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COMPARISON BETWEEN SINGLE AND COMBINED POST-TREATMENT WITH S-METHYL-N,N-DIETHYLTHIOLCARBAMATE SULFOXIDE AND TAURINE FOLLOWING TRANSIENT FOCAL CEREBRAL ISCHEMIA IN RAT BRAIN

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Abstract—We have recently reported on the efficacy of an N-methyl-D-aspartate (NMDA) receptor partial antagonist, S-Methyl-N,N-diethylthiolcarbamate sulfoxide (DETC-MeSO), in improving outcome following stroke, including reduced infarct size and calcium influx, suppressing the endoplasmic reticulum (ER) stress-induced apoptosis as well as improving behavioral outcome. DETC-MeSO was shown to suppress the protein kinase R-like endoplasmic reticulum kinase (PERK) pathway, one of the major ER stress pathways. Several studies including ours have provided evidence that taurine also has neuroprotective effects through reducing apoptosis and inhibiting activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE-1) pathways. We hypothesized that a combined treatment with DETC-MeSO and taurine would ameliorate ischemia-induced brain injury by inhibiting all three ER stress pathways. Twenty four hours following reperfusion of a 2-h ischemic stroke, rats received either 0.56-mg/kg DETC-MeSO or 40-mg/kg of taurine, either alone or in combination, subcutaneously for 4 days. Our study showed that combined DETC-MeSO and taurine, but not DETC-MeSO alone at the dose used, greatly reduced the infarct size, improved performance on the neuro-score test and attenuated proteolysis of αII-spectrin. Meanwhile, the level of the pro-apoptotic protein, Bax, declined and the anti-apoptotic protein, B-cell lymphoma 2 (BCL-2), expression was markedly increased. Combination therapy decreased both caspase-12 and caspase-3 activation by preventing the release of Cytochrome-c from mitochondria, indicating attenuation of apoptosis in ischemic infarct. Glucose-regulated protein (GRP)78 as a marker of the unfolded protein response decreased and levels of the key ER stress protein markers p-PERK-ATF4, p-eIF2α and cleaved-ATF-6 were found to significantly decline. NeuN expression levels indicated that more neurons were protected in the presence of DETC-MeSO and taurine. We also showed that combined treatment can prevent gliosis and increase p-AKT a pro-survival marker in the penumbra. Therefore, we conclude that combined treatment with both DETC-MeSO and taurine synergistically inhibits all three ER stress pathways and apoptosis and therefore can be a novel and effective treatment after ischemic stroke. Published by Elsevier Ltd. on behalf of IBRO.

Key words: stroke, DETC-MeSO, taurine, apoptosis, endoplasmic reticulum stress.

INTRODUCTION

Ischemic stroke is by far the more common type of stroke, causing 87% of all strokes (Go et al., 2014) and leading to serious disability and death. Despite improvements in clinical care, stroke morbidity and mortality is still high and new neuroprotective pharmacological strategies are required (Go et al., 2014). Currently, the only widely approved treatment for ischemic strokes is the tissue plasminogen activator (tPA) which should be the first-line treatment for patients within 4.5 h from the onset (Zivin, 2009). However, its use remains limited and only about 10% of eligible patients can be treated with tPA (Minnerup et al., 2011). Therefore, there is an urgent need for developing safer therapies that can be offered

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Abbreviations: ANOVA, analysis of variance; ASK-1, apoptosis signal-regulating kinase 1; ATF, activating transcription factor; BCL-2, B-cell lymphoma 2; CHOP, C/EBP-homologous protein; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GRP, glucose-regulated protein; HEPES, 4-(2-hydroxyethyl)-1-piperazinee thanesulfonic acid; IRE-1, inositol requiring enzyme 1; LCBF, local cerebral blood flow; MCAO, middle cerebral artery occlusion; NMDA, N-methyl-D-aspartate; PERK, protein kinase R-like endoplasmic reticulum kinase; tPA, tissue plasminogen activator; TTC, 2,3,5-triphenyltetrazolium chloride; UPR, unfolding protein response; XBP1, X box-binding protein 1.

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to a higher percentage of patients. Although some pharmacological agents exist (Zivin, 2009; Turner and Vink, 2012; George et al., 2013; Gharibani et al., 2013; Modi et al., 2014; Mohammad-Gharibani et al., 2014; Venna et al., 2014; Xu et al., 2015), it is very important to establish how these agents could be neuroprotective in post-stroke individually or in a combined manner. Following stroke, the ischemic core and the peri-infarct zone (ischemic penumbra) suffer from different degrees of cellular damage and it is widely accepted that cell death in the ischemic core is triggered by necrosis while that which occurs in the penumbra is predominantly mediated by apoptosis (Kiewert et al., 2010). Taurine as a neurotransmitter, neuromodulator, membrane stabilizer, and major intracellular free amino acid has been employed in experimental therapies against neuronal damage, hypoxia, and epilepsy in several studies (Birdsall, 1998; Louzada, 2004; Sun and Xu, 2008; Sun et al., 2011). We and other investigators have previously shown that post-stroke treatment with taurine can reduce rat neurological deficits, brain infarct volume, and also caspase-3 activities in the ischemic penumbra following middle cerebral artery occlusion (MCAO) (Guan et al., 2011; Sun et al., 2011; Gharibani et al., 2013). Moreover, taurine inhibited the endoplasmic reticulum (ER) stress-induced apoptosis by activating transcription factor 6 (ATF6) and the inositol requiring enzyme 1 (IRE1) pathway, but not the protein kinase R-like endoplasmic reticulum kinase (PERK) pathway (Pan et al., 2010; Gharibani et al., 2013). Our recent findings have demonstrated that S-Methyl-N,N-diethylthiol carbamate sulfoxide (DETC-MeSO), a metabolite of disulfiram and an Food and Drug Administration (FDA) approved drug for alcoholism (Hart and Faiman, 1994), can be a good candidate against glutamate-induced toxicity *in vitro* (Mohammad-Gharibani et al., 2014). Moreover, we have shown that post-stroke treatment with DETC-MeSO as a partial antagonist of N-methyl-D-aspartate (NMDA) receptor can prevent the calcium influx occurring through overactivated NMDA receptors and hence reduce apoptosis and ER stress as well as improve the neurological behavior of rats (Mohammad-Gharibani et al., 2014). In contrast to taurine, DETC-MeSO inhibited ER stress-induced apoptosis arising through the PERK pathway. There should be minimal clinical concern about using taurine as it is a natural free amino acid, while using DETC-MeSO as a pharmaceutical medication could have more possible side-effects after stroke. Therefore, in this study we tested the effect of a lower dose of DETC-MeSO with or without taurine in post-stroke treatment using the MCAO model.

EXPERIMENTAL PROCEDURES

Ethics

All animal procedures were carried out in accordance with the guidelines for care and use of animals and were approved by the institutional animal care and use committee of the Florida Atlantic University, Boca Raton, Florida. Adult male Sprague–Dawley rats (n = 41, 260–300 g, Harlem Chicago, IL, USA) were used in this study. After transport, animals were rested for several days before inclusion in any experiment. Animals were group housed in a conventional rodent room on a 12/h day–night cycle and provided with a standard diet of rodent pellets and water *ad libitum*.

Focal middle cerebral artery occlusion

Animals were fasted overnight before surgery and then anesthetized by intra-peritoneal injection of ketamine hydrochloride (80 mg/kg; Putney) and xylazine hydrochloride (20 mg/kg; Vedco) (Gharibani et al., 2013; Mohammad-Gharibani et al., 2014). As described previously, transient focal brain ischemia was induced by the intraluminal suture occlusion technique (Gharibani et al., 2013; Modi et al., 2014; Mohammad-Gharibani et al., 2014). Briefly, after the left common carotid artery and the left external carotid artery were exposed through a midline neck incision, a 4-0 monofilament nylon suture coated with silicon (Doccol Co., Albuquerque, NM, USA) was inserted in the external carotid artery and gently advanced approximately 17 mm from the carotid bifurcation into the internal carotid artery. During the surgery the core temperature was maintained at 37 ± 0.5 °C by a thermostatically controlled heating pad regulated via a rectal temperature probe (CMA 450). Local cerebral blood flow (LCBF) was monitored in the cerebral cortex of the left hemisphere in the supply territory of the middle cerebral artery by laser doppler flowmeter (Perimed Inc., North Royalton, OH, USA). Reduction in LCBF was detected when the filament was appropriately inserted. Rats were excluded from the study if they did not show a LCBF reduction of at least 70% (Mohammad-Gharibani et al., 2014). Reperfusion was accomplished by withdrawing the filament 2 h after MCAO (Longa et al., 1989; Sun et al., 2011).

Study design

Animals (n = 41) were randomly assigned for sham, control and experimental groups. In experimental groups animals received either DETC-MeSO (n = 9, 0.56 mg/kg in 1 mL saline 0.09%) or taurine (n = 9, 40 mg/kg in 1 mL saline 0.09%), either alone or in combination (n = 9, 0.56 mg/kg in 0.5 mL saline 0.09% + taurine 40 mg/kg in 0.5 mL saline 0.09%) 24 h after reperfusion, subcutaneously. Animals received treatment for 4 days before sacrifice. In the control group (n = 9) vehicle (1 mL saline 0.09%) was injected subcutaneously 24 h after reperfusion for 4 days before sacrifice. All injections were done by an investigator who was blinded for group assignments. Sham-operated group (n = 5) received the same surgical procedure without insertion of the silicon filament. After surgery, animals were allowed to recover from anesthesia and given food and water *ad libitum*. The animals were daily examined for body temperature and weight and those who had a body temperature more than 39 °C after 24 h were excluded from the experiment (Li et al., 1999; Mohammad-Gharibani et al., 2014).

Assessment of infarct volume. At 4 days after stroke animals were deeply anesthetized by Isoflurane (Phoenix). Their brains were rapidly removed and by an

adult rat brain slicer (Matrix, Zivic Instruments, Pittsburgh, PA, USA) brains were sectioned coronally into six 2-mm coronal slices (2, 4, 6, 8, 10, and 12 mm from the frontal pole). Brain slices were incubated for 5 min in a pre-warmed 37 °C 2% (w/v) solution of 2,3,5-triphenyltetrazolium chloride (TTC, J.T. Baker, Faridabad, India) under dark room conditions (in incubator) for staining (Kramer et al., 2010). TTC stains mitochondria and turns the normal tissue deep red, while the infarcted tissue remains a pale white color (Bederson et al., 1986; Rich et al., 2001). Thus, reduced TTC staining identifies regions of diminished mitochondrial function in the ischemic tissue. To assess lesion volume, TTC-stained slices were scanned using an HP ScanJet 5530 and analyzed by Image-J analysis software (http://rsb-web.nih.gov/ij/). Lesion volume was determined as the percent of the total ipsilateral hemispheric volume as described previously (Swanson et al., 1990; O'Donnell et al., 2006). Briefly, to eliminate the effect of brain edema, the corrected infarct volume was calculated as follows: [(VR − VLn)/VR] 100 in which VR is the volume of the right hemisphere and VLn is the volume of nonlesioned tissue in the left hemisphere (Schäbitz et al., 1999, 2000; O'Donnell et al., 2006). After the TTC staining, while the sections were on ice, the ischemic penumbra of the left hemisphere (as shown in Fig. 1A) were quickly dissected and snap frozen (Ashwal et al., 1998).

Assessment of neurological deficit. The neurological deficit was assessed by neuro-score assessment according to the description of Menzies et al. (1992) with small modifications as we have described previously (Gharibani et al., 2013; Mohammad-Gharibani et al., 2014). This assessment has been shown to be correlated with infarct volume in a variety of previous studies (Menzies et al., 1992; Schäbitz et al., 2001; Mohammad-Gharibani et al., 2014). Assessments were carried out every day for 4 days after MCAO by an observer blinded to the experimental grouping of the animals. Briefly, this six-point neuro-score is a rating scale between 0 to 5 as follows: 0 = indicating no spontaneous movement, 1 = circling spontaneously toward the paretic side, 2 = circling toward the paretic side if pulled by tail, 3 = with severe consistently reduced resistance to lateral push toward the paretic side, 4 = with consistent flexion of the forelimb contralateral to the injured hemisphere and 5 = with both forelimbs extended toward the floor.

Western blot analysis. Cytosolic and whole-cell extracts from the ischemic penumbra of the ipsilateral hemisphere were prepared as described previously (Mohammad-Gharibani et al., 2014). Briefly, whole-cell lysate samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin) (Thermo scientific, Rockford, IL, USA) containing 1% (v/v) mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 2% (v/v) phosphatase inhibitor cocktail

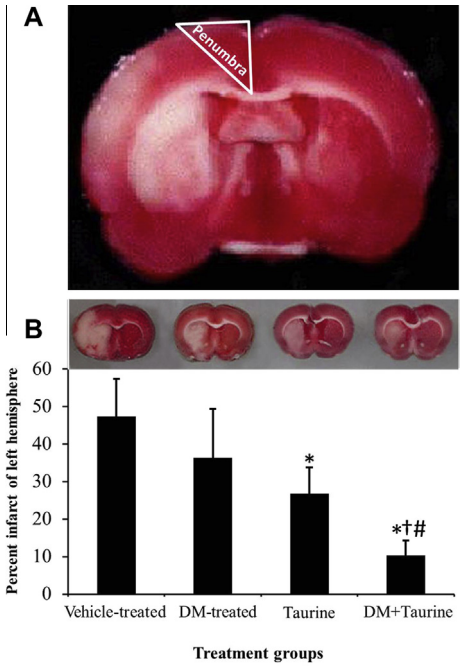

Fig. 1. TTC staining of a brain section and effect of DETC-MeSO and taurine on the infarct size of the ischemia-induced brain injury in MCAO stroke model. (A) This micrograph shows how to sample the penumbra (white triangle) from a TTC incubated slice for Western blot. (B) TTC results of the infarct volume of the core and the penumbra of brain slices from the vehicle, DETC-MeSO, taurine and DETC-MeSO + taurine-treated groups at 4 days after reperfusion. Representative 6-mm coronal brain sections with 2% TTC staining were presented on the upper panel. Quantitative analysis revealed that both taurine and combined DETC-MeSO + taurine-treated groups after 4 days produced a significant reduction in the infarction percent versus vehicle-treated group. DETC-MeSO + taurine-treated group showed significant infarct size reduction with all groups. Sham-operated group showed no infarct zone. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO- (0.56 mg/kg) and taurine (40 mg/kg)-treated groups, respectively by ANOVA and Tukey post hoc tests ($N = 9$, $p < 0.05$).

(Thermo scientific). For cytosolic cell subfractionation, we followed Solaroglu et al. (2006). Firstly, the whole-cell lysates were obtained by homogenizing the brain sample with a homogenizer in five volumes of buffer A (20 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.1 mM PMSF, 1 mM dithiothreitol (DTT), 1% mammalian protease inhibitor cocktail and 2% phosphatase inhibitor cocktail; pH 7.9). Samples were further centrifuged at 750*g* at 4 °C for 15 min to separate the sample into supernatant A and pellet A. Supernatant A, containing the cytosolic/mitochondrial protein, was further centrifuged at 16,000*g* for 30 min at 4 °C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction. Protein concentrations were then determined by the Bradford protein assay. Proteins in cell lysates were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After proteins were transferred to a nitrocellulose membrane, the membrane was then blocked in blocking buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk or bovine serum albumin) for 1.5 h at room temperature. After blocking

membranes they were incubated overnight with the following antibodies. Abcam, Boston, MA, USA: glucose-regulated protein 78 (GRP78), glial fibrillary acidic protein (GFAP), αII-spectrin, ATF4 and p-IRE1 (1:2000); Cell Signaling, Danvers, MA, USA: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000), Bax, B-cell lymphoma 2 (BCL-2) (1:1000), caspase-3, AKT, p-AKT and Cytochrome-c (1:500); Santa Cruz, Dallas, TX, USA: CHOP (C/EBP-homologous protein)/GADD153 (growth arrest and DNA damage-inducible gene 153), caspase-12 (1:500); Imgenex, San Diego, CA, USA: ATF6 (1:1500); Sigma: m-calpain (1:2000); Millipore, Billerica, MA, USA: NeuN (1:1000). Secondary mouse and rabbit antibodies were purchased from Abcam (1:3000). Membranes were then incubated with ECL horseradish peroxidase-conjugated anti-Rabbit or anti-mouse IgG (1:3000; GE Healthcare, Hatfield, UK) for 90 min in room temperature. GAPDH was used as internal controls for cytosolic and whole-cell lysate samples. Extensive washes with blocking buffer were performed between each step. The protein immuno-complex was visualized using ECL detection reagents purchased from Thermo Scientific. Quantitative western blot results were obtained by densitometric analysis using Image processing and Analysis in Java (Image J). Sham-operated ischemic penumbra served as baseline for left hemisphere samples (impaired).

Data expression and statistical analysis

All data were expressed as the mean ± SEM. For TTC and western blot experiments, the statistical significance of the data was determined with *t*-test or a one-way analysis of variance (ANOVA) combined with Dunnett post hoc or Tukey test for comparison between groups. For the neuro-score test, we used Kruskal–Wallis ANOVA, followed by Dunn's test.

RESULTS

Infarct volume

Rats were sacrificed 4 days after MCAO and the brains were sliced. Cerebral ischemic areas were visualized by TTC and their volume was determined as described earlier in materials and methods. Fig. 1B (upper panel) shows representative TTC stained 6-mm sections starting from the frontal pole of TTC staining in rats subjected to MCAO in the vehicle-treated group versus DETC-MeSO, taurine and DETC-MeSO + taurine-treated groups. A clear infarct can be seen in the left hemisphere of the rats treated with vehicle, whereas the infarct is noticeably reduced in other experimental groups.

The lower panel of Fig. 1B represents the mean of the percent infarct volumes in different treatment groups. In taurine- and taurine + DETC-MeSO-treated groups, a significant reduction was seen in infarct size in comparison to the vehicle-treated group. However, the maximum reduction was seen in rats post-treated with the combination of DETC-MeSO and taurine, in which the mean size of the ischemic area was reduced by approximately 5-fold compared to the vehicle-treated group (9.3 ± 3.4% to 47.6 ± 10.2%, respectively, $P < 0.05$). There is also a significant difference in the size of the ischemic area (lesion area) between treatment groups. Greater improvement was seen in the rats post-treated with the combination of DETC-MeSO and taurine, than the other treatment groups, while no reduction was seen in DETC-MeSO-treated group. The sham-operated group showed no ischemic injury as determined by TTC staining.

Neurological deficit

The absence of any performance deficit was tested before surgery. Rats were tested every day for 4 days in groups as described in materials and methods. As shown in Fig. 2, the sham-operated animals showed no deficit in performance. Only animals that received DETC-MeSO + taurine showed a significant decrease in neurological deficits only at day 4 after reperfusion compared to vehicle-treated group. Animals that received DETC-MeSO or taurine showed no decrease in neurological deficits until the day 4 after reperfusion compared to the vehicle-treated group. No significant difference was seen between treatment groups.

Western blot

DETC-MeSO and taurine attenuate caspase-12 expression and proteolysis of αII-spectrin in the ipsilateral ischemic penumbra. Disturbance in calcium homeostasis and calpain activation may contribute to ischemic neuronal injury and apoptosis. Since m-calpain is activated by elevated intracellular calcium and it is required for caspase-12 activation and αII-spectrin cleavage (Nakagawa and Yuan, 2000), we examined both markers in our experiment. Following 2-h MCAO, significant accumulation of m-calpain was seen in all groups in comparison to the sham-operated group (Mohammad-Gharibani et al., 2014), however results did not show any changes in m-calpain regulation in treated groups (data not shown). Therefore we decided to test

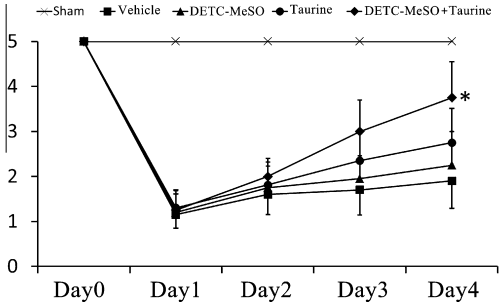

Fig. 2. Effect of DETC-MeSO and taurine on neuro-score assessment following ischemia-induced brain injury in MCAO stroke model after 4 days. Recovery was seen only in the group which received DETC-MeSO + taurine everyday started 24 h after reperfusion. All sham-operated animals showed no deficit with score 5. Values in the graphs represent mean ± SEM. * denote statistical significance between DETC-MeSO + taurine (0.56 mg/kg and 40 mg/kg, respectively) and vehicle-treated group, by Kruskal–Wallis ANOVA, followed by Dunn's test ($N = 9$, $p < 0.05$).

the m-calpain substrate, αII-spectrin and activation of caspase-12 which is dependent on m-calpain activation. In the ipsilateral ischemic penumbra (left hemisphere), MCAO caused a significant accumulation of the non-specific 150-kDa band (generated by calcium-activated m-calpain and/or caspase-3) and of the m-calpain-specific 145-kDa band as compared with the sham-operated group (Fig. 3A). Levels of the 150/145-kDa bands in the vehicle-treated group were significantly higher than in the sham-operated group (62.3 ± 8.3%), however in the single treated groups of DETC-MeSO and taurine, levels of the 150/145-kDa bands were significantly lower than in the vehicle-treated group. In treated groups which had received the combination of DETC-MeSO and taurine, levels of the 150/145-kDa bands were almost 2-fold lower than the other treated groups. On the other hand, the 120-kDa band was detected in sham-operated rats due to basal processing of αII-spectrin by caspase-3. This observation has been reported previously by Pike et al. (2004) and Zhang (2002). MCAO injury resulted in a significant increase in the 120-kDa band above levels of the sham-operated group. Levels of the caspase-3-specific 120-kDa fragment were 19.2 ± 4.3% and 18 ± 7.9% in the DETC-MeSO- and taurine-treated groups respectively, which was markedly lower than the vehicle-treated group (28.9 ± 5.6%). Our data showed that the level of the 120-kDa band in the penumbra of the combined treated group significantly decreased in comparison to either the single DETC-MeSO- or taurine-treated group (10.1 ± 2.9%).

It has been shown that m-calpain is responsible for cleaving procaspase-12, a caspase localized in the ER outer membrane, to generate active caspase-12 (Nakagawa and Yuan, 2000). Fig. 3B shows that following 2-h MCAO, significant up-regulation of caspase-12 and cleaved caspase-12 was seen in all groups in comparison to the sham-operated group. Caspase-12 protein expression was 2.9 ± 0.43% and 2.2 ± 0.37% in the DETC-MeSO- and taurine-treated group respectively, which was markedly lower than the vehicle-treated group (3.96 ± 0.6%). Combined DETC-MeSO and taurine down-regulated the expression of caspase-12 in comparison to DETC-MeSO- and vehicle-treated groups. Levels of the cleaved caspase-12 in single treatment of either DETC-MeSO or taurine showed no changes in comparison to the vehicle-treated group. In contrast, levels of the cleaved caspase-12 in the penumbra of the combined treated group declined almost 6-fold in comparison to either the single DETC-MeSO- or taurine-treated group.

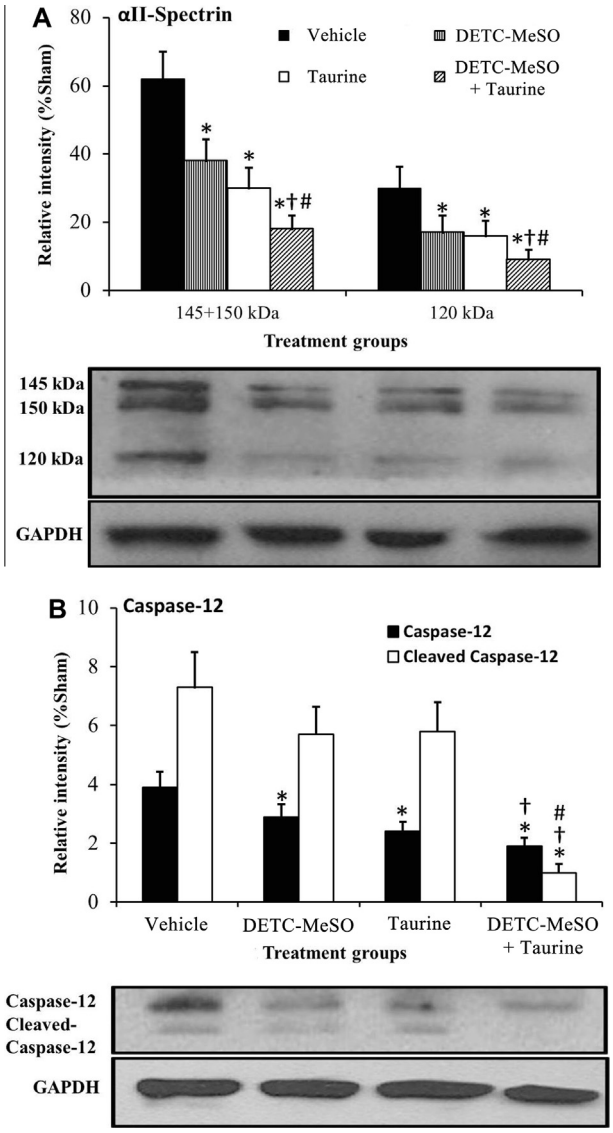

Fig. 3. Activation and cleavage of m-calpain and caspase-12. (A) Activation of m-Calpain was measured by cleavage of αII-Spectrin. Full-length αII-spectrin (280 kDa) is vulnerable to calcium-activated m-calpain and caspase-mediated cleavage, generating fragments of 150/145 kDa and 120 kDa. MCAO causes accumulation of full-length αII-spectrin protein (not shown) and calpain-mediated 145 kDa and caspase-3 mediated 120 kDa in the penumbra of the ipsilateral hemisphere but not the contralateral. Calpain- and caspase-mediated cleavage (145 and 120 kDa, respectively) were decreased significantly in the penumbra of all treated groups in comparison to the vehicle-treated group. Combined treated group showed a greater reduction of cleavage in comparison to the other treated groups. (B) Activated caspase-12 (cleaved) in the penumbra of MCAO brain was analyzed by Western blot after 4 days. Both caspase-12 and cleaved caspase-12 in the penumbra showed a dramatic decrease in the combined treated group in comparison to the other groups. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO- (0.56 mg/kg) and taurine (40 mg/kg)-treated groups, respectively by ANOVA and Tukey post hoc tests ($N = 5$, $p < 0.05$).

DETC-MeSO and taurine can decrease apoptosis by down-regulation of apoptotic markers. Expression levels of apoptosis-related proteins were investigated by Western blot analysis. The BCL-2 protein is known to promote cell survival, while Bax a proapoptotic protein, can translocate from the cytosol into the outer mitochondrial membrane after brain ischemia to release Cytochrome-c which activates caspase-3, the final stage of apoptosis (Sun et al., 2011). Our results demonstrate that DETC-MeSO (0.56-mg/kg 24 h after reperfusion for 4 days) could not increase the ratio of BCL-2/Bax in the penumbra of the DETC-MeSO-treated versus vehicle-treated group (Fig. 4A). However, in both taurine and DETC-MeSO + taurine groups a marked increase was seen in the ratio of BCL-2/Bax in comparison to the

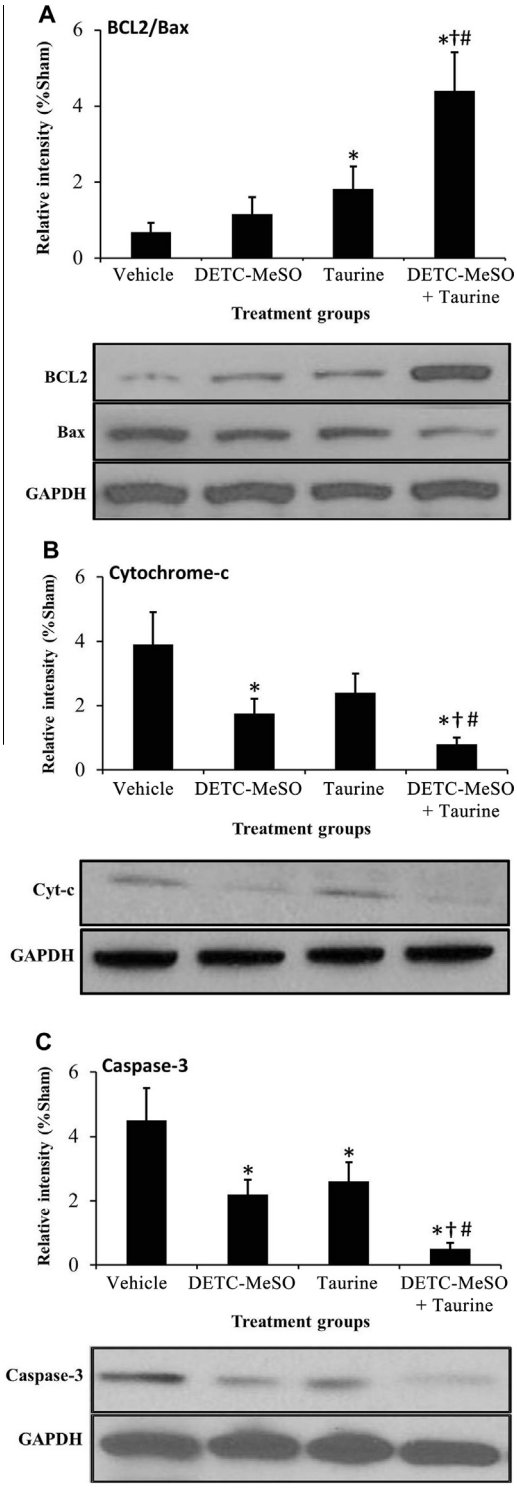

Fig. 4. Effect of DETC-MeSO and taurine on expression of Bax, Bcl-2 and caspase-3 in MCAO stroke model. (A) Bax and Bcl-2 expression in the penumbra of MCAO brain were analyzed by Western blot. The graph shows the ratio of Bcl-2 to Bax in the penumbra of MCAO brain in combined DETC-MeSO + taurine-treated group markedly increased versus other treated groups. (B) and (C) Cytochrome-c release from mitochondria into cytoplasm followed by caspase-3 expression reduced in the combined treated group. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO (0.56 mg/kg) and taurine (40 mg/kg)-treated groups, respectively by ANOVA and Tukey post hoc tests ($N = 5$, $p < 0.05$).

vehicle-treated group. In combined DETC-MeSO + taurine-treated group the level of the ratio of BCL-2/Bax was significantly up-regulated in comparison to the other treated groups. Up-regulation of BCL-2 could decrease the release of Cytochrome-c from the mitochondria (Fig. 4B) into the cytoplasm in the treated groups which in turn decreased caspase-3 in treated groups (Fig. 4C) in comparison to the vehicle-treated group. More importantly, the combined DETC-MeSO + taurine-treated group showed significant differences in release of both Cytochrome-c and caspase-3 versus the other treated groups.

DETC-MeSO and taurine can modulate the unfolded protein response and ER stress-induced apoptosis. Our data showed that following MCAO, GRP78 increased in all groups versus the sham-operated group. As we can see in Fig. 5A, both treatment groups of taurine and DETC-MeSO + taurine could decrease the expression of GRP78 in comparison to the vehicle-treated group. By contrast, DETC-MeSO did not demonstrate any changes of GRP78 expression after treatment. The combination DETC-MeSO + taurine-treated group showed a significant decline in GRP78 expression in comparison to the other treated groups. PERK, ATF6 and IRE1 are the three major ER stress-induced signaling pathways, activated by GRP78. We tested all three signaling pathways in ER stress. ATF6 after dissociation from GRP78, translocates from the ER to the Golgi apparatus where it is cleaved to its active form (Chen et al., 2002). Treatment with DETC-MeSO had no effect on the level of cleaved ATF6 in the penumbra of the infarct of MCAO rats, while taurine and DETC-MeSO + taurine both showed down-regulation of cleaved ATF6 (Fig. 5B). Then we tested the PERK pathway in the brain of rats subjected to MCAO occlusion. Only after combined treatment with DETC-MeSO + taurine, the levels of ATF4 in the penumbra dramatically declined in comparison to the vehicle and other treated groups (Fig. 5C), indicating that combined treatment can inhibit the PERK pathway. To determine whether IRE1 pathway can be affected, we tested the expression of p-IRE1 (activated form of IRE-1) in the penumbra of the infarct of MCAO rats with and without treatment. The results showed that phosphorylated IRE1 is highly expressed in the penumbra of the infarct in the MCAO rat brain in comparison to the sham-operated group. Our study showed that DETC-MeSO administration individually for 4 days had no effects on the expression of p-IRE (Fig. 5D). However, both treatment groups of taurine and DETC-MeSO + taurine prevented IRE-1 activation in the ischemic penumbra of infarct area. The combined treatment with DETC-MeSO + taurine could down-regulate p-IRE-1 expression to almost 50% of the levels in the individual taurine treatment group.

PERK, IRE-1 and ATF6 all converge on the promoter of the gene encoding the protein CHOP, which regulates the BCL-2 family (Kim et al., 2008). As shown in Fig. 5E, the expression of CHOP was up-regulated in the penumbra of the MCAO model by comparison to the sham-operated group. Western blot analyses showed that all

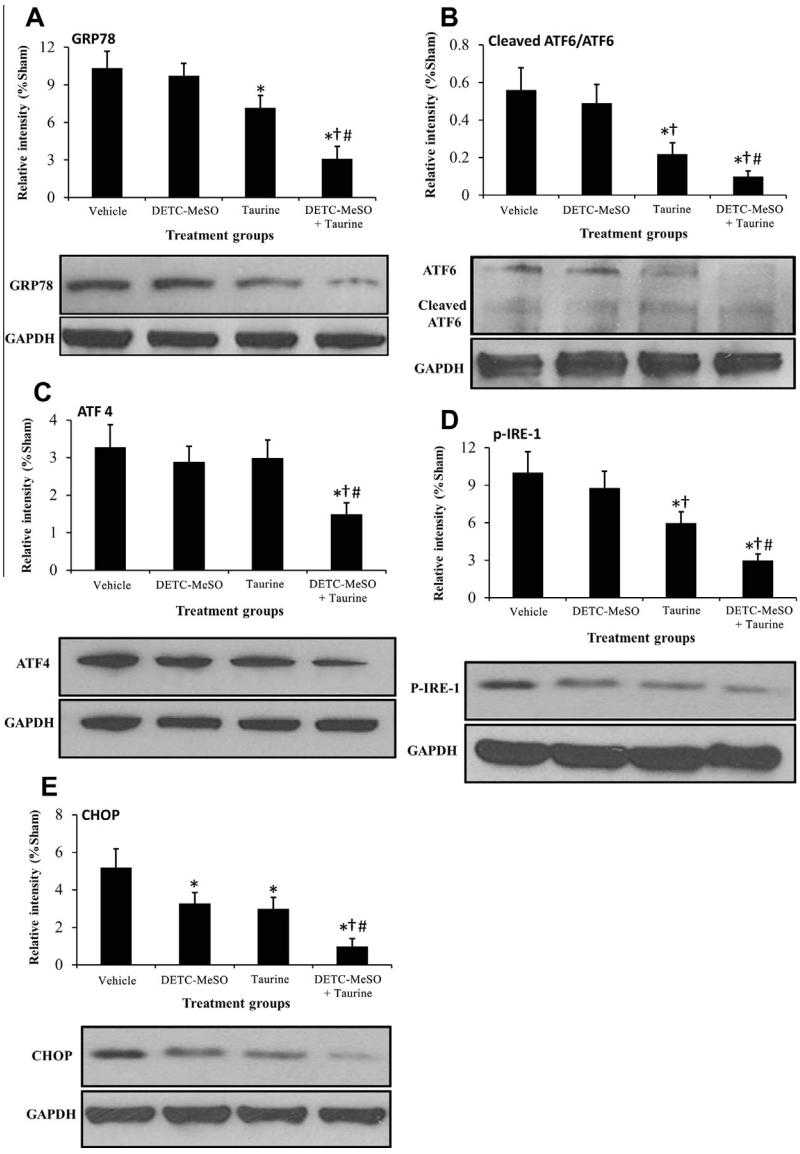

Fig. 5. Effect of DETC-MeSO on expression of UPR (GRP78) and ER stress proteins in the MCAO stroke model. (A) GRP78 expression as a marker of UPR in the penumbra of MCAO brain was analyzed by Western blot. GRP78 expression markedly decreased in the penumbra of combined DETC-MeSO + taurine-treated group versus the other treated groups. All three pathways of ER stress were checked in the penumbra of MCAO after 4 days treatment. Combined treatment of DETC-MeSO + taurine significantly declined all three pathways of ER stress including cleaved ATF6 (B), ATF4 (C) and p-IRE-1 (D) in comparison to the other treatment groups. E: Western blot analyses showed that all treatment groups can significantly decrease levels of CHOP in the ischemic penumbra of rat's brain. However, combined therapy more effectively inhibited CHOP and apoptosis induced by ER stress in comparison to the other treatment groups. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO- (0.56 mg/kg) and taurine-(40 mg/kg) treated groups, respectively by ANOVA and Tukey post hoc tests ($N = 5$, $p < 0.05$).

treatment groups can significantly decrease levels of CHOP in the ischemic penumbra of the rat brain. More importantly, we saw an almost 4-fold decrease in CHOP expression in the ischemic penumbra of combined DETC-MeSO + taurine-treated group versus other treatment groups, demonstrating that combined therapy has a more effective capacity inhibiting apoptosis induced by ER stress in the MCAO stroke model (Fig. 5E).

DETC-MeSO and taurine can attenuate gliosis in MCAO. Following MCAO, resting glia generate gliosis (Pérez-Álvarez et al., 2012) in the injury site. To evaluate the effect of our treatment on gliosis after MCAO, we analyzed GFAP, a marker of astrocytes, by western blot in the penumbra of the left hemisphere of treated and vehicle-treated groups. Our data showed that GFAP is increased significantly in the penumbra of MCAO rats by comparison to the sham-operated group (Fig. 6A),while using either taurine or combined DETC-MeSO + taurine significantly decreased GFAP expression in the ischemic penumbra in comparison to the vehicle-treated group. Individually DETC-MeSO did not show any changes in GFAP expression. Combined treatment with DETC-MeSO + taurine decreased gliosis in the penumbra by almost 2-fold versus the individual taurine-treated group. This could also be further

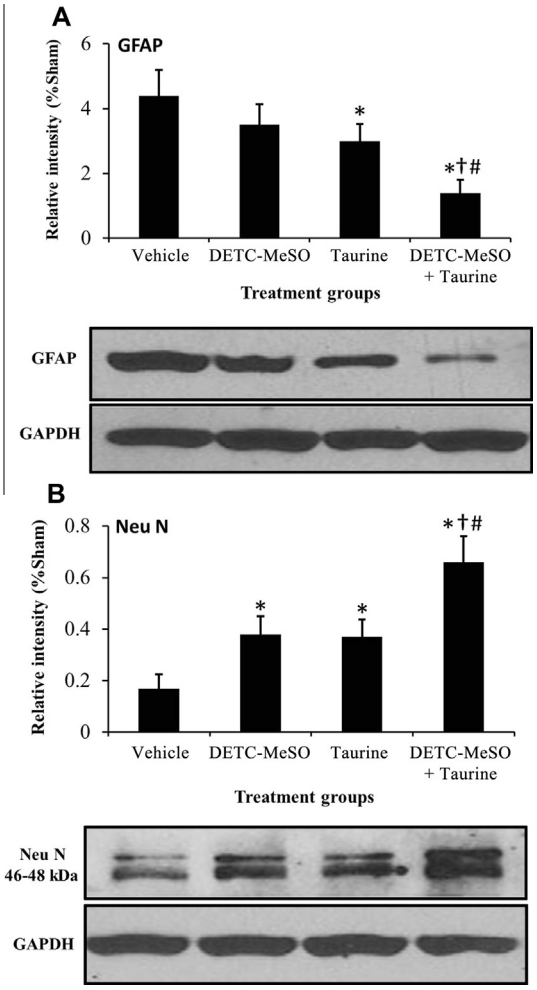

Fig. 6. Protective effect of DETC-MeSO and taurine on glia and neurons in MCAO stroke model. (A) Expression of GFAP, a marker of glia, in the penumbra of MCAO brain analyzed by Western blot. Combined DETC-MeSO and taurine significantly decreased GFAP expression in the penumbra of the combined treated group in comparison to the other treated groups. (B) Levels of NeuN, a marker of neurons, in the penumbra of MCAO brain were analyzed by Western blot. NeuN was decreased significantly in the penumbra of MCAO vehicle-treated group in comparison to sham-operated group. Loss of NeuN expression in all treated groups is significantly lower than the vehicle-treated group, indicating DETC-MeSO, taurine and combined DETC-MeSO + taurine are able to protect neurons from cell death. However, the combined treated group protects neurons from cell death more than the other treated groups. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO- (0.56 mg/kg) and taurine- (40 mg/kg) treated group, respectively by ANOVA and Tukey post hoc tests ($N = 9$, $p < 0.05$).

confirmed by analyzing NeuN, a marker which is expressed in all of the nuclei of most neuronal cell types but not in non-neuronal cells (Herculano-Houzel and Lent, 2005). As we see in Fig. 6B, the vehicle-treated group has almost lost 75% of NeuN-positive cells in the penumbra following MCAO. Our data showed that all treatment groups increased the percent of NeuN expression in the penumbra of MCAO rats in comparison to the vehicle-treated group. In addition, the level of NeuN expression in the penumbra of the combined DETC-MeSO + taurine group shows that this treatment can protect neurons from cell death significantly more than the other treatment groups.

DETC-MeSO and taurine can promote p-AKT level in MCAO. In our experiments the activated form of AKT (phosphorylated AKT or p-AKT) showed a dramatic up-regulation in the penumbra of the taurine and DETC-MeSO + taurine-treated groups in comparison to the vehicle-treated group (Fig. 7), whereas p-AKT expression showed no changes when DETC-MeSO alone was administered. A significant increase was seen in the level of p-AKT in the combined treatment group versus all treated groups.

DISCUSSION

In recent years, several studies including ours have shown that taurine as a semi-essential amino acid is beneficial in several neurological disorders such as stroke and traumatic brain injury (Huxtable, 1992; Birdsall, 1998; El Idrissi and Trenkner, 1999; Taranukhin et al., 2008; Wu et al., 2009; Pan et al., 2010, 2011; Sun et al., 2012; Gharibani et al., 2013). We have also recently reported using DETC-MeSO as an active metabolite of disulfiram and a potent and selective carbamoylating agent for sulfhydryl groups in glutamate receptors (Jin et al., 1994; Nagendra, 1997) can ameliorate neural damage following ischemic stroke (Mohammad-Gharibani et al., 2014). Taurine and DETC-MeSO individually have shown that they can down-regulate apoptotic factors in the brain damaged areas. On the other hand, as we have shown, taurine or DETC-MeSO administered individually had influences on decreasing ER stress-induced apoptosis and infarct size of the brain after ischemic stroke. However since either one has its own mechanism of action, we hypothesized that combined post-treatment using these molecules

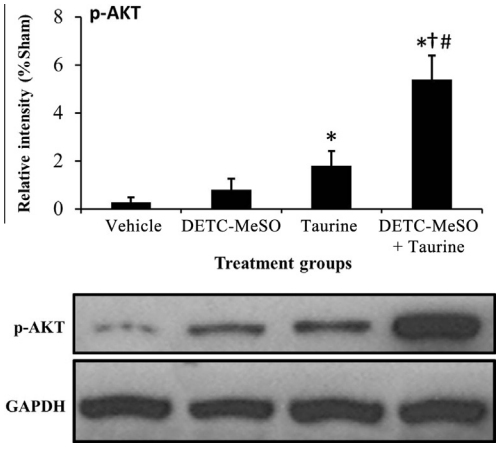

Fig. 7. Effect of DETC-MeSO and taurine on expression of p-AKT in the MCAO stroke model. (A) This figure shows p-AKT up-regulation in the penumbra of the combined treated group in comparison to the other treated groups. Values in the graphs represent mean ± SEM. *, † and # denote statistical significance compared to vehicle, DETC-MeSO- (0.56 mg/kg) and taurine (40 mg/kg)-treated group, respectively by ANOVA and Tukey post hoc tests ($N = 5$, $p < 0.05$).

can show more improvement after MCAO in rats. To our best knowledge, no data exist of combined treatment of taurine and DETC-MeSO in a model of MCAO in rat. For this reason, we administered the same dose of taurine as we had used in previous studies. However a lower dose of DETC-MeSO was used in this study to reduce any undesirable or unknown side effects associated with excessive DETC-MeSO on the nervous system (Gozlan and Ben-Ari, 1995). Some of these rare side effects associated with DETC-MeSO are consistent with those expected due to excessive blockage of glutamate function in the central nervous system (Branchey et al., 1987; Nagendra, 1997; Ningaraj et al., 2001). The present findings demonstrate that combination therapy comprising taurine and the partial NMDA receptor antagonist, DETC-MeSO, is highly effective in ameliorating brain infarct size and functional deficits following stroke. Furthermore, combined administration of taurine and DETC-MESO was able to reduce all three pathways of ER stress and ER-induced apoptosis in the ischemic penumbra.

Combined DETC-MeSO and taurine attenuates apoptosis induced by excitotoxicity and excessive calcium influx in the ipsilateral ischemic penumbra

It has been shown in cerebral ischemia that taurine can exert its neuroprotective function through both extracellular mechanisms by inhibiting calcium influx and by intracellular mechanisms by protecting the mitochondrion and ER through preventing mitochondrial and ER dysfunction resulting from cytoplasmic calcium overload (Huxtable, 1992; El Idrissi and Trenkner, 1999; Foos and Wu, 2002; Pan et al., 2010; Gharibani et al., 2013). In this study, we have confirmed that taurine not only may decrease extracellular calcium influx in the ischemic penumbra, but also can effectively inhibit some apoptotic markers, such as Bax and caspase-3. αII-Spectrin is the major structural component of the membrane of the cytoskeleton in axons and presynaptic terminals and a substrate for both m-calpain and caspase-3 (Pike et al., 2001, 2004). Activation of m-calpain by a massive increase of intracellular Ca^{2+} can cleave αII-spectrin into the 150/145-kDa bands. In the taurine-treated group levels of the 150/145-kDa bands were significantly lower than in the vehicle-treated group. Activated caspase-3 cleaves αII-spectrin to proteolytic fragments of 120-kDa band. Our data showed that the 120-kDa band was decreased in the penumbra of the taurine-treated group in comparison to the vehicle-treated group. This shows that taurine can decrease caspase-3 activation in the penumbra of the taurine treated group which is also confirmed by our investigation on the other apoptotic markers, such as Cytochrome-c. On the other hand, it has been shown that DETC-MeSO can bind to the NMDA receptors and prevent over-activation of these receptors and Ca^{2+} influx from excess glutamate (Nagendra, 1997; Nagendra et al., 1997). As we showed in our previous study using 5.6-mg/kg of DETC-MeSO could significantly reduce the cleavage of αII-spectrin into 150/145-kDa in the penumbra (Mohammad-Gharibani et al., 2014). This study reveals that even a lower dose of DETC-MeSO (0.56-mg/kg) can decrease αII-spectrin cleavage by preventing the calcium influx through binding to the NMDA receptor in the ischemic penumbra. Treatment with the lower dose of DETC-MeSO also showed that the 120-kDa band was decreased in the penumbra of the DETC-MeSO-treated group in comparison to the vehicle-treated group explaining that it can decrease caspase-3 activation in the penumbra of the taurine-treated group.

Interestingly, using combined therapy of taurine and DETC-MeSO synergistically reduced the level of αII-spectrin cleavage into 150/145-kDa and 120-kDa by inhibiting m-calpain activation and apoptotic markers, respectively. These data demonstrate that both taurine and DETC-MeSO together can prevent intracellular calcium overload more effectively. The significant increase of BCL2 as an anti-apoptotic marker relative to Bax as a pro-apoptotic marker in the group of combined treatment versus the other treated groups individually could be one of the main reasons for releasing less Cytochrome-c from mitochondria followed by markedly lower caspase-3 expression.

Combined DETC-MeSO and taurine attenuates apoptosis by increasing a major cell survival factor AKT in the ipsilateral ischemic penumbra

AKT, also known as protein kinase B (PKB, is a serine/threonine-specific protein kinase) plays a key role in increasing survival and inhibiting apoptosis. A number of studies including ours have indicated that activated AKT (p-AKT) promotes neuroprotection during cerebral ischemia (Kilic et al., 2005; Hasegawa et al., 2006). Previously, we documented that 5.6-mg/kg of DETC-MeSO increased p-AKT in the penumbra eliciting neuroprotection in MCAO after 4 days. However, using lower dose of DETC-MeSO (0.56-mg/kg) caused no changes in the level of p-AKT expression in the penumbra. It was reported that p-AKT is able to induce some anti-apoptotic markers, such as BCL-2 (Eberle et al., 2007). This result is consistent with the level of BCL2 in DETC-MeSO-treated group in which no increase was seen in contrast to the other treated groups. The level of p-AKT in either taurine or combined DETC-MeSO + taurine-treated group was significantly higher than the vehicle and DETC-MeSO-treated groups. DETC-MeSO alone could not alter the level of p-AKT in the penumbra. It was however found that DETC-MeSO in combination with taurine could increase the p-AKT level to almost 3 times that of the individual taurine treatment. These data are also supported by the ratio of BCL2/Bax expression in taurine and DETC-MeSO + taurine-treated groups. It is known that the AKT serine/threonine kinases are critical mediators of cell survival in response to Ca^{2+} influx (Dudek et al., 1997; Yano et al., 1998). For taurine and DETC-MeSO + taurine-treated groups, data on αII-spectrin (Ca^{2+} influx substrate) and the anti-apoptotic marker BCL-2 support the role of p-AKT up-regulation in protecting cells in the penumbra of these treated groups.

Combined DETC-MeSO and taurine attenuates apoptosis induced by ER stress in the ipsilateral ischemic penumbra

The ER is an organelle that plays important roles in the maintenance of intracellular calcium homeostasis and in the folding and processing of newly synthesized proteins. Various conditions such as alterations in calcium homeostasis, glucose deprivation, and hypoxia lead to the accumulation of unfolded proteins in the ER, resulting in ER stress (Reddy et al., 2003). Caspase-12 contributes to inflammatory pathways and is a representative molecule related to ER stress-induced apoptosis signaling pathways in neuronal death resulting from ischemia/reperfusion (Shibata et al., 2003). Shibata et al. found co-localization of caspase-12 immunoreactivity and DNA fragmentation detectable by the TUNEL method 5–23 h after reperfusion. However, we found that caspase-12 can be seen even after 4 days in the penumbra. As has been shown previously, caspase-12 was cleaved into a size of 42 kDa in ER stress-induced cell death following hypoxia/reoxygenation in primary neuronal culture (Pan et al., 2011; Gharibani et al., 2013). Notably, our results reveal that MCAO can increase the caspase-12 cleavage after 4 days in the penumbra. However, we showed that combined therapy can markedly decrease this cleavage to less than 16% of the other treatment groups. Ameliorating of calcium homeostasis by DETC-MeSO and taurine together may be one of the reasons for downregulation of caspase-12 and preventing its cleavage.

In this investigation, we aimed to identify which particular ER stress-induced pathways are predominantly affected by our treatment in the brain of the MCAO model. GRPs are commonly used as an indicator for the unfolding protein response (UPR). GRP78 is one of the subclasses of the heat shock protein (HSP)-70 (Reddy et al., 2003) which is induced during oxidative stress, chemical toxicity, and treatment with Ca^{2+} ionophores. GRP78 has various functions in the cell including participating in protein folding in the ER, the UPR and inhibition of apoptosis (Ouyang et al., 2012). GRP78 is mostly located in the ER lumen but can also be detected as a transmembrane protein outside the ER. For example at the cell-surface GRP78 has been shown to be an important receptor for pro-survival growth signaling and control of viability, while in the mitochondrion regulates mitochondrial energy balance during ER stress (Ni et al., 2011). As we reported previously, GRP78 showed a significant increase in MCAO 4 days after reperfusion (Gharibani et al., 2013; Mohammad-Gharibani et al., 2014). As in our previous report with 5.6-mg/kg DETC-MeSO, in this experiment we detected no changes in GRP78 expression after treatment with 0.56-mg/kg DETC-MeSO in the whole-cell lysate of the penumbra. However, in this experiment both taurine and DETC-MeSO + taurine decreased the expression of GRP78 in comparison to the vehicle-treated group. Also a significant reduction of almost 2–3-fold was seen in the combined treated group in comparison to taurine and DETC-MeSO-treated group, respectively. These data are supported by our results indicating a significant decrease in mitochondrial apoptosis markers following both taurine and DETC-MeSO + taurine administration.

The UPR dissociates GRP78 from its sensors (ATF6, PERK and IRE-1) inside the ER lumen due to accumulation of unfolded proteins. PERK, ATF6 and IRE1 are three ER-resident transmembrane proteins and serve as the proximal sensors of the ER stress response. Our previous study demonstrated that DETC-MeSO (5.6-mg/kg) has beneficial effects on the protection against ER stress in the penumbra of the MCAO infarct. We previously showed that post treatment with DETC-MeSO with a dose of 5.6-mg/kg can prevent ER stress through the PERK pathway; however the dose of 0.56-mg/kg showed no inhibition of PERK in this experiment. PERK has been reported to be responsible for repressing global protein synthesis by phosphorylation of eIF2a (Harding et al., 2000; Kumar et al., 2001) which inhibits general translation to reduce further accumulation of proteins in the ER lumen (Harding et al., 1999). Phosphorylated eIF2a can indirectly control gene transcription by positively regulating ATF4 (Szegezdi et al., 2006). ATF4 translocates to the nucleus to drive transcription of ER genes and gene products (including GRP78) involved in amino acid biosynthesis, redox reaction and protein secretion as well as pro-apoptotic mechanisms including synthesis of the transcription factor CHOP (Harding et al., 2003). Since ATF4 is down-stream protein in the PERK pathway, it is appropriate to measure expression levels of ATF4 in order to determine the extent of the PERK pathway response in our experimental groups. We found that in the MCAO model of stroke there was no change in ATF4 expression in either DETC-MeSO or the taurine-treated group. Notably in the combined treated group, ATF4 was markedly decreased. These data are consistent with GRP78 expression in the combined treated group which showed a significant down-regulation in comparison to the other treated groups.

Our data indicate that post treatment with taurine could prevent UPR by downregulation of ATF6 and IRE-1 pathways, but not the PERK pathway. Following dissociation of GRP78, ATF6 can translocate to the Golgi apparatus to be activated by cleavage (Chen et al., 2002), and translocate to the nucleus where it induces the expression of chaperone proteins and the pro-survival transcription factor X box-binding protein 1 (XBP1) (Yoshida et al., 2000; Adachi et al., 2008). ATF6 signaling appears to be predominantly pro-survival with little evidence linking it to cell death (Morishima et al., 2011). We showed in our experiment that DETC-MeSO did not alter ATF6 cleavage in the penumbra of MCAO stroke model. Taurine however did inhibit ATF6 cleavage significantly. This is consistent with GRP78 expression in the taurine-treated group where we saw less GRP78 expression in penumbra, and then less ATF6 translocating into the Golgi apparatus to be cleaved.

Upon dissociation of GRP78 in the ER lumen, IRE1 is activated by dimerisation and autophosphorylation. Following its activation, p-IRE1 enables translation and generation of a basic leucine zipper family transcription factor, spliced XBP1 (XBP1s) (Yoshida et al., 2001).

XBP1s activate the transcription of various proteins involved in the maintenance of ER homeostasis such as ER chaperones GRP78, as well as transcription factors such as CHOP (Lee et al., 2003). Activated IRE-1 also binds to tumor necrosis factor (TNF) receptor-associated factor 2, activating apoptosis signal-regulating kinase 1 (ASK-1). ASK-1 can activate ASK-1-CHOP pathway which leads to apoptosis. Levels of p-IRE-1 in the penumbra of MCAO stroke model were measured to test whether DETC-MeSO has an effect on the IRE1 pathway. Results indicate that there are no changes in p-IRE-1 by DETC-MeSO (0.56-mg/kg) treatment in the penumbra. However, there was a significant alteration of p-IRE-1 protein levels in taurine-treated groups in the penumbra in comparison to the vehicle and DETC-MeSO-treated groups. Interestingly, our novel approach of combined treatment using both DETC-MeSO and taurine not only yielded marked decreases in all three ER stress PERK, ATF6 and IRE-1 pathways in the same fashion, but also showed that this reduction is significant for individual treatments with either DETC-MeSO or taurine.

CHOP as a pro-apoptotic transcription factor is a point of convergence for all three arms of the UPR pathways and CHOP induces cell death through the regulation of BCL-2 family members in favor of pro-apoptotic BCL-2 pathways (Matsumoto et al., 1996) such as Bax-dependent apoptotic cascade (Lai et al., 2007). Our data showing that CHOP that was up-regulated in the penumbra of the MCAO model demonstrated a significant decrease in the penumbra of both taurine and DETC-MeSO-treated groups. However, this decrease was more significant in the combined treated group to approximately 30%.

Together, these results provide evidence that activation of the ATF6, PERK and IRE-1 pathways can be inhibited by combined DETC-MeSO and taurine and through these three pathways may inhibit ER-induced apoptosis. Furthermore the results indicating suppression of CHOP by combined DETC-MeSO and taurine treatment provide substantial evidence that these two drugs can contribute to an effective inhibition of ER stress induced by MCAO, in comparison to the DETC-MeSO or taurine individually.

Combined DETC-MeSO and taurine attenuates gliosis in the ipsilateral ischemic penumbra

Previously we showed that after MCAO, gliosis is strongly activated in the infarct boundaries (Mohammad-Gharibani et al., 2014). It was shown that the activated astrocytes rapidly surround the infarct, and thus separate the necrotic tissue from the viable brain and probably prevent the spread of damage (Li et al., 2005). Reactive astrocytes form a physical barrier that isolates the lesion site from normal brain tissue, and help the restoring of the blood–brain barrier. However, this physical barrier, tends to inhibit neurite outgrowth and neural regeneration (Sofroniew, 2009), so down-regulation of this phenomenon shows less damage or further improvement. In this study, we showed that GFAP expression after MCAO markedly increased in the penumbra in comparison to sham-operated group, which is consistent with previous reports (Wang et al., 2010). In our previous findings we demonstrated that DETC-MeSO at a dose of 5.6-mg/kg could reduce gliosis in the penumbra of MCAO 4 days after reperfusion, however in this study DETC-MeSO with a dose of 0.56-mg/kg could not alter GFAP expression in the penumbra. On the other hand, taurine was reported to prevent glial alteration in retina of diabetic rats (Zeng et al., 2009). Here, we show for the first time that taurine can prevent gliosis by down-regulation of GFAP after MCAO in the penumbra. In combined therapy with DETC-MeSO (lower dose) and taurine, the result is more impressive. DETC-MeSO + taurine together were able to reduce gliosis by almost 100% compared to taurine treatment individually. Interestingly, although DETC-MeSO showed no improvement individually, when it was administered in combination with taurine, it elicited a very large reduction in GFAP expression. Since most pro-survival markers such as, BCL-2, p-AKT and GRP78 in addition to ER markers showed a significant increase in the penumbra following combined DETC-MeSO + taurine administration, GFAP down-regulation could be due to fewer damaged neurons and thus fewer neuronal interactions with astrocytes for gliosis formation.

Combined DETC-MeSO and taurine improved neuro-score assessment due to reduction in brain infarct volume of the ipsilateral ischemic penumbra

Our investigation of neuronal populations through measuring levels of NeuN, a marker which is expressed in all of the nuclei of most neuronal cell types but not in non-neuronal cell types, by western blot showed that all treatment groups can decrease the loss of neuron populations after MCAO. Although all experimental groups were able to save neurons to some extent, the combined treated group showed more survival in all neuronal populations almost by 25% more that the individual treatment groups.

In our previous experiment, we demonstrated that DETC-MeSO (5.6 mg/kg) could markedly reduce the volume of the lesion in MCAO after 4 days whereas by contrast in this experiment the group which received 0.56 mg/kg DETC-MeSO showed no improvement after 4 days. This result may indicate that the lower dose of DETC-MeSO is not efficient in preventing damage from MCAO in rats. These data may be somewhat controversial as we saw that the lower dose of DETC-MeSO can augment some survival processes and factors, such as decreasing calcium influx, Cytochrome-c, caspase-3 and caspase-12. But these alterations do not seem sufficient to reduce the infarct size or to improve the neuro-score of the rats after 4 days. However, in either taurine or DETC-MeSO + taurine-treated groups, a reduction of infarct size was seen after 4 days. It is important to mention that only in these two groups, ER stress pathways leading to apoptosis were markedly decreased. Interestingly, the infarct size of the combined treated group was 100% less than taurine-treated group. This improvement with the combined treatment group could be due to the reduction in all three pathways (PERK, ATF6 and IRE-1) of ER stress, by comparison to the taurine-treated group in

which only two (ATF6 and IRE-1) of three pathways was reduced.

After MCAO onset, rats were tested daily for the neuro-score test. It has been shown previously that the neuro-score test correlates with infarct volume (Menzies et al., 1992; Schäbitz et al., 2001; Gharibani et al., 2013; Mohammad-Gharibani et al., 2014). Animals that received DETC-MeSO + taurine showed significantly fewer neurological deficits at 4 days after reperfusion compared to the other experimental groups. TTC for the infarct size and the neuro-score test showed greater improvement in the combined treatment group which had received DETC-MeSO + taurine for 4 days. These data confirm a correlation between infarct volume and neuro-score test as reported previously by Schäbitz et al. (2001).

CONCLUSIONS

This experiment showed that combined treatment of DETC-MeSO + taurine can exert protective effects on damaged neurons in the MCAO model through suppression of ER stress to a greater extent than the individual taurine or DETC-MeSO administration. Moreover, combined treatment showed significant inhibition of apoptosis by activation of the PERK, IRE-1 and ATF6 pathways. We demonstrated upregulation of AKT phosphorylation which can prevent ischemia-induced apoptosis (Taranukhin et al., 2008) and attenuate ER stress (Yung et al., 2007). Administration of combined DETC-MeSO + taurine showed that an increase in CHOP and Bax was prevented in the penumbra of MCAO stroke model more than with the individual administration of taurine or DETC-MeSO, indicating that DETC-MeSO + taurine can decrease apoptosis both in mitochondrial Ca^{2+}-induced apoptosis (up-regulation of BCL-2/Bax and down-regulation of caspase-3) and ER-induced apoptosis (down-regulation of CHOP). These data support the hypothesis that both DETC-MeSO as a partial antagonist of the NMDA receptor, and taurine as a semi-essential amino acid together can decrease infarction by decreasing ER stress in the rat MCAO stroke model and improve behavioral performance reflected in the neuro-score test.

DISCLOSURES

None.

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REFERENCES

Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K (2008) ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct Funct 33:75–89.

Ashwal S, Tone B, Tian HR, Cole DJ, Pearce WJ (1998) Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. Stroke 29:1037–1046 [discussion 1047].

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17:1304–1308.

Birdsall TC (1998) Therapeutic applications of taurine. Altern Med Rev 3:128–136.

Branchey L, Davis W, Lee KK, Fuller RK (1987) Psychiatric complications of disulfiram treatment. Am J Psychiatry 144:1310–1312.

Chen X, Shen J, Prywes R (2002) The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. J Biol Chem 277:13045–13052.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Eberle J, Kurbanov BM, Hossini AM, Trefzer U, Fecker LF (2007) Overcoming apoptosis deficiency of melanoma-hope for new therapeutic approaches. Drug Resist Updat 10:218–234.

El Idrissi A, Trenkner E (1999) Growth factors and taurine protect against excitotoxicity by stabilizing calcium homeostasis and energy metabolism. J Neurosci 19:9459–9468.

Foos TM, Wu J-Y (2002) The role of taurine in the central nervous system and the modulation of intracellular calcium homeostasis. Neurochem Res 27:21–26.

George S, Kadam SD, Irving ND, Markowitz GJ, Raja S, Kwan A, Tu Y, Chen H, Rohde C, Smith DR, Comi AM (2013) Impact of trichostatin A and sodium valproate treatment on post-stroke neurogenesis and behavioral outcomes in immature mice. Front Cell Neurosci 7:123–130.

Gharibani PM, Modi J, Pan C, Menzie J, Ma Z, Chen P-C, Tao R, Prentice H, Wu J-Y (2013) The mechanism of taurine protection against endoplasmic reticulum stress in an animal stroke model of cerebral artery occlusion and stroke-related conditions in primary neuronal cell culture. Adv Exp Med Biol 776:241–258.

Go AS et al (2014) Heart disease and stroke statistics – 2014 update: a report from the American Heart Association. Circulation 129. e28–e292.

Gozlan H, Ben-Ari Y (1995) NMDA receptor redox sites: are they targets for selective neuronal protection? Trends Pharmacol Sci 16:368–374.

Guan W, Zhao Y, Xu C (2011) A combined treatment with taurine and intra-arterial thrombolysis in an embolic model of stroke in rats: increased neuroprotective efficacy and extended therapeutic time window. Transl Stroke Res 2:80–91.

Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271–274.

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 5:897–904.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 11:619–633.

Hart BW, Faiman MD (1994) In vivo pharmacodynamic studies of the disulfiram metabolite S-methyl N,N-diethylthiolcarbamate sulfoxide: inhibition of liver aldehyde dehydrogenase. Alcohol Clin Exp Res 18:340–345.

Hasegawa Y, Morioka M, Hasegawa S, Matsumoto J, Kawano T, Kai Y, Yano S, Fukunaga K, Kuratsu J-I (2006) Therapeutic time window and dose dependence of neuroprotective effects of sodium orthovanadate following transient middle cerebral artery occlusion in rats. J Pharmacol Exp Ther 317:875–881.

Herculano-Houzel S, Lent R (2005) Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain. J Neurosci 25:2518–2521.

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev 72:101–163.

Jin L, Davis MR, Hu P, Baillie TA (1994) Identification of novel glutathione conjugates of disulfiram and diethyldithiocarbamate in rat bile by liquid chromatography–tandem mass spectrometry. Evidence for metabolic activation of disulfiram in vivo. Chem Res Toxicol 7:526–533.

Kiewert C, Mdzinarishvili A, Hartmann J, Bickel U, Klein J (2010) Metabolic and transmitter changes in core and penumbra after middle cerebral artery occlusion in mice. Brain Res 1312:101–107.

Kilic E, Kilic U, Soliz J, Bassetti CL, Gassmann M, Hermann DM (2005) Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. FASEB J 19:2026–2028.

Kim I, Xu W, Reed JC (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov 7:1013–1030.

Kramer M, Dang J, Baertling F, Denecke B, Clarner T, Kirsch C, Beyer C, Kipp M (2010) TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses. J Neurosci Methods 187:84–89.

Kumar R, Azam S, Sullivan JM, Owen C, Cavener DR, Zhang P, Ron D, Harding HP, Chen JJ, Han A, White BC, Krause GS, DeGracia DJ (2001) Brain ischemia and reperfusion activates the eukaryotic initiation factor 2alpha kinase, PERK. J Neurochem 77:1418–1421.

Lai E, Teodoro T, Volchuk A (2007) Endoplasmic reticulum stress: signaling the unfolded protein response. Physiology (Bethesda) 22:193–201.

Lee A-H, Iwakoshi NN, Glimcher LH (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol 23:7448–7459.

Li F, Omae T, Fisher M, Dietrich WD, Kuluz JW (1999) Spontaneous hyperthermia and its mechanism in the intraluminal suture middle cerebral artery occlusion model of rats editorial comment. Stroke 30:2464–2471.

Li Y, Chen J, Zhang CL, Wang L, Lu D, Katakowski M, Gao Q, Shen LH, Zhang J, Lu M, Chopp M (2005) Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells. Glia 49:407–417.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84–91.

Louzada PR (2004) Taurine prevents the neurotoxicity of -amyloid and glutamate receptor agonists: activation of GABA receptors and possible implications for Alzheimer's disease and other neurological disorders. FASEB J 18:511–518.

Matsumoto M, Minami M, Takeda K, Sakao Y, Akira S (1996) Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett 395:143–147.

Menzies SA, Hoff JT, Betz AL (1992) Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery 31:100–106 [discussion 106–107].

Minnerup J, Wersching H, Ringelstein EB, Schilling M, Schäbitz W-R, Wellmann J, Berger K (2011) Impact of the extended thrombolysis time window on the proportion of recombinant tissue-type plasminogen activator-treated stroke patients and on door-to-needle time. Stroke 42:2838–2843.

Modi JP, Gharibani PM, Ma Z, Tao R, Menzie J, Prentice H, Wu J-Y (2014) Protective mechanism of sulindac in an animal model of ischemic stroke. Brain Res 12:91–99.

Mohammad-Gharibani P, Modi J, Menzie J, Genova R, Ma Z, Tao R, Prentice H, Wu J-Y (2014) Mode of action of S-methyl-N,N-diethylthiocarbamate Sulfoxide (DETC-MeSO) as a novel therapy for stroke in a rat model. Mol Neurobiol 50:655–672.

Morishima N, Nakanishi K, Nakano A (2011) Activating transcription factor-6 (ATF6) mediates apoptosis with reduction of myeloid cell leukemia sequence 1 (Mcl-1) protein via induction of WW domain binding protein 1. J Biol Chem 286:35227–35235.

Nagendra SN (1997) Carbamoylation of brain glutamate receptors by a disulfiram metabolite. J Biol Chem 272:24247–24251.

Nagendra SN, Faiman MD, Davis K, Wu JY, Newby X, Schloss JV (1997) Carbamoylation of brain glutamate receptors by a disulfiram metabolite. J Biol Chem 272:24247–24251.

Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150:887–894.

Ni M, Zhang Y, Lee AS (2011) Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. Biochem J 434:181–188.

Ningaraj NS, Chen W, Schloss JV, Faiman MD, Wu J-Y (2001) S-methyl-N,N-diethylthiocarbamate sulfoxide elicits neuroprotective effect against N-methyl-D-aspartate receptor-mediated neurotoxicity. J Biomed Sci 8:104–113.

O'Donnell ME, Lam TI, Tran LQ, Foroutan S, Anderson SE (2006) Estradiol reduces activity of the blood-brain barrier Na-K-Cl cotransporter and decreases edema formation in permanent middle cerebral artery occlusion. J Cereb Blood Flow Metab 26:1234–1249.

Ouyang Y-B, Lu Y, Yue S, Xu L-J, Xiong X-X, White RE, Sun X, Giffard RG (2012) MiR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo. Neurobiol Dis 45:555–563.

Pan C, Giraldo GS, Prentice H, Wu J-Y (2010) Taurine protection of PC12 cells against endoplasmic reticulum stress induced by oxidative stress. J Biomed Sci 17(Suppl 1):S17.

Pan C, Prentice H, Price AL, Wu J-Y (2011) Beneficial effect of taurine on hypoxia- and glutamate-induced endoplasmic reticulum stress pathways in primary neuronal culture. Amino Acids 42:845–855.

Pérez-Álvarez MJ, Maza MDC, Anton M, Ordoñez L, Wandosell F (2012) Post-ischemic estradiol treatment reduced glial response and triggers distinct cortical and hippocampal signaling in a rat model of cerebral ischemia. J Neuroinflamm 9:157.

Pike BR, Flint J, Dutta S, Johnson E, Wang KK, Hayes RL (2001) Accumulation of non-erythroid alpha II-spectrin and calpain-cleaved alpha II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. J Neurochem 78:1297–1306.

Pike BR, Flint J, Dave JR, Lu X-CM, Wang KKK, Tortella FC, Hayes RL (2004) Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived alphaII-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab 24:98–106.

Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS (2003) Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278:20915–20924.

Rich PR, Mischis LA, Purton S, Wiskich JT (2001) The sites of interaction of triphenyltetrazolium chloride with mitochondrial respiratory chains. FEMS Microbiol Lett 202:181–187.

Schäbitz WR, Li F, Irie K, Sandage BW, Locke KW, Fisher M (1999) Synergistic effects of a combination of low-dose basic fibroblast growth factor and citicoline after temporary experimental focal ischemia. Stroke 30:427–431 [discussion 431–432].

Schäbitz WR, Sommer C, Zoder W, Kiessling M, Schwaninger M, Schwab S (2000) Intravenous brain-derived neurotrophic factor reduces infarct size and counter regulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke 31:2212–2217.

Schäbitz WR, Hoffmann TT, Heiland S, Kollmar R, Bardutzky J, Sommer C, Schwab S (2001) Delayed neuroprotective effect of insulin-like growth factor-I after experimental transient focal cerebral ischemia monitored with MRI. Stroke 32:1226–1233.

Shibata M, Hattori H, Sasaki T, Gotoh J, Hamada J, Fukuuchi Y (2003) Activation of caspase-12 by endoplasmic reticulum stress induced by transient middle cerebral artery occlusion in mice. Neuroscience 118:491–499.

Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638–647.

Solaroglu I, Tsubokawa T, Cahill J, Zhang JH (2006) Anti-apoptotic effect of granulocyte-colony stimulating factor after focal cerebral ischemia in the rat. Neuroscience 143:965–974.
Sun M, Xu C (2008) Neuroprotective mechanism of taurine due to up-regulating calpastatin and down-regulating calpain and caspase-3 during focal cerebral ischemia. Cell Mol Neurobiol 28:593–611.
Sun M, Gu Y, Zhao Y, Xu C (2011) Protective functions of taurine against experimental stroke through depressing mitochondria-mediated cell death in rats. Amino Acids 40:1419–1429.
Sun M, Zhao Y-M, Gu Y, Xu C (2012) Therapeutic window of taurine against experimental stroke in rats. Transl Res 160:223–229.
Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293.
Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7:880–885.
Taranukhin AG, Taranukhina EY, Saransaari P, Djatchkova IM, Pelto-Huikko M, Oja SS (2008) Taurine reduces caspase-8 and caspase-9 expression induced by ischemia in the mouse hypothalamic nuclei. Amino Acids 34:169–174.
Turner RJ, Vink R (2012) Combined tissue plasminogen activator and an NK1 tachykinin receptor antagonist: an effective treatment for reperfusion injury following acute ischemic stroke in rats. Neuroscience 220:1–10.
Venna VR, Li J, Hammond MD, Mancini NS, McCullough LD (2014) Chronic metformin treatment improves post-stroke angiogenesis and recovery after experimental stroke. Eur J Neurosci 39:2129–2138.
Wang C-C, Chio C-C, Chang C-H, Kuo J-R, Chang C-P (2010) Beneficial effect of agmatine on brain apoptosis, astrogliosis, and edema after rat transient cerebral ischemia. BMC Pharmacol 10:11.
Wu J-Y, Wu H, Jin Y, Wei J, Sha D, Prentice H, Lee H-H, Lin C-H, Lee Y-H, Yang L-L (2009) Mechanism of neuroprotective function of taurine. Adv Exp Med Biol 643:169–179.
Xu L-J, Ouyang Y-B, Xiong X, Stary CM, Giffard RG (2015) Post-stroke treatment with miR-181 antagomir reduces injury and improves long-term behavioral recovery in mice after focal cerebral ischemia. Exp Neurol 264:1–7.
Yano S, Tokumitsu H, Soderling TR (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature 396:584–587.
Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol Cell Biol 20:6755–6767.
Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881–891.
Yung H, Korolchuk S, Tolkovsky AM, Charnock-Jones DS, Burton GJ (2007) Endoplasmic reticulum stress exacerbates ischemia-reperfusion-induced apoptosis through attenuation of Akt protein synthesis in human choriocarcinoma cells. FASEB J 21:872–884.
Zeng K, Xu H, Mi M, Zhang Q, Zhang Y, Chen K, Chen F, Zhu J, Yu X (2009) Dietary taurine supplementation prevents glial alterations in retina of diabetic rats. Neurochem Res 34:244–254.
Zhang C (2002) Comparison of calpain and caspase activities in the adult rat brain after transient forebrain ischemia. Neurobiol Dis 10:289–305.
Zivin JA (2009) Acute stroke therapy with tissue plasminogen activator (tPA) since it was approved by the U.S. Food and Drug Administration (FDA). Ann Neurol 66:6–10.

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