Possible Pharmacodynamic Interaction of Azelnidipine with Citicoline Against Ischemic Brain Injury: Behavioral, Biochemical and Histological Alterations

Varun Gupta¹, Zein Eddin Bader¹,², Aakriti¹, and Anil Kumar¹

Abstract

Background: Currently, no drug has been approved for the management of postischemic neuronal damage. Existing studies show that calcium channel blockers have neuroprotective properties, while citicoline is involved in maintaining neuronal integrity.

Purpose: This study was envisaged to investigate the effect of azelnidipine (novel calcium channel blocker) alone and in combination with citicoline (phosphatidyl-choline analogue) against ischemic brain damage in Wistar rats.

Methods: Previously standardized bilateral common carotid artery occlusion model was used to induce cerebral ischemic injury in Wistar rats. Pretreatment with azelnidipine (1.5 mg/Kg and 3 mg/Kg; p.o.) or citicoline (250 mg/Kg; i.p.) was done every 24 h starting 7 days before the bilateral common carotid artery occlusion surgery. Pharmacological assessments (behavioral, biochemical, mitochondrial, molecular, and histological) were done after 48 h of the reperfusion period.

Results: Azelnidipine and citicoline were found to protect the brain from progressive neuronal damage as seen by improved sensorimotor behavior (locomotion, rota rod, and beam balance performance) and reduced oxidative stress (decreased malondialdehyde (MDA), nitrite, increased glutathione (GSH), superoxide dismutase (SOD)). Impairment of mitochondrial enzyme system and increase in the infarct area were found to be arrested by individual treatments with azelnidipine and citicoline. These effects were further potentiated synergistically as the combination of citicoline and azelnidipine was found to decrease glutamate levels, caspase-3 activity and histological alterations as compared to their individual effects.

Conclusion: Azelnidipine and citicoline synergistically decrease excitotoxic and oxidative damage against ischemic brain injury in Wistar rats and, therefore, propose a clinically relevant combination for the prevention of postischemic neuronal damage.

Keywords

Ischemia, azelnidipine, citicoline, excitotoxicity, oxidative stress

Introduction

Ischemic stroke is a major contributor toward global mortality and morbidity.¹ Although significant research efforts are being made toward finding a relevant therapeutic answer for the repair of postischemic brain, no major breakthrough has been achieved so far. Various pathological cascades initiated after a primary ischemic insult gradually exaggerate cerebral injury and eventually lead to an elevated and progressive brain damage. These cascades primarily include glutamate/Ca²⁺ mediated excitotoxicity, mitochondrial dysfunction, oxidative stress, neuroinflammation and apoptosis, etc.² Hypertension remains to be one of the most critical risk factors of ischemic brain stroke or cerebral ischemia³ and most of the patients with a history or proneness toward stroke are prescribed antihypertensive drugs to neutralize this risk factor.

Amongst a variety of antihypertensive drugs available, calcium channel blockers belong to the most widely and commonly prescribed class of drugs due to their proven efficacy and safety. Besides regulating blood pressure, calcium channel blockers have been shown to have various pharmacological actions on the vasculature and target organs including heart and brain. In this context, few L-type calcium channel blockers...
channel blockers such as nimodipine and nifedipine have been shown to exert neuroprotective properties on various brain cell subtypes subjected to different challenges involving apoptotic, antioxidant, and anti-inflammatory mechanisms.\(^{4,5}\) Besides this, nonhypotensive doses of amlodipine have been shown to reduce stroke size in mouse model of ischemic injury.\(^{15}\) However, when citicoline was tested in rat brain slices, it was found to reduce ATP levels, thereby proposing to protect the neurons from astrocytic glutamate uptake, besides increasing neuronal ischemia-induced neuronal glutamate release and stimulates neuroprotection of brain by antioxidant, membrane stabilizing, antiapoptotic, mitochondrial reparative, and antixcitotoxic mechanisms. In this study, we demonstrate the effects of azelnidipine and citicoline, alone or in combination with mitochondrial function and synergistic effect on glutamate toxicity, besides their potential to arrest oxidative stress, apoptosis, infarction, and motor dysfunction.

**Methods**

**Animals**

Wistar rats (male; 240–280 g), from CAH, Panjab University, were employed in the study. Normal rat chow and water were provided *ad libitum*. The study (ethics approval nos. PU/IAEC/S/14/124 and PU/45/99/CPCSEA/IAEC/2018/111) was performed according to CPSCEA, Government of India, guidelines.

**Surgical Induction of Global Cerebral Ischemia**

Bilateral common carotid artery occlusion (BCCAO) was used to induce cerebral ischemia and reperfusion injury as previously reported.\(^{19,20}\) Under the effect of ketamine (80 mg/Kg; *i.p.*.) and xylazine (10 mg/Kg; *i.p.*.) induced anesthesia, both common carotid arteries were occluded for 30 min, followed by a 48 h reperfusion period. Normal physiological functions were maintained throughout the surgery.

**Study Design**

Randomly, the animals were arranged into 7 groups (*n = 8*; Table 1). Rats in each group were treated according to the experimental design followed by behavioral assessments and later sacrificed to collect samples for biochemical, mitochondrial, molecular, and histological studies (Figure 1). Azelnidipine was procured from Pure Chem Pvt. Ltd (Gujarat, India), whereas citicoline was obtained from Sun Pharma Laboratories Ltd (India). Azelnidipine was formulated into a suspension using 0.25% w/v sodium carboxy methyl cellulose for oral administration, whereas citicoline was administered intraperitoneally, once daily for a total of 9 days with treatments starting 7 days before the induction of ischemia reperfusion injury. Detailed protocol is shown in Figure 1.
### Table 1. Experimental Groups

| S. No. | Treatment Group (mg/Kg) | Treatment                                                                 |
|--------|-------------------------|--------------------------------------------------------------------------|
| 1      | Naive                   | Untreated animals                                                        |
| 2      | Sham                    | BCCAO surgical procedure without occlusion                               |
| 3      | IR                      | BCCAO for 30 min; 48 h reperfusion; vehicle (p.o.)                        |
| 4      | Azd (1.5)               | IR + Azelnidipine (1.5 mg/Kg) (p.o.) for 9 days                          |
| 5      | Azd (3)                 | IR + Azelnidipine (3 mg/Kg) (p.o.) for 9 days                            |
| 6      | Citi (250)              | IR + Citicoline (250 mg/Kg) (i.p.) for 9 days                            |
| 7      | Azd (1.5) + Citi (250)  | IR + Azelnidipine (1.5 mg/Kg) (p.o.) + Citicoline (250 mg/Kg) (i.p.) for 9 days |

**Abbreviations:** IR, ischemia reperfusion; BCCAO, bilateral common carotid artery occlusion.

### Behavioral Assessments

**Locomotor Activity**

Gross locomotor function in the animals was assessed by using a digital actophotometer and represented as the number of total photo beams crossed in 5 min as previously reported.\(^{20,21}\)

**Rota Rod Performance**

A rota rod apparatus (IMCORP, India) was used to assess motor coordination and grip performance as previously reported.\(^ {20,21}\) Fall off time was recorded in each 5 min trial.

**Beam Balance Test**

Using a wooden bar of 2 cm width and an inclination of 60° angle from the ground, rats were scored on the basis of their performance when placed on the beam as a measure of gait abnormality and gross vestibular motor function, as reported earlier.\(^ {20,22,23}\)

### Biochemical Estimations

**Sample Collection and Preparation**

After the assessment of behavioral functions, animals were sacrificed by cervical dislocation, brains were harvested, and cortex was processed for the preparation of cytosolic fraction as reported previously.\(^ {20}\) Tests for biochemical and molecular parameters were done using this cytosolic fraction.

**Measurement of Lipid Peroxidation, Nitrite, Reduced Glutathione, and Superoxide Dismutase**

The assessment of lipid peroxidation according to the method of Wills,\(^ {24}\) nitrite using the method of Green,\(^ {25}\) reduced glutathione using the method of Ellman,\(^ {26}\) superoxide
dismutase by using the method of Kono, and total protein by Bradford method was done as reported previously.

**Assessment of Mitochondrial Parameters**

**Isolation of Mitochondria**
Differential centrifugation was employed for the isolation of brain mitochondria using a slightly modified method of Brown. Cortex from the isolated brains was processed in appropriate buffer solutions to prepare the mitochondrial fraction as reported earlier.

**Mitochondrial Enzyme Complexes**
Enzyme activities of mitochondrial complex I (NADH dehydrogenase), II (succinate dehydrogenase), and IV (cytochrome C oxidase) were studied using the methods of King and Howard, King, and Sottocasa, respectively.

**Mitochondrial Viability**
Viability of the isolated mitochondrial fractions after respective experimental treatments was estimated using MTT dye which employs the presence of hydrogenases from viable mitochondria to give a colorimetric reaction.

**Molecular Estimations**

**Assessment of Glutamate and Caspase-3**
Caspase-3 colorimetric kit (R&D systems, USA) was used for the assessment of caspase-3 activity in the cytosolic fraction of brain samples, whereas glutamate measurement was done by using rat glutamate colorimetric kit (EnzyChrom; BioAssay Systems, USA).

**Infarct Area**
A standard triphenyl tetrazolium chloride (TTC) staining procedure was employed for the assessment of relative infarct area in the isolated brain sections from different experimental groups according to the procedure reported previously.

**Histopathology**
Hematoxylin and eosin staining (H&E) of the microsections from isolated brain samples of different experimental groups was done using a procedure reported previously to study histopathological changes after different treatments.

**Statistical Analysis**
Graph Pad Prism software (Graph Pad Prism La Jolla, CA, USA) was used to statistically analyze all the data which was expressed as mean ± SEM. Two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was employed for behavioral parameters, whereas one-way ANOVA followed by Tukey’s post hoc test was used for the analysis of biochemical, mitochondrial, molecular, and histological assessments. P < 0.05 was considered to be the criterion for statistical significance in all tests.

**Results**

**Effect of Azelnidipine and Citicoline Treatment on Locomotor Activity, Rota Rod Performance, and Beam Balance Score**
All treatment groups had similar baseline locomotor activity, rota rod performance, and beam balance score when assessed at 0 h. Ischemia reperfusion injury significantly reduced the locomotor activity and rota rod performance, besides increasing the beam balance score as compared to the sham group after 48 h. Azelnidipine (3 mg/Kg) and citicoline were found to significantly improve the behavioral function of ischemic rats individually, whereas a pronounced effect was seen when azelnidipine (1.5 mg/Kg) was given in combination with citicoline (Figure 2).

**Impact of Azelnidipine and Citicoline on Postischemic Oxidative Stress**
BCCAO lead to a significant elevation in the levels of pro-oxidant marker MDA and nitrite while causing depletion in GSH and superoxide dismutase (SOD) enzyme activities in the ischemia-reperfusion (IR) group as compared to sham group. 9 days’ individual treatment with azelnidipine (1.5

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**Figure 2.** Effect of Azelnidipine and Citicoline on Behavioral Function

**Abbreviations:** Azd, Azelnidipine; Ct, Citicoline.

**Notes:** *P* < 0.05 vs sham, *P* < 0.05 vs IR, *P* < 0.05 vs Ct (250), and *P* < 0.05 vs Azd (1.5).
and 3 mg/Kg) and citicoline significantly lowered MDA and nitrite as compared to IR treated group. A significant increase in SOD was seen in azelnidipine (1.5 and 3 mg/Kg) and citicoline (250 mg/Kg) groups as compared to the IR group, but only citicoline (250 mg/Kg) and not azelnidipine could significantly increase GSH in IR-treated animals. Treatment using a combination of azelnidipine (1.5 mg/Kg) and citicoline significantly decreased MDA and nitrite, and restored GSH and SOD as compared to their individual effects (Figure 3).

**Effect of Azelnidipine and Citicoline Treatment on Mitochondrial Parameters**

Mitochondrial enzyme activities (I, II, and IV) along with mitochondrial viability were found to be significantly reduced in rats subjected to IR injury as compared to corresponding sham group. Viability and enzyme activities were found to be significantly improved in groups treated with azelnidipine (1.5 and 3 mg/Kg) and citicoline (250 mg/Kg) as compared to ischemic controls. The individual effects of these treatments were further observed to be significantly enhanced when a combination of azelnidipine (1.5 mg/Kg) with citicoline was administered to rats (Figure 4).

**Effect of Azelnidipine and Citicoline on Caspase-3**

BCCAO treatment for 30 min (IR) significantly increased caspase-3 activity as compared to respective sham controls. 9-day treatments with azelnidipine (1.5 and 3 mg/Kg) and citicoline significantly attenuated caspase-3 activity as compared to the IR group. Combination treatment for 9 days with azelnidipine (1.5 mg/Kg) and citicoline significantly reduced caspase-3 activity as compared to their individual effects (Figure 5).
Effect of Azelnidipine and Citicoline on Glutamate Levels

IR treatment significantly increased glutamate levels as compared to the respective sham controls. Significant reduction in glutamate levels was seen in azelnidipine (1.5 and 3 mg/Kg) and citicoline-treated groups as compared to the ischemic controls. Further reduction in the levels of glutamate was observed in groups treated with a combination of azelnidipine (1.5 mg/Kg) and citicoline as compared to their individual effects in ischemic animals (Figure 6).

Effect of Azelnidipine and Citicoline on Infarct Area

In comparison to sham controls, ischemic rat brains showed a significant increase in infarct area. Azelnidipine (3 mg/Kg) and citicoline treatments carried out individually decreased the infarct area significantly as compared to the ischemic controls. Further, treatment for 9 days with a combination of azelnidipine (1.5 mg/Kg) and citicoline (250 mg/Kg) significantly decreased the infarct area as compared to their individual effects (Figure 7[a] and [b]).

Figure 8 Effect of Azelnidipine and Citicoline Treatment on Histopathological Changes in the Cortical Region of Ischemic Rat Brains

**Abbreviations:** IR, ischemia reperfusion; Azd, azelnidipine; Ct, citicoline.

**Notes:** Photomicrographs (40X) of H&E stained brain coronal sections. **Black arrow,** healthy neurons and **red arrow,** damaged neurons. Calibration bar = 50 μm.

Effect of Azelnidipine and Citicoline on Histopathological Analysis

Hematoxylin and eosin staining of the coronal sections from cortex regions of the brain showed healthy and well-integrated neurons in naïve and sham group (black arrows). However, many pyknotic neurons were observed with increased vacuolation and shrunken chromatin (red arrows) in IR group. 9 days’ treatment with azelnidipine (3 mg/Kg) and citicoline (250 mg/Kg) significantly decreased the occurrence of pyknotic nuclei, and more healthy neurons were seen as compared to the IR group. Further, the administration of azelnidipine (1.5 mg/Kg) in combination with citicoline (250 mg/Kg) for 9 days significantly preserved the histological morphology with healthy neurons showing distinct nucleus and cytoplasm, decreased vacuolation, and lowered necrotic and apoptotic neuronal death (Figure 8).

Discussion

This study is based on the repurposing of 2 clinically available drugs which have been hypothesized to act in synergism for their neuroprotective abilities in the event of cerebral ischemic injury. Selective L-type calcium channel blocker azelnidipine and phosphatidylcholine analogue citicoline have been screened for their effects alone as well as in combination using 2 vessel occlusion model of cerebral ischemia in Wistar rats. The study has been supported by relevant molecular, neurobehavioral, mitochondrial, biochemical, and histopathological evaluations.

In the current study, animals subjected to IR treatment exhibited severe behavioral dysfunction as assessed by rota rod activity, actophotometer, and beam balance tests. 9 days’ pretreatment with azelnidipine (3 mg/Kg) and citicoline (250 mg/Kg) significantly improved neurobehavioral function as compared to IR group, whereas insignificant results were seen with low dose azelnidipine. Behavioral function improved further when a combination of low-dose azelnidipine was given with citicoline. Besides many research reports that demonstrate protective effect of citicoline through antioxidant mechanism, azelnidipine has been shown to be...
neuroprotective against ischemic injury because of inhibition of lipid peroxidation. Therefore, to evaluate the effect of azelnidipine and citicoline on endogenous pro-oxidant and antioxidant systems, we further studied MDA, nitrite, GSH, and SOD activities. Azelnidipine and citicoline significantly decreased the oxidative stress markers and increased SOD as compared to IR group, but only citicoline and not azelnidipine could increase the endogenous antioxidant GSH. However, in combination, a significant decrease in pro-oxidant markers and increase in both antioxidant markers was observed as compared to the effects of azelnidipine and citicoline individually in ischemic animals. In addition to this, the activity of mitochondrial enzyme complexes (I, II, and IV), along with mitochondrial viability, was found to be restored by azelnidipine and citicoline, when administered alone or in combination as compared to the ischemic controls where these activities were diminished. This effect can be attributed to the calcium channel blocking effects of azelnidipine, thereby decreasing the mitochondrial calcium load and decreasing the excitotoxic damage that leads to mitochondrial dysfunction. On part of citicoline, this effect could be due to the hypothesis that citicoline has the ability to restore the levels of a mitochondrial inner membrane exclusive phospholipid, cardiolipin, which helps in the integrity of mitochondria and hence its function. Because mitochondrial dysfunction contributes toward the leakage of cytochrome c and apoptotic mediators into the cytosol, we next checked cytosolic levels of caspase-3. Caspase-3 was found to be increased in the IR group significantly as compared to sham animals. This increase was found to be significantly attenuated by azelnidipine and citicoline, when administered alone or in combination, suggesting antiapoptotic effects of the duo. Posts ischemic excitotoxicity is one of the first mechanisms that initiate neuronal damage. 2 most important mediators of excitotoxicity are calcium and glutamate. The effect of selective L-type VDCC blocker, azelnidipine, on cytosolic glutamate levels was therefore studied along with citicoline, which has shown to decrease neuronal release of glutamate and increase its astrocytic uptake. It was found that the combination of both drugs significantly decreased cytosolic glutamate levels as compared to their individual effects, reflecting their synergism in action. Further, to visualize the effects produced by these drugs on the brain after an ischemic insult, we performed TTC staining and H&E staining of the coronal brain sections. In TTC staining, it was evident that infarct area increased significantly after IR injury, which was found to be significantly improved by high-dose azelnidipine and citicoline treatment. A combination group of both drugs further decreased the infarct area but the effect remained to be insignificant as compared to their individual effects. In histological studies, we found that H&E-stained sections of the IR group had plenty of pyknotic and inflamed neurons with disturbed cytoarchitecture. Treatment with azelnidipine and citicoline, alone or in combination, preserved the cytoarchitecture of neurons and also maintained their integrity. Pyknosis and vacuolization was reduced and increased number of healthy neurons could be seen.

Conclusion

This study therefore highlighted the pharmacological justification for use of 2 promising drugs in combination for stroke-prone subjects who have had a history of stroke, transient ischemic attack, or are subjected to stroke risk factors. This combination serves a dual purpose of managing hypertension, which is the most important stroke risk factor, and also of arresting the neuronal damage which occurs after an ischemic insult by targeting excitotoxic, antioxidative, antiapoptotic, and mitochondrial mechanisms, independent of antihypertensive effects of azelnidipine.

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Author Contributions

Varun Gupta executed the in vivo experiments, performed sample analysis, and wrote the manuscript. Zein Eddin Bader performed histopathological experiments. Aakriti performed blinded behavioral analysis. Anil Kumar designed the study, edited, and approved the final manuscript.

Ethical Statement

The experimental design and protocol was approved by the Institutional Animal Ethics Committee (Approval No. PU/IAEC/S/14/124; November, 2014) and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSCEA) guidelines for the rational use and care of experimental animals.

This article complies with International Committee of Medical Journal editor’s (ICMJE) uniform requirements for manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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