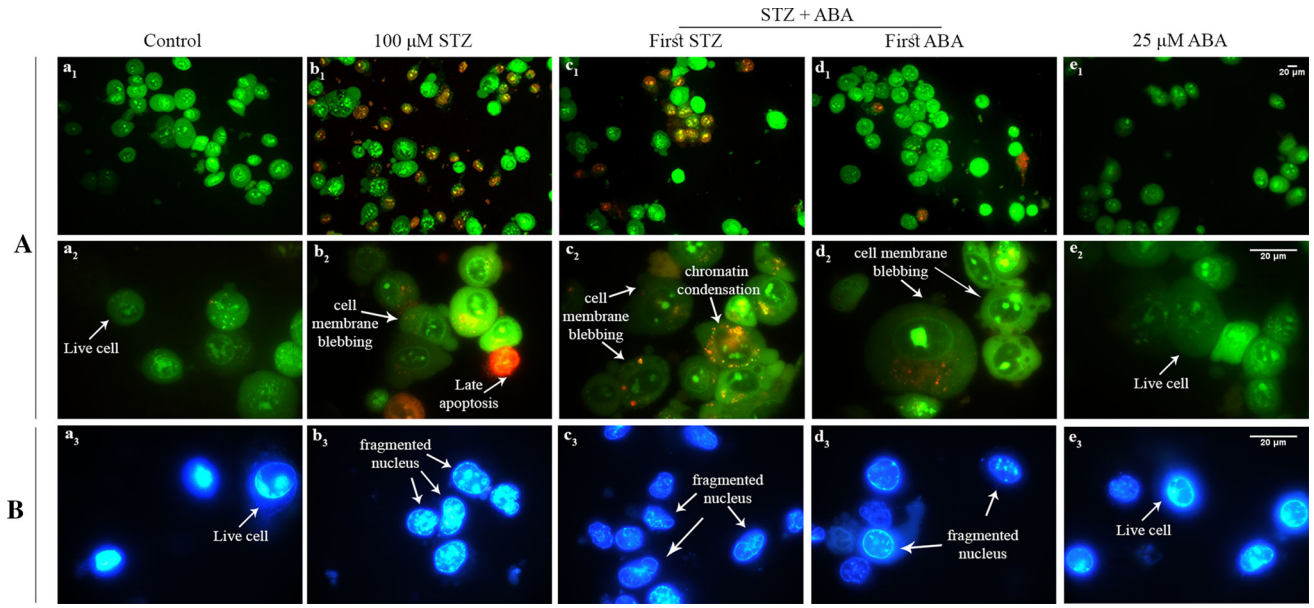

Fig. 4 Photomicrograph of apoptotic changes in astrocytes treated with 100 µM STZ and 25 µM ABA for 24 h using **a** acridine orange/ethidium bromide double staining method and **b** Hoechst assay visualized by fluorescence microscopy and photographed. **a** Acridine orange emits green fluorescence by binding with double-stranded DNA of live cells. Untreated astrocytes and treated with ABA demonstrate normal cellular structure in Group I and V (a_1–a_2 and e_1–e_2). Early apoptosis features, including membrane blebbing and chromatin condensation, and late apoptosis are frequently observed in the presence of STZ in Groups II, III and IV (b_1–b_2, c_1–c_2 and d_1–d_2). **b** Astrocytes stained with Hoechst 33,342. Astrocytes with signs of apoptosis (fragmented nuclei) are regularly seen in Groups II, III and IV (b_3, c_3 and d_3). *Scale bar* (a_1–e_3), 20 µm

and Akt (Ser473), Reelin protein expression in primary human astrocytes in the presence and absence of STZ.

Based on the literature, resin of *B. serrata* can positively affect the development of brain and dendritic branching density, mental condition and cognitive functions in rats (Gokaraju et al. 2013; Yassin et al. 2013). Among boswellic acid isomers constituent of *B. serrata* resin, BBA has increased the microtubule length and axonal outgrowth through enhancement of microtubule polymerization dynamics and mass in hippocampal neurons (Karima et al. 2010). While the effects of BBA on microtubule polymerization and axonal extension have been studied, the efficacy of ABA on astroglial cells is not known. Furthermore, recent evidence suggests that AD is associated with atrophy of astrocytes, the main homeostatic cells of the brain, which leads to disruptions in synaptic plasticity, neurotransmitter homeostatic balance and eventually neural death through raised excitotoxicity (Verkhratsky et al. 2010). Therefore, we investigated the potential effects of ABA on primary human astrocytes. Previous studies have described the involvement of Reelin in induction of astroglial branching (Chai et al. 2015), protection against both the neuronal death and dendritic spine loss in AD mice and also being required for neural migration (Pinto-Lord et al. 1982; Pujadas et al. 2014). Our results demonstrated that the exposure of astrocytes to ABA considerably increased Reelin 410 and 180 kDa which evaluated by novel anti-Reelin antibody [EPR3330(2)] (ab139691). Inducing the enhancement of astroglial branching is one of the Reelin's characteristics (Fatemi 2008). Thus, ABA may have an effect on Reelin signaling pathway, which is worth to be further investigated. Additionally, ABA promoted cell viability and proliferation and up-regulated Survivin protein expression. Survivin is a chromosomal passenger complex that modulates life and death through its bifunctional roles in inhibiting apoptosis and promoting cell proliferation. It has been indicated that Survivin level is high in G2 and M phases of the cell cycle, which is essential for cell division (Colnaghi et al. 2006; Wheatley and McNeish 2005).

Rajasekar et al. (2014) showed that STZ induces Alzheimer's hallmarks by abrogating downstream Akt and GSK-3 (a/b) signaling pathway and producing Aβ plaques in astroglial cells. This scenario fits with our results which indicated that STZ decreased Akt phosphorylation in astrocyte. Several previous studies have concluded that AD might be associated with the Reelin deficiency and showed that the levels of full-length Reelin and its fragments are decreased in the hippocampus of AD human brain and transgenic mice (Chin et al. 2007; Herring et al. 2012; Kocherhans et al. 2010). Reelin inhibits GSK3β by activating phosphorylation of Akt at Ser473 and Thr308 and consequently reducing pathological hallmarks of AD including hyperphosphorylated Tau, Aβ plaques (Beffert

et al. 2002; Cantley 2002; Wang et al. 2010) and finally neuronal death (Philpott and Facci 2008). In the present study for the first time, the results showed that 410 kDa species of Reelin and its 180 kDa isoform are reduced in STZ-affected astrocytes. Moreover, it is known that phosphorylation of Tau at Ser396 and Ser404 prevents Tau function that leads to dissociation of Tau from microtubules and impairment of microtubule assembly (Evans et al. 2000). In addition, increased phosphorylation of Tau has been observed in the lack of Reelin expression, which leads to neuronal degeneration (D'Arcangelo et al. 1999; Hiesberger et al. 1999). Our results showed that STZ increases phosphorylation of Tau at Ser396 in astrocytes. In this regard, it can be inferred that besides the impairment in the Reelin signaling cascade in neurons, interruption in astroglial Reelin/Akt/Tau signaling pathways may have a roll in AD. However, further investigations are required.

Furthermore, our results indicated reduction in cell viability in STZ-affected astrocytes and on the contrary, increase in caspase-dependent programmed cell death by enhancing cleaved caspase three. It can be inferred that STZ can have an influence on activation of caspase three which leads to reduced cell proliferation. Mariani et al. (2005) and Tota et al. (2012) suggest reactive oxygen is involved in the pathophysiology of AD. Rajasekar et al. (2014) demonstrated that STZ causes oxidative stress in astrocytes, which may be a more compelling pathological event affecting degeneration of neighboring neurons. Likewise, the results of our experiments depicted a significant increase in ROS generation in STZ-affected astrocytes, which complement the Rajasekar et al. (2014) work. This data suggest that astroglial oxidative stress may have a role in the pathophysiology of AD. Previously, it was indicated that pentacyclic triterpenes such as boswellic acids show strong antioxidant activity by reducing the generation of free radicals which can be beneficial in AD management (Assimopoulou et al. 2005; Bandehali Naeini et al. 2014). In this study, we observed a significant drop in ROS generation in astrocytes treated with ABA. Further study on Akt/Tau phosphorylation showed that ABA increased Akt (Ser473) phosphorylation which leads to decrease in hyperphosphorylated Tau (Ser404) in astrocytes.

Regarding to the above-mentioned promising results about the protective impacts of ABA on astrocytes, we also investigated its effects in the present of STZ. Despite the fact that STZ decreased the cell viability and the expression of Reelin protein in astrocytes, we observed considerable suppression of STZ effect in the presence of ABA. ABA promoted cell proliferation, Survivin and Reelin protein expression, as well as diminishing ROS generation in the presence of STZ. In addition, analysis of apoptosis assays revealed that ABA reduced the number of apoptotic astrocytes affected by STZ. Moreover, it was seen that phosphorylation of Akt (Ser473) was increased, while phosphorylated Tau at Ser404 was significantly decreased. These results were seen in both post-treated and pre-treated with 25 μM ABA (Groups III and IV); however, the results were considerably better when cells were pre-treated with ABA (Group IV). Taking these results into account, it seems that ABA down-regulated phosphorylation of Tau (Ser404) through Reelin/Akt pathway in astrocytes affected by STZ.

In conclusion, our study elucidated while STZ decreases cell proliferation and induces toxicity in primary human astrocytes through increasing activation of caspase three, ROS generation, hyperphosphorylation of Tau (Ser396), down-regulating Reelin and p-Akt (Ser473) expression, ABA abrogated these effects of STZ. These findings indicate the possibility of involvement of astroglial mediated toxicity in AD pathophysiology and beneficial use of ABA in the management of AD. These data also suggest that this may be a way, in which ABA can be exerted with a novel significant therapeutic potential. However, clinical experiments are needed.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Standard Compliance with ethical standards of Ethics Committee of University of Tehran within which the work was undertaken and the requirement generally comply with the Ethics Board regulations.

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