Trimetazidine Prevents Oxidative Changes Induced in a Rat Model of Sporadic Type of Alzheimer´s Disease

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Received: 24 Feb. 2014; Accepted: 25 Jul. 2014

Abstract- Oxidative stress plays a major role in the pathogenesis of Alzheimer's disease (AD) of sporadic origin. The expression of DHCR24 (Seladin-1), marker for neuronal oxidative stress and degeneration, has been reported to be altered in the brains of AD patients. In the present study, we investigated the effect of trimetazidine (TMZ) on the hippocampal oxidative parameters and the expression of DHCR24 (Seladin-1) in an animal model of sporadic AD. Male rats were pre-treated with TMZ (25 mg/kg) after which injected with intracerebroventricular-streptozotocin (ICV-STZ)/Saline. Following 2, 7 and 14 days, animals of different groups were sacrificed with their brain excised to detect the hippocampal lipid peroxidation, superoxide dismutase (SOD), catalase activity, DHCR24 (Seladin-1) expression and possible histopathological changes. ICV-STZ administration induced significant oxidative changes in the hippocampus. Meanwhile, TMZ pre-treatment showed to ameliorate the oxidative stress, which was demonstrated by a significant rise in the hippocampal SOD and catalase activity, as well as a significant decrease in the malondialdehyde (MDA) level. TMZ administration also increased the expression of DHCR24 (Seladin-1) gene in the hippocampus. In conclusion, our findings indicated a neuroprotective effect of TMZ possibly related to its antioxidant activity resulting in the up-regulation of DHCR24 (Seladin-1). Such TMZ effects may be beneficial in minimizing oxidative stress in sporadic Alzheimer´s disease and possible prevention of disease progression.

Acta Medica Iranica, 2015;53(1):17-24.

Keywords: Alzheimer´s disease; Streptozotocin; Trimetazidine; Oxidative stress

Introduction

Alzheimer´s disease (AD) is a frequent form of dementia in which vast majorities (90–95%) of afflicted patients are sporadic in origin (1). The disease occurs through a multifactorial process with the oxidative stress playing a major role in its pathogenesis (2,3). Evidence suggests increased regional levels of oxidative stress in the brain of AD subjects (4,5). It has been shown that the oxidative process develop in AD patients' brains before the beta-amyloid begins to aggregate (6). Despite a large oxidative capacity, the ability of the brain to resist oxidative stress is limited (7). Besides, the plasma levels of antioxidants are shown to be dramatically decreased in the course of AD (8).

Given the precipitous predicts for dementia over the coming decades and on the basis of above insights, potential pharmacological approaches to effectively modulate the oxidative stress are of extreme importance in the therapeutic and preventive interventions against AD. Although the therapeutic effects of natural polyphenols, as antioxidants, have been reported in AD, few clinical trials have indicated promising results (9).

Trimetazidine (TMZ) [1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride], which its antioxidant properties were introduced by Aubert on 1989, is shown to be well capable to pass the blood-brain barrier and to reduce the production of free radicals (10-12). It is an anti-ischemic agent (13-15) and is also shown to induce axonal regeneration and myelination effectively in healthy and injured nerves (16). Nevertheless, despite several investigations on TMZ's anti-ischemic and antioxidant properties, the general consensus on its efficacy in the management of

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AD is lacking.

Intra-cerebroventricular (ICV) injection of a sub-diabetogenic dose of streptozotocin (STZ) causes brain oxidative stress (17) and metabolic changes (18) resembling Alzheimer's disease. This intervention is known to replicate a reliable rat model for the sporadic type of AD (19-22). Since ICV-STZ treatment effectively alters the brain antioxidative defense system, it may be a useful approach to studying new possible antioxidant treatments for neurodegenerative disorders (23). With the view of the brain atrophy and neuronal loss in AD which preferentially affect the hippocampus and association cortices (1), we aimed to investigate the effect of TMZ pre-treatment on hippocampal oxidative parameters and the expression of DHCR24 in an animal model of sporadic AD. DHCR24 is a marker counteracting the oxidative stress and degeneration in central neurons (24), and its downregulation have reported in the hippocampus, amygdala and entorhinal cortices of AD patients (25,26).

Materials and Methods

Animals

Male Wistar rats, weighing 280-300g were maintained under the standard conditions (12 dark/light cycle, temperature 23±2°, humidity 45%-55%). They had free access to diet and water. All experiments and animal care procedures were approved by the committee of animal ethics of Tehran University of Medical Sciences.

Drugs

All drugs used in the experiment, Trimetazidine, Streptozotocin, ketamine, and xylazine were purchased from Sigma Inc.

Experimental groups

72 rats were randomly divided into 12 groups each comprising 6 rats. Sham-operated groups (Saline-2, Saline-7, Saline-14, Saline+TMZ-2, Saline+TMZ-7, Saline+TMZ-14) received 10µl of intracerebroventricular (ICV) normal saline on days -2 and 0 (Figure 1) following the intraperitoneal injection of 0.5 ml normal saline or 0.5 ml trimetazidine (25mg/kg). The STZ groups (STZ-2, STZ-7, STZ-14, STZ+TMZ-2, STZ+TMZ-7, STZ+TMZ-14) received the bilateral ICV injection of STZ (3 mg/kg of body weight in saline, 1.5 mg/site, 5 µl/site) on days -2 and 0 following intraperitoneal injection of 0.5 ml normal saline or 25mg/kg trimetazidine (0.5 ml). Oxidative stress parameters were studied in the animals' hippocampus on days 2, 7 and 14 following the ICV-STZ/Saline administration.

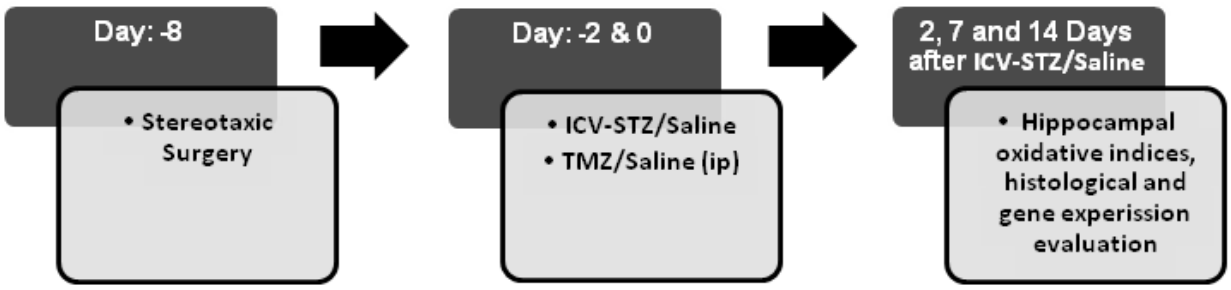

Figure 1. The different steps of the experimental procedure. (ICV-STZ): Intracerebroventricular-Streptozotocin

Surgical procedure

Animals were anesthetized using the intraperitoneal injection of ketamine (100mg/kg) and xylazine (20mg/kg), after which placed on a stereotaxic frame with their skulls exposed. Two stainless steel 23 gauge guide cannulae were implanted into the lateral ventricles. The stereotaxic coordinates for the lateral ventricle (27) were measured accurately as 0.8 mm posterior to bregma; 1.5 mm lateral to the sagittal suture; 3.6 mm beneath the surface of the brain. Animals were allowed one week to recover from surgery before any pharmacological interventions. Streptozotocin (STZ; 3 mg/kg of body weight in saline) was injected on days 1 and 3 after one-week recovery using a Hamilton syringe at the rate of 1µl/min. While the sham groups underwent an identical surgical procedure, they received the same volume of saline rather than STZ. At the end of the experiment, and on days 2, 7 and 14 following the ICV-STZ/Saline infusion, the animals were sacrificed, and their brains were immediately removed and washed in cold phosphate-buffered saline. The left hemisphere was used for histological evaluations while the right hippocampus was used to assess the oxidative stress indices and RNA extraction.

Determination of the oxidative stress markers

At the end of the experiment, on days 2, 7 and 14 following the ICV-STZ/Saline infusion, animals were sacrificed; their brains were immediately removed and washed in cold phosphate-buffered saline. The right hippocampus was homogenized in ice-cold Tris buffer (10 mmol/L Tris, pH 7.4). After centrifugation (8500 g, 25 min, at 2°C), the supernatant was used to estimate catalase and superoxide dismutase activity, as well as the malondialdehyde (MDA) level. Protein concentration was determined by the method of bovine serum albumin (BSA) which produces a standard reaction with folin phenol reagent.

Measurement of the hippocampal MDA

Lipid peroxidation was estimated colorimetrically measuring the malondialdehyde (MDA) according to the Satoh's method (28). Briefly, 2 ml of the hippocampal homogenate was added to 2 ml of freshly prepared trichloroacetic acid (10% w/v), and the mixture was cooled in an ice bath for 15 min. Following centrifugation (276 g for 25 min at 0°C), 2 ml of the supernatant solution was mixed with 2 ml of freshly prepared thiobarbituric acid (0.67% w/v). After about 10 min heating in a boiling water bath, the preparation was cooled instantly in an ice bath. The developed color was measured at 532 nm against a reagent blank.

Measurement of the hippocampal SOD activity

Superoxide dismutase activity was assessed according to the method described by Misra and Fridovich (29). Briefly, 0.5 ml hippocampal homogenate was mixed with 0.5 ml distilled water, 0.25 ml ice-cold ethanol, and 0.15 ml chloroform. Then, 1.5 ml of carbonate buffer and 0.5 ml of EDTA solution were added to 0.5 ml supernatant. The reaction was started by adding 0.4 ml adrenaline (3mmol/L) and the optical density alteration per minute was recorded at 480 nm.

Measurement of the hippocampal catalase activity

Catalase activity was assessed on days 2, 7 and 14 after the ICV-STZ/Saline administration as described by Aebi (30). In this assay, 1 ml of H2O2 (30 mmol/L) was mixed with 2 ml diluted homogenate to initiate the reaction. The absorbance decrement was measured at 240 nm. In such method, the catalase activity is expressed as mmol H2O2 consumed/min per mg protein.

RNA extraction and RT-PCR

Total hippocampal RNA was extracted using a total high pure RNA extraction kit according to the manufacturer's instructions (Roch Life technologies, USA). The RNA integrity and concentration was evaluated using electrophoresis (1% agarose gel) and spectrophotometry, respectively. One microgram of total RNA was converted to complementary DNA using prime scriptTM RT reagent Kit (Takara Bio INC, Japan, code RR037A) according to the manufacturer's manual. RT-PCR was performed in a 20µl reaction mixture containing 1µl cDNA, 10µl SYBR Premix EX Taq (Takara Bio INC, Japan, code RR820A) and 200 nm primers for each gene.

Reactions were run at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 20 sec and 72°C for 15 sec using the Corbett Life science Rotor-GeneTM 6000. A standard curve method was used to achieve DHCR24 (Seladin-1), and GAPDH mRNA levels. Comparative quantization of each target gene was performed based on the cycle threshold, which was normalized to GAPDH using the ΔΔCT method.

Seledin-1: forward, GGGTGTTTGTGTGCCTCTTCC; reverse, GCTCCTTCCACTCCCGTACC

GAPDH: forward, TGGTGCCAAAAGGGTCATC; reverse, CTTCCACGATGCCAAAGTTG

Histological study

After decapitation, the left hemispheres were fixated in 4% paraformaldehyde-phosphate buffer (0.1 M, pH 7.2), and paraffin embedded. To prepare the histology slides, 10µm coronal sections were taken and stained with cresyl violet.

Statistical analysis

All values were presented as mean ± SEM (n=6). Data were analyzed using one-way analysis of variance (ANOVA) followed by Student Newman Keuls's post hoc test. The null hypothesis was rejected at the 0.05 level of significance

Results

The effect of TMZ pre-treatment on the hippocampal MDA level

Figure 2 shows the changes of MDA level in the hippocampus on days 2, 7, and 14 following ICV-STZ/Saline infusion in the presence or absence of TMZ (25 mg/kg). There was no significant difference for MDA level between the sham groups. However, a significant rise in the MDA level was seen on days 7 and 14 [F (11,60)= 21.559, p= 0.000, nmol/mg protein]

following the ICV-STZ administration as compared to the sham groups. The TMZ (25 mg/kg) administration significantly decreased the MDA levels in STZ+TMZ-7 and STZ+TMZ-14 groups as compared to the STZ groups (Figure 2).

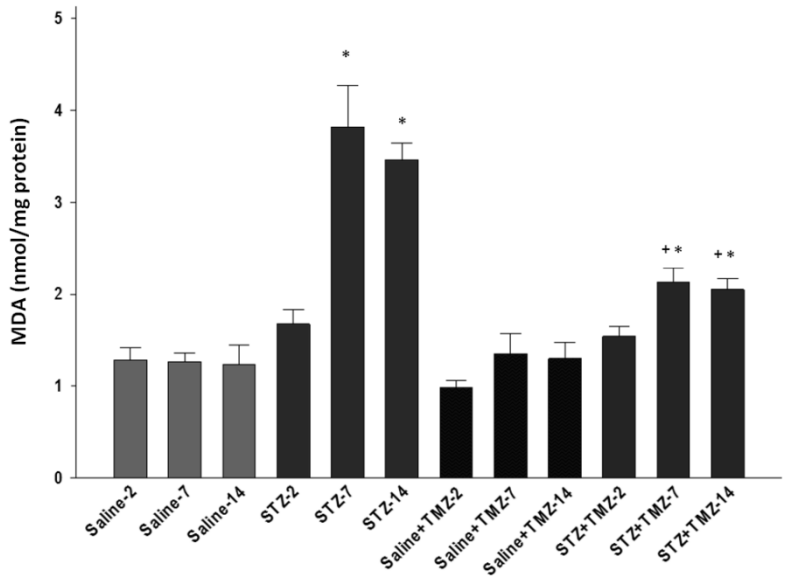

Figure 2. Alteration of MDA level in the presence or absence of TMZ (25 mg/kg) in ICV-STZ/Saline infused rats. Values are expressed as mean± SEM. **P*<0.05 *vs*. Sham groups; ^{+}P<0.05 *vs*. ICV-STZ groups (n=6)

The effect of TMZ pre-treatment on the hippocampal SOD activity

While no significant difference between the sham groups with regard to the SOD activity was observed, this variable was significantly increased in Saline+TMZ as compared to the sham groups. Meanwhile, the activity of this enzyme was significantly decreased in STZ groups [F (11,60)= 28.609, *P*= 0.000, U/mg protein, Figure 3] as compared to the sham groups. TMZ (25 mg/kg) pre-treatment significantly prevented the decline in the SOD activity in STZ+TMZ as compared to STZ groups.

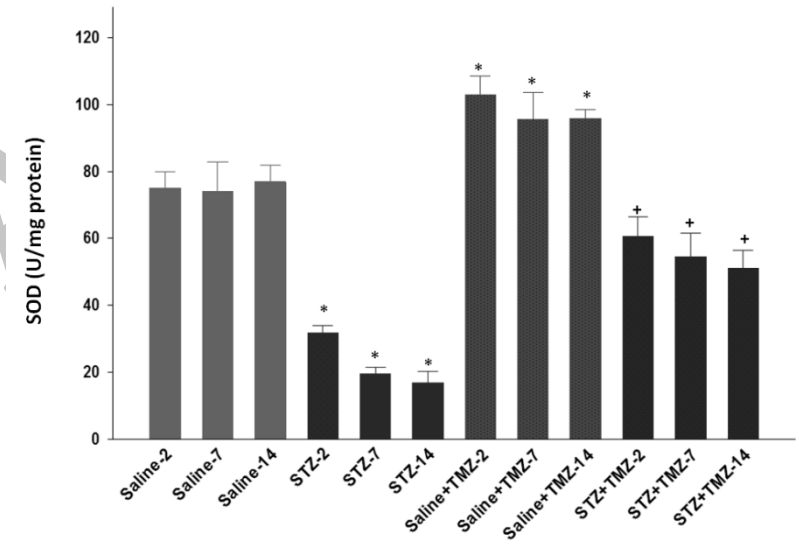

Figure 3. Alteration of SOD activity in the presence or absence of TMZ (25 mg/kg) in ICV-STZ/Saline infused rats. Values are expressed as mean± SEM. **P*<0.05 *vs*. Sham groups; ^{+}P<0.05 *vs*. ICV-STZ groups (n=6)

The effect of TMZ pre-treatment on the hippocampal catalase activity

In the absence of any significant difference in catalase activity between sham groups, the activity of this enzyme was significantly diminished in STZ as compared to the sham groups. While TMZ supplementation failed to significantly increase the catalase activity in the Saline+TMZ as compared to sham groups, the TMZ pre-treatment could effectively prevent the declined catalase activity in STZ+TMZ-7 and STZ+TMZ-14 groups [F (11,60)= 12.340, *P*= 0.000, mmol H2O2 consumed/min/mg protein, Figure 4] as compared to the STZ groups.

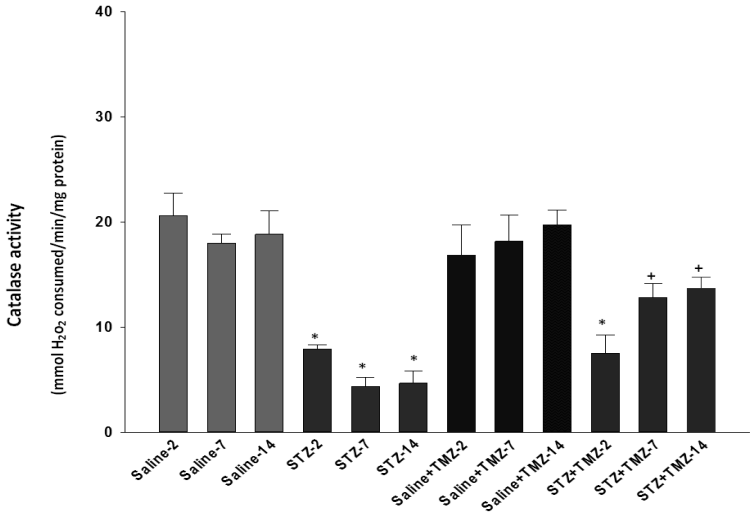

Figure 4. Alteration of Catalase activity in the presence or absence of TMZ (25 mg/kg) in ICV-STZ/Saline infused rats. Values are expressed as mean± SEM. **P*<0.05 *vs*. Sham groups; ^{+}P <0.05 *vs*. STZ groups (n=6)

The effect of TMZ pre-treatment on DHCR24 (Seladin-1) expression

While no significant difference in DHCR24 (Seladin-1) mRNA expression was observed between the sham groups, this variable was significantly decreased (fold change=0.4± 0.16 *vs.* 1.12± 0.14, *P*<0.05) in STZ-14 group. Meanwhile, the fold change in gene expression (0.87± 0.12, *P*<0.05) was significantly higher in STZ+TMZ-14 as compared to the STZ-14 group.

The effect of TMZ pre-treatment on hippocampal histology

The cresyl violet stained hippocampal sections showed a marked loss of cell bodies in STZ-14 group (51.97±2.26 *vs.* 110±17.7; *P*<0.05) as compared to the sham groups. Meanwhile, pre-treatment with TMZ in STZ+TMZ-14 group attenuated the cell loss (74.39±3, Figure 5).

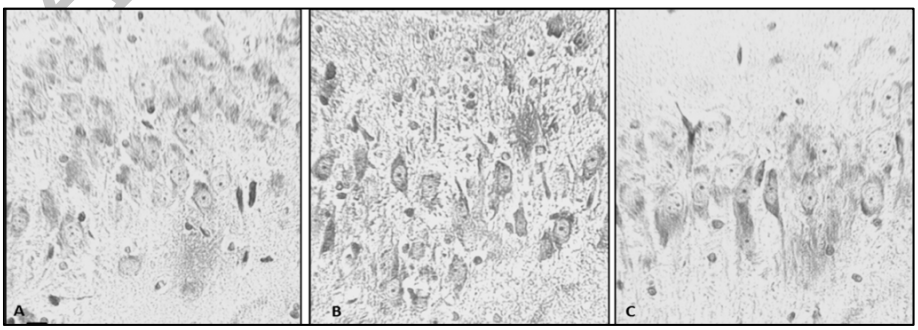

Figure 5. Photomicrographs are showing morphological features of hippocampal pyramidal cell layer by cresyl violet staining in different groups. (A) Hippocampal section of a sham group presented the healthy pyramidal neurons that are densely packed. (B) STZ-injected group after 14 days showed degenerating shrunken dark pyramidal cells with an oval or triangular nucleus. (C) Hippocampal section of TMZ treated shows the intact cells in the CA3 region. Magnification: A–C: ×400

Discussion

According to our findings, TMZ pre-treatment (25 mg/kg) was shown to ameliorate the ICV-STZ-induced oxidative stress demonstrated with a significant rise in the hippocampal SOD and catalase activity as well as a significant decrease in the MDA level. Our results are in line with the previous reports showing the beneficial effects of 25 mg/kg of TMZ in an animal model of brain ischemia (31). In addition, Iqbal evaluated pre-treatment of TMZ in the animal model of brain ischemia demonstrating the neuroprotective properties of TMZ (32).

In the present study, TMZ (25 mg/kg) pre-treatment effectively prevented the SOD activity decrement. The above observation is in agreement with that of Dhote and Balaraman (31) reporting a significant restoration of the SOD activity by TMZ following a focal cerebral ischemia–reperfusion injury in rat. They concluded that TMZ neuroprotective activity is mediated by its antioxidant properties. Further to the above, our results indicated that TMZ administration ameliorate lipid peroxidation in the hippocampus which was demonstrated by the reduced MDA levels in STZ+TMZ-7 and STZ+TMZ-14 groups. These findings are in agreement with our previous study on this model (33). The findings of the current study are also consistent with those of Dhote and Balaraman (31).

In the absence of any marked alterations in catalase activity in the saline+TMZ groups, TMZ significantly prevented the decline in catalase activity in STZ+TMZ-7 and STZ+TMZ-14 groups. The beneficial effects of TMZ may be partly attributable to the scavenged free radicals or restoration of the SOD activity in STZ+TMZ-7 and STZ+TMZ-14 groups. Recently, the beneficial effect of TMZ on H2O2-induced impairment of biological functions in endothelial progenitor cells have been reported (34).

In addition, in STZ-2 group, the catalase and SOD activities decreased, and the MDA level was shown not to be altered. Moreover, the histological evaluation revealed no damage in this group. One possibility is that the ICV-STZ induced progressive deficits, and sharp changes might only be observed during the second and third weeks following STZ administration. It has been postulated that the STZ-induced impairment of glucose metabolism may be a potential source for the oxidative stress (17). It should be noted that our brain lacks enriched defensive antioxidant mechanisms (7).

DHCR24 (Seladin-1) demonstrates resistance to both β-amyloid-induced toxicity and oxidative stress (26), are down-regulated in brain regions affected by AD (35). It is assumed that DHCR24 (Seladin-1) is a marker for oxidative stress and degeneration in central neurons (24). Our study indicates an increase in DHCR24 (Seladin-1) mRNA following TMZ administration. These finding suggests that some protective effects of TMZ may at least partly be attributed to the increased DHCR24 (Seladin-1). Our findings are in line with an earlier investigation (36) revealing that the enhanced DHCR24 (Seladin-1) activity could possibly be considered advantageous in AD therapy.

The ICV-STZ injection caused a significant decrease in the hippocampal CA3 neurons whereas TMZ protected these neurons against STZ and attenuated the cell loss. These findings reveal potential neuroprotective effects of TMZ possibly attributed to its antioxidant activity and DHCR24 (Seladin-1) up-regulation.

In conclusion, the present study suggests that TMZ may potentially prevent the oxidative stress-induced structural impairments in the rat hippocampus. Its neuroprotective effect possibly related to the antioxidant activity resulting in the up-regulation of DHCR24 (Seladin-1). Such TMZ effects may be useful in pharmacologic approaches against the oxidative stress in sporadic Alzheimer´s disease.

Acknowledgment

This study was supported with a grant from Tehran University of Medical Sciences.

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