J Appl Biomed
DOI: 10.32725/jab.2019.007
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

14-3-3ζ protein protects against brain ischemia/reperfusion injury and induces BDNF transcription after MCAO in rat

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Abstract
Brain ischemia is a leading cause of death and disability worldwide that occurs when blood supply of the brain is disrupted. Brain-derived neurotrophic factor (BDNF) is a protective factor in neurodegenerative conditions. Nevertheless, there are some problems when exogenous BDNF is to be used in the clinic. 14-3-3ζ is a pro-survival highly-expressed protein in the brain that protects neurons against death. This study evaluates 14-3-3ζ effects on BDNF transcription at early time point after ischemia and its possible protective effects against ischemia damage. Human 14-3-3ζ protein was purified after expression. Rats were assigned into four groups, including sham, ischemia, and two treatment groups. Stereotaxic cannula implantation was carried out in the right cerebral ventricle. After one week, rats underwent middle cerebral artery occlusion (MCAO) surgery and received 14-3-3ζ (produced in our laboratory or standard form as control) in the middle of ischemia time. At 6 h of reperfusion after ischemia, brain parts containing the hippocampus, the cortex, the piriform cortex-amygdala and the striatum were collected for real time PCR analysis. At 24 h of reperfusion after ischemia, neurological function evaluation and infarction volume measurement were performed. The present study showed that 14-3-3ζ could up-regulate BDNF mRNA at early time point after ischemia in the hippocampus, in the cortex and in the piriform cortex-amygdala and could also improve neurological outcome and reduce infarct volume. It seems that 14-3-3ζ could be a candidate factor for increasing endogenous BDNF in the brain and a potential therapeutic factor against brain ischemia.

Keywords: 14-3-3ζ; BDNF; Brain ischemia; MCAO; Purification

Highlights:
- 14-3-3ζ protein induces BDNF transcription after MCAO in the brain
- 14-3-3ζ protein reduces infarction volume and neurological severity scores in MCAO model in rat
- 14-3-3ζ protein is a candidate factor for increasing endogenous BDNF in the brain an a potential therapeutic factor against ischemic neurodegeneration

Introduction

Ischemic brain injury with high mortality and disability in survivors, takes place in stroke, and is also a common cause of death and neurologic dysfunctions after cardiac arrest (Benjamin et al., 2018; Chalkias and Xanthos, 2012; Hankey, 2017; Nolan et al., 2008; Sacco et al., 2013). Although tissue plasminogen activator, endovascular thrombectomy, and hypothermia have been successfully used clinically, many researches have been conducted and are also underway to introduce a protective method against cerebral ischemia (Chalkias and Xanthos, 2012; Hankey, 2017).

14-3-3 proteins are conserved regulatory proteins with average molecular weight of 30 kD found in all eukaryotes. There are seven known 14-3-3 isotypes in mammalian (β, γ, ε, η, ζ, σ, τ/θ) and their highest tissue expression is in the brain. They interact with various targets in the brain and play role in vital processes via scaffolding, chaperoning and regulating their target's localization (Berg et al., 2003; Cau et al., 2018; Ferl et al., 2002). 14-3-3 proteins are up-regulated in ischemic astrocytes and in human and rat brains with infarction (Chen et al., 2005; Kawamoto et al., 2006; Pirim, 1998; Umahara et al., 2007). 14-3-3ζ is encoded by *YWHAZ* gene and is known as a major pro-survival protein (Kasinski et al., 2014). Cerebrospinal fluid level of 14-3-3ζ associates with severity of ischemic

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http://doi.org/10.32725/jab.2019.007
Submitted: 2018-09-24 • Accepted: 2019-04-04 • Prepublished online: 2019-04-15
J Appl Biomed 17/2: 99–106 • EISSN 1214-0287 • ISSN 1214-021X

damage in rat and is introduced as a surrogate biomarker for acute brain injury (Siman et al., 2005). Depletion of zeta isoform evokes cell death in mouse hippocampal culture (Murphy et al., 2008). Up regulation of zeta isoform is protective *in vitro* and *in vivo* under conditions having common patho-mechanism, to ischemia (Brennan et al., 2013; 2015). It has been reported that, 14-3-3ζ regulates brain-derived neurotrophic factor (BDNF) transcription in neuronal cells (Neasta et al., 2011).

BDNF is a nerve growth factor produced in different regions in the brain with the highest levels in the hippocampus and cerebral cortex and is essential for neuronal function and survival throughout life (Nagahara and Tuszynski, 2011; Zuccato and Cattaneo, 2009). BDNF is considered as a key target for drug development in neurodegenerative diseases (The BDNF StudyGroup, 1999; Hernandez-Chan et al., 2015; Levivier et al., 1995; Lu et al., 2013; Nagahara et al., 2009; Shruthi et al., 2017; Silva et al., 2015). Intraventricular delivery (Beck et al., 1994; Schäbitz et al., 1997) and intravenous bolus (Müller et al., 2008; Schäbitz et al., 2000, 2007) of BDNF, has been shown to be protective in cerebral ischemia in rat, whereas blockade of endogenous BDNF activity was reported to intensify cerebral ischemia (Larsson et al., 1999). Since effective delivery of BDNF to the brain is a challenge, strategies to induce endogenous production of BDNF in the brain are helpful for its clinical development.

We hypothesized that: (A) 14-3-3ζ may increase BDNF mRNA after cerebral ischemia; (B) 14-3-3ζ treatment may protect against cerebral ischemic damage. Intracerebroventricular (i.c.v.) delivery of 14-3-3ζ was performed during middle cerebral artery occlusion (MCAO) in rat and the effects on BDNF mRNA expression and neurological behavior and infarct volume after ischemia were assessed.

Materials and methods

Ethics statement

All animal experiments were reviewed and approved by our university animal care committee (IR.SBMU.RETECH.REC.1395.263). All experiments were performed in accordance with relevant guidelines and regulations.

Synthesis and sub cloning of human 14-3-3ζ gene

The coding sequence of the human 14-3-3ζ gene (Accession Number: NM-003406) was selected and optimized into the most preferred codons of the *E. coli* codon usage system without any changes in amino acid sequence. The resulting sequence was synthesized into the pGEM-B1 vector (Bioneer, Korea). The pGEM-B1-14-3-3ζ was used to amplify 14-3-3ζ coding sequence by PCR using following set of primers: forward, 5'-ATTCGGATCCTATGGACAAAAACGAACTGGT-3'; reverse, 5'-TACAGTCGACGTTTTCACCACCTTCACCCAGC-3'. Amplified sequence was then ligated into the BamHI and SalI restriction enzyme sites of the prokaryotic expression vector pET26b and the process was confirmed by DNA sequencing.

Expression and purification of 14-3-3ζ protein

E. coli Bl21 (DE3) was transformed with pET26b-14-3-3ζ construct. A single clone was selected and grown at 37 °C at 200 rpm in 2 ml LB medium containing 30 μg/ml kanamycin to OD600 = 1. Every 2 ml culture was diluted into 50 ml of fresh LB media containing 30 μg/ml kanamycin and cultured at 37 °C at 200 rpm to OD600 = 0.6. The 14-3-3ζ His- tagged protein was induced using 1 mM isopropyl-β-D-thiogalactoside (IPTG). Bacterial cells were pelleted by centrifugation at 10,000 × g, after 5 h growing at 37 °C at 200 rpm. The pellet was suspended in binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, pH 8) and cells were lysed by sonication on ice. The lysate was centrifuged at 15.000 × g for 15 min at 4 °C. The supernatant was loaded onto a Ni^{2+}-NTA resin column (Invitrogen) pre-equilibrated with binding buffer. The column was washed by washing buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 8). Stepwise imidazole gradient of elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 50 to 500 mM imidazole, pH 8) was used for protein elution. A 15 kDa molecular weight cutoff dialysis tubing (D0530 Sigma, Germany) was used and eluted fractions were dialyzed against PBS, pH 7.4 at 4 °C. The recombinant protein was confirmed by Western blot, using anti-6X-His-tag antibody (1:1000, Abcam, UK). The purified protein was named KBKζ.

Animal study design

A total of 39 adult male Wistar rats (250–300 g, aged 5 to 7 weeks, Pasteur Institute, Karaj, Iran) were housed under standard 12 h light/dark cycle and with *ad libitum* access to food and water at 25 °C. Animals were studied in two groups: 18 for real time PCR analysis (3 intact rats, 3 sham-operated animals received vehicle, 4 ischemic rats received vehicle, 4 ischemic rats treated with 14-3-3ζ made in our laboratory, and 4 ischemic rats treated with 14-3-3ζ provided from Creative BioMart, USA, as standard control); 21 for neurological severity score and infarction size assessments (3 sham-operated animals, 6 ischemic animals, 6 ischemic animals treated with our laboratory 14-3-3ζ and 6 ischemic rats treated with standard 14-3-3ζ). Ischemic animals underwent MCAO surgery. Rats in sham group were subjected to the same surgical procedures except the filament insertion.

Cannula implantation

Animals were anaesthetized by i.p. injection of the mixture of 100 mg/kg of ketamine and 10 mg/kg of xylazine. A 22 gauge guide cannula was implanted into the right lateral ventricle using a sterotax instrument [from the bregma: –0.9 mm anteroposterior (AP), ±1.5 mm Mediolateral (ML), –3.0 mm dorsoventral (DV)]. The implanted cannula was fixed to anchored screws on the skull by dental cement and fitted with a mandarin. The procedure was verified by 2 μl injection of methylene blue solution into the lateral ventricle of one rat. Cannulated rats were allowed to recover for one week before MCAO.

Middle cerebral artery occlusion

One week after cannulation, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and transient occlusion of the middle cerebral artery was performed using the suture occlusion technique described by Longa et al. (1989). The right common carotid artery (CCA), the right external carotid artery (ECA) and the right internal carotid artery (ICA) were exposed briefly. The CCA and ECA were ligated. A 3-0 mono filament nylon suture, coated with silicon was introduced through an arteriectomy in the CCA, gently advanced into the ICA lumen 20 to 22 mm, until resistance was felt. At this point, suture has blocked the origin of MCA and all sources of blood flow were occluded. The body temperature was maintained at 37 °C throughout the operation. After 1 h of MCAO, reperfusion was performed by withdrawal of the filament.

Intraventricular microinjection

In the middle of MCAO (30 min of ischemia), rats were injected with standard 14-3-3ζ (5 μg per 5 μl per rat, Creative Bio-

Mart, rats were named standardζ group) or KBK 14-3-3ζ (5 µg per 5 µl per rat, made in our laboratory, rats were named KBKζ group), into the right lateral brain ventricle via an injection cannula tubed to a Hamilton micro syringes. Protein volumes of 5 µl were infused over 3 min. The injection cannula was left in the brain for at least 1 min after the injection, before being pulled so that backflow of the injected proteins is prevented. In real time PCR group, Ischemic control rats and sham-operated rats received vehicle in the same way as 14-3-3ζ treated groups. Since the standard form of the protein was purchased as a solution in PBS, KBKζ was also prepared in PBS, and this solvent was used as vehicle in control groups.

Real time quantitative PCR

Rats were sacrificed under deep anesthesia with chloral hydrate (800 mg/kg, i.p.) at 6 h of reperfusion after 1 h MCAO or sham operation. Four parts from the brain right hemispheres including the hippocampus, the cortex, the piriform cortex-amygdala (Pir-Amygdala), and the striatum were separated and saved in –80 °C immediately until further processing.

Total RNA was isolated using RNeasy Mini Kit (QIAGENE, USA) following the manufacturer's instructions. RNA quality and concentration were determined using agarose gel electrophoresis and a Multi-Mode Reader (SYNERGY HTX, Bio Tek). Every RNA sample (1 µg) was reverse transcripted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (FERMENTASE, USA), following the manufacturer's recommendations. Volumes corresponding to 1/20 of the resulting cDNA were subjected to qPCR by using the following set of primers: BDNF (forward, 5'-GGC TGA CAC TTT TGA GCA CGT-3'; reverse, 5'-CTC CAA AAG GCA CTT GAC TGC TC-3'), GAPDH (forward, 5'-AGT TCA ACG GCA CAG TCA AG-3'; reverse, 5'-TAC TCA GCA CCA GCA TCA CC-3') and AccuPower 2 × GreenStar qPCR Master (BIONEER, Korea). PCR reactions were run in duplicates on the real-time PCR system (Step One Plus, Applied Biosystems). The conditions were: holding stage at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min followed by melt curve stage at 95 °C for 15 s and 60 °C for 1 min and 95 °C for 15 s. Gene expression was assessed using the ΔΔCt method (Livak and Schmittgen, 2001). Results were normalized to GAPDH expression and expressed as log base 2 of the relative gene expression vs. ischemia as calibrator (Yuan et al., 2006).

Neurological function evaluation

At 24 h of reperfusion after 1 h MCAO, rats were subjected to neurological function evaluation (Long et al., 2013). Scoring system includes five parts and a maximum score of 18 (0 = normal, 1–6 = minor injury, 7–12 = moderate injury, 13–18 = sever injury, 18 = complete loss of neurological function). Rats with scores > 6, were considered successful ischemic.

Infarct volume assessment

After scoring neurological function, rats were sacrificed under deep anesthesia with chloral hydrate (800 mg/kg, i.p.) and the brains were separated and immersed in saline at 4 °C for 10 min. Thereafter, six 2-mm coronal sections were made from the frontal to temporal side and were stained with 2% 2,3,5-triphenyltetrazoliumchloride (TTC) at 37 °C for 30 min (Lin et al., 2013). The areas of the infarction (pale color) were measured using image J software (NIH image). Corrected infarct volume = Left hemisphere volume – (Right hemisphere volume – Infarct volume).

Statistical analysis

Data were analysed by GraphPad Prism 7 software, using one-way ANOVA. Dunnett was used as the post test for multiple comparisons and statistical significance was measured as $P < 0.05$.

Results

Generation of pure 14-3-3ζ protein

The recombinant protein, fused to 6 His-tag in its C-terminal was expressed inside bacterial cells, and showed a molecular mass of approximately 30 kDa (Fig. 1).

Assessment of BDNF mRNA expression

Fig. 2 shows the BDNF mRNA expression data as log base 2 of relative BDNF mRNA expression (ratio) vs. ischemia group as calibrator. The results revealed that BDNF mRNA was increased significantly in ischemic vs. sham operated rats in the hippocampus, in the cortex and also in the pir-amygdala (log 2 expression ratio ± SD of Ischemia vs. Sham: 1.03 ± 0.37, $P < 0.05$; 0.87 ± 0.08, $P < 0.05$; 0.73 ± 0.24, $P < 0.05$, respectively).

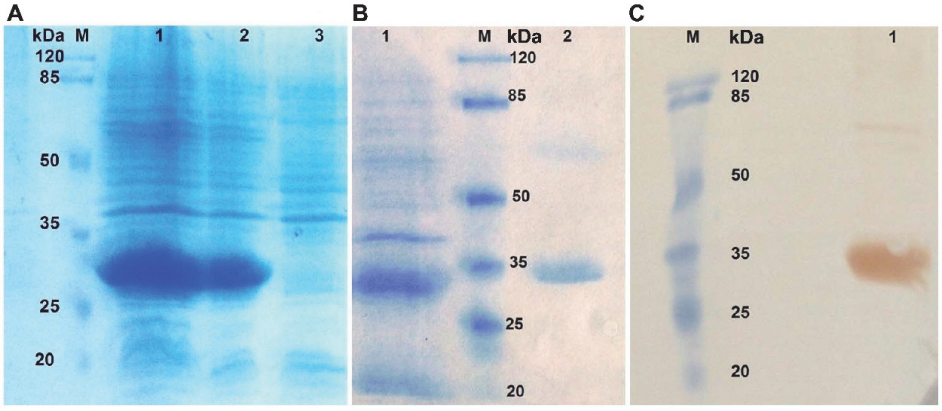

Fig. 1. Generation of 14-3-3ζ protein.

(**A**) Expression of 14-3-3ζ protein in *E. coli* BL21 (DE3) cells was induced by 1 mM IPTG at 37 °C and supernatant of bacterial cell lysate was loaded on SDS/PAGE gel and stained with Coomassie brilliant blue R250 after electrophoresis. Lane M, protein size marker; Lane 1, 5 h after induction; Lane 2, 3 h after induction; Lane 3, before induction. (**B**) Purification of recombinant His-tagged protein. Lane 1, supernatant of cell lysate; Lane M, protein size marker; Lane 2, purified 14-3-3ζ. (**C**) Western blot analysis of purified His-tagged 14-3-3ζ with anti His-tag antibody. Lane M, protein size marker; Lane 1, purified 14-3-3ζ.

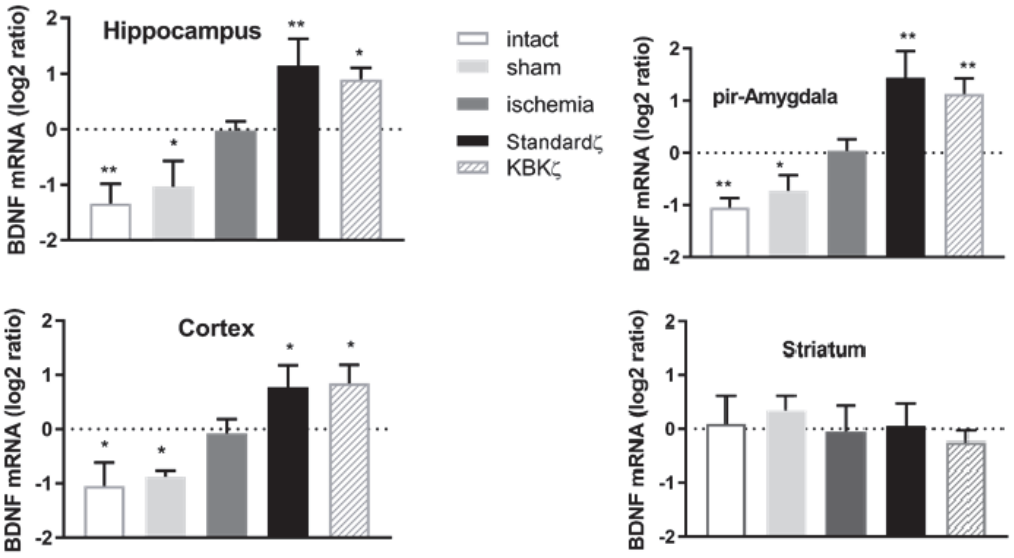

Fig. 2. qPCR analysis.

BDNF mRNA expression in four parts of brain, separated from five groups of animals [Intact (n = 3), sham (6 h after sham operation + PBS, n = 3), Ischemia (6 h reperfusion after 1 h MCAO + PBS, n = 4), standardζ (6 h reperfusion after 1 h MCAO + standard 14.3.3ζ, n = 4) and KBKζ (6 h reperfusion after 1 h MCAO + our lab 14.3.3ζ, n = 4)]. BDNF mRNA has been induced by ischemia in ischemia vs. sham and by standardζ and KBKζ in treated vs. ischemia groups. Results are expressed as log base 2 ratio vs. ischemia as calibrator and are the average ± SD of independent nonpooled samples assayed in duplicate.

* $P < 0.05$ by One-Way ANOVA and Dunnet post-test for comparing every group to ischemia group as control.

** $P < 0.01$.

The results also revealed that BDNF mRNA was up-regulated further by standard 14-3-3ζ in the hippocampus, in the cortex and also in the pir-amygdala (log 2 expression ratio ± SD of Standardζ vs. Ischemia: 1.14 ± 0.39, $P < 0.01$; 0.78 ± 0.32, $P < 0.05$; 1.44 ± 0.41, $P < 0.01$, respectively). KBK 14-3-3ζ also up-regulated BDNF mRNA in the hippocampus, in the pir-amygdala and in the cortex (log 2 expression ratio ± SD of KBKζ vs. Ischemia: 0.90 ± 0.16, $P < 0.05$; 0.84 ± 0.27, $P < 0.05$; 1.30 ± 0.23, $P < 0.01$, respectively).

Thus, ischemia up-regulated BDNF mRNA by 2.042-fold (log base 2 ratio = 1.03) in the hippocampus and also standard 14-3-3ζ and KBK14-3-3ζ increased it by 2.20 and 1.86-fold respectively. A 1.82-fold up-regulation of BDNF mRNA was observed by ischemia in the cortex, and 14-3-3ζ and KBK13-3-3ζ caused its further induction by 1.71 and 1.79-fold respectively. In the pir-amygdala, ischemia caused over expression of BDNF mRNA 1.65-fold and 14-3-3ζ and KBK13-3-3ζ up-regulated it by 2.71 and 2.46-fold respectively.

No significant elevation of BDNF mRNA was observed by ischemia in ischemic compared to sham operated animals in the striatum. Neither standard 14-3-3ζ nor KBK13-3-3ζ up-regulated BDNF mRNA in treated compared to untreated rats, in this part of brain (Fig. 2).

Protection of 14-3-3ζ against focal cerebral ischemia

Treatment with standard 14-3-3ζ and KBK14-3-3ζ significantly improved neurological function. Neurological severity score mean was 10 (8 to 11) in standardζ group and 11 (8 to 12) in KBKζ group, while it was 14 (13 to 15) in ischemia group (Table 1).

Neurological severity score reduction was confirmed by infarct volume data. Recombinant 14-3-3ζ (standard and KBK) significantly reduced total infarct size in the right hemisphere (Fig. 3).

Table 1. Neurological severity scores. Standard and KBK 14-3-3ζ reduced neurological severity scores.

Animal group	Rats/Score scale			Total	Mean	P value
	1–6	6–12	12–18			
Ischemia	0	0	6	6	14	
Sham	3	0	0	3	3	*
Standardζ	0	6	0	6	10	*
KBKζ	0	5	1	6	11	*

* Significant difference compared to ischemia group by One-Way ANOVA and Dunnet post-test for comparing every group to ischemia group as control.

Discussion

In our knowledge it is the first time that the positive effect of 14-3-3ζ recombinant protein on BDNF transcription in the ischemic brain and also its protective effect in this pathogenic condition is reported. We purified 14-3-3ζ for conducting the experiments and the standard form of the protein was used as control to verify the prepared protein.

The findings of the present study showed that: 1. BDNF mRNA was increased in the hippocampus, in the cortex and in the piriform cortex-amygdala, ipsilaterally, by 1 h focal cerebral ischemia after 6 h of reperfusion. 2. 14-3-3ζ treatment up-regulated already increased BDNF mRNA in the hippocampus, in the cortex and in the piriform cortex-amygdala, ipsilaterally, at 6 h of reperfusion after MCAO in treated animals compared to untreated animals. 3. i.c.v. administration of 14-3-3ζ improved neurological function and reduced cerebral infarct volume in this rat focal cerebral ischemia-reperfu-

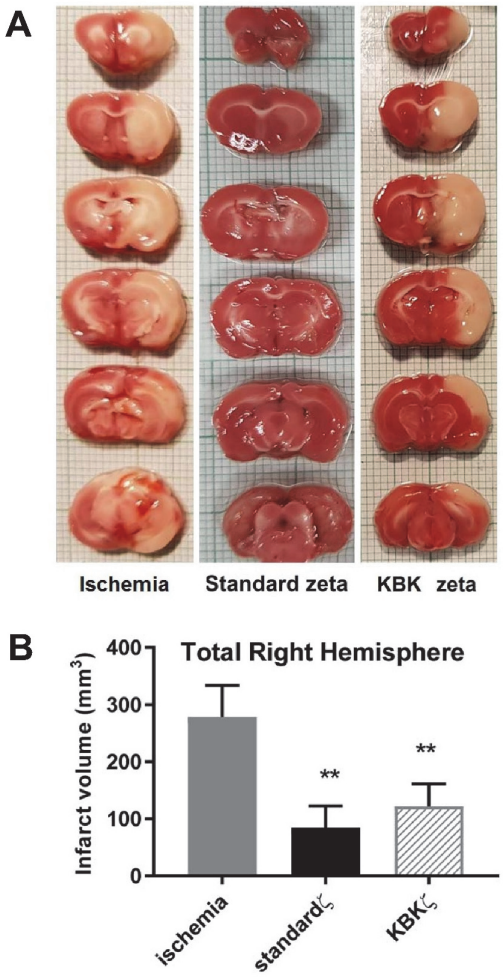

Fig. 3. Infarct volume analysis.

(**A**) Representative TTC-stained brain coronal sections from three groups of rats [Ischemia (24 h reperfusion after 1 h MCA0, *n* = 6), standardζ (24 h reperfusion after 1 h MCAO + standard 14.3.3ζ, *n* = 6) and KBKζ (24 h reperfusion after 1 h MCAO + our lab 14.3.3ζ, *n* = 6). (**B**) Assessment of total right brain hemisphere infarct volume from each group. Standard 14.3.3ζ and KBK14.3.3ζ decreased total infarct volume in right hemisphere. Results are the average ± SD.
* $P < 0.05$ by One-Way ANOVA and Dunnet post-test for comparing every group to ischemia group as control.
** $P < 0.01$.

sion model. The same results were concluded using standard 14-3-3ζ and KBK 14-3-3ζ.

Induction of BDNF mRNA ipsilaterally, in the cortex, hippocampus and piriform cortex-amygdala, but not in the striatum, at 6 h reperfusion after ischemia, was observed in the present study and it confirmed other reports (Arai et al., 1996; Dmitrieva et al., 2016; Kokaia et al., 1995; Lindvall et al., 1992; Mengozzi et al., 2012; Rickhag et al., 2007; Schmidt-Kastner et al., 2001). BDNF is an immediate early gene whose transcription could take place in the absence of *de novo* protein synthesis and various stimuli can induce rapid and transient synthesis of its mRNA (Okuno, 2011). *In situ* hybridization data have shown that BDNF mRNA has been induced in the cerebral cortex ipsilaterally surrounding the ischemic core and bilaterally in the hippocampus at early time points after MCAO and then gradually decreased to the basal level by 24 h. Based on their reports, no BDNF mRNA induction has been observed in the striatum (Arai et al., 1996; Kokaia et al., 1995). BDNF mRNA is absent in the striatum and BDNF protein is synthesized and anterogradely transported to the adult striatum from the cell bodies located in cerebral cortex, substantia nigra, amygdala, and thalamus (Baydyuk and Xu, 2014). Striatum is also a part of ischemic core in the MCAO model.

14-3-3ζ could up-regulate the already increased level of BDNF mRNA in the hippocampus, cortex and piri-amygdala at early time point after ischemia. Brain ischemia disrupts neuronal Ca^{2+} homeostasis and cytoplasmic Ca^{2+} is increased dramatically and causes neuronal death after ischemia (Neumar, 2000). Elevation of intracellular calcium leads to the activation of cAMP/PKA, Ras/ERK, and CaM kinase pathways (Zheng et al., 2012). Direct interaction between 14-3-3ζ and RACK1 has been reported to regulate BDNF transcription. It has been shown that this interaction is necessary for BDNF regulation in response to cAMP/PKA signaling and mediates translocation of RACK1 from cytosol to the nucleus and depletion of 14-3-3ζ inhibits BDNF transcription in SH-SY5Y cells and in hippocampal neurons in response to cAMP (Neasta et al., 2011). 14-3-3ζ has been shown to directly bind to Raf and activate MEK/ERK, finally leading to phosphorylation and activation of CREB (Freed et al., 1994). Finally, from this interaction, another group reported that 14-3-3ζ over expression activates the MEK/ERK pathway and consequently increases the binding of CREB to CRE, thus leading to transcriptional up-regulation of genes under regulation of CRE containing promoter in their system. By targeting MEK/ERK/CREB pathway, they observed the inhibition of gene of their interest (Chang et al., 2016). CREB is located in transcription regulatory system of BDNF gene (Chang et al., 2016; Zuccato and Cattaneo, 2009). CREB is a key transcription factor that modulates stimulus-dependent transcription. Activity-dependent BDNF expression is regulated by intracellular Ca^{2+} and CREB (Numakawa et al., 2010). Therefore, the finding of the present study that 14-3-3ζ administration was associated with elevation of BDNF mRNA in ischemia is line with regulatory effects of 14-3-3ζ reported by other groups.

BDNF exerts its protective effect through pro-survival mechanisms, regulating synaptic plasticity and neurogenesis (Nagahara and Tuszynski, 2011; Zuccato and Cattaneo, 2009). Recently, an excellent study has reported its protective effect on the cortex and hippocampus using single ΨεRACK treatment in an ischemic preconditioning model by which BDNF expression is up regulated (Neumann et al., 2015). Up regulation of BDNF mRNA in the brain after ischemia is an inherent mechanism; its pattern has been stated to be associated with its protectivity (Arai et al., 1996; Kokaia et al., 1995). It is also a mechanism, ischemic preconditioning takes advantage from. Based on the finding of the present study, 14-3-3ζ has a positive effect on this inherent protective mechanism. Thus, it can be expected that 14-3-3ζ may ameliorate ischemia damages by increasing BDNF expression.

14-3-3ζ could reduce infarct size and reduce neurological severity score in this model of focal ischemia. In addition to the effects that 14-3-3ζ may have on BDNF production, it may exert its protective effect through other mechanisms, too. 14-3-3ζ as a major pro-survival protein (Kasinski et al., 2014), sequesters pro-apoptotic proteins like apoptosis signal-regulate kinase 1 (Ask 1) and Fkhrl1 (a member of Forkhead transcription factors) and antagonize their death-promoting activities (Berg et al., 2003). Knockdown of 14-3-3ζ causes endoplasmic reticulum stress and cell death in mouse hippocampal cultures. ER stress takes places as a consequence of disruption of cellular Ca^{2+} homeostasis. Transgenic overexpression of 14-3-3ζ is protective against ER stress *in vivo* (Brennan et al., 2013). ER stress involved genes are reported as part of dif-

ferentially expressed genes after MCAO (Wang et al., 2017). Brennan et al. also reported protection of zeta protein overexpression against excitotoxicity-induced nerodegeneration *in vivo* (Brennan et al., 2013). Smani et al. (2018) have recently reported downregulation of 14-3-3 isoforms by kainic acid in vivo and proposed sustaining enough level of this proteins as a target of therapeutic intervention against neurodegeneration for neuronal injury induced by excitotoxicity. 14-3-3 proteins have shown neuroprotective effects by regulating apoptosis via targeting pro-apoptotic proteins (Fan et al., 2012; Masters et al., 2001; Noh et al., 2006; Zhu et al., 2014). Since 14-3-3ζ interacts with a variety of molecules, different mechanism could be proposed to explain its neuroprotectivity in our study.

Since BDNF has short half-life in plasma (~1 h) and also poorly penetrates blood-brain barrier, its clinical potential as treatment against ischemia has been limited. The results of the present study introduce administration of 14-3-3ζ as an alternative method for increasing endogenous BDNF in the brain.

Despite BDNF that is a positively charged protein, 14-3-3ζ is an acidic protein. Clearance of anionic molecules through glomerular infiltration is much smaller than that of the neutral and cationic molecules (Rennke et al., 1978). On the other hand, it has been reported that 14-3-3ζ could not cross the blood brain barrier (BBB) even in ischemia condition when the barrier is damaged (Zhu et al., 2014). These authors used this isoform intravenously 2 h before ischemia. In our study, 14-3-3ζ was administered intraventriculary and its ability to cross the BBB remained unknown. Furthermore, BDNF protein level at later time points after ischemia was not assessed. Then, more studies are needed to reveal whether 14-3-3ζ could pass intact BBB or enter the brain in ischemic condition when is used intravenously as well as if up-regulation of BDNF mRNA would lead to its protein level elevation at later time points.

Kawamoto et al proposed the possible involvement of 14-3-3 proteins in astrogliosis and intermediate filament (IF) formation (Kawamoto et al., 2006). Asterogliosis is a defensive astroglical reaction against ischemia and animals lacking IF in their reactive astrocytes have shown larger infarction after ischemia (Li et al., 2008) but nevertheless, it may have some negative effects on regeneration (Pekny and Pekna, 2014). Transgenic mice over expressing 14-3-3ζ have shown normal brain phenotype and hippocampal morphology and the same level of astrocyte and microglia markers (Brennan et al., 2013). Although in the present study zeta protein was increased only transiently, the possible harmful effect of its increased level should be studied and 14-3-3 protein significance in cancer formation (Hermeking, 2003) should be considered in other studies.

Conclusions

14-3-3ζ could increase BDNF mRNA 6 h after MCAO in rat. It also could reduce infarct size and neurological severity scores in this model of focal ischemia. In conclusion this study introduces 14-3-3ζ protein as a potential factor for increasing endogenous BDNF in the brain and also a candidate therapeutic protein against cerebral ischemia.

Conflict of interests

The authors have no conflict of interests to declare.

Acknowledgements

This article was extracted from Naeemeh Khalesi's PhD thesis and was supported by the School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences (Grant Number: 8402), and was carried out at the Cellular and Molecular Biology Research Center, the directors of which we gratefully.

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