RESEARCH ARTICLE

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Effect of the Spin Trapping Compound PBN and Thiotriazoline on the Outcome from Experimental Middle Cerebral Artery Occlusion in Rats

Ihor Belenichev[1*], Sergej Pavlov[1], Elena Sokolik[1], Ivan Mazur[1], Nina Buhtiyarova[1], Lyudmyla Kucherenko[1]

Abstracts: The article aims to provide a comparative estimation of thiotriazoline's and PBN's neuroprotective effect on the outcome from experimental middle cerebral artery occlusion in rats. 60 Wistar rats were subjected to transient, middle cerebral artery occlusion and were randomly assigned to 3 treatment groups (n=10 each): (1) Control, (2) PBN, (3) Thiotriazoline. All rats were subjected to 90 minutes of MCA occlusion by insertion of a silicone-coated, 4-0 nylon monofilament via the external carotid artery. Investigated preparations were administered enteraly within 1 hr after operation. Functional deficits were quantified by daily neurological examinations (Garcia et al., 1995); rats' behavior was quantified in the test of Passive Avoidance Conditioned Response (PACR). Infarct volume was assessed after 7 days. Nitrotyrosine value was measured after 3 days. PBN and thiotriazoline reduced total infarct volume by 36.8%, and 24.7%, respectively, relative to controls. PBN and thiotriazoline therapy reduced infarct volume in the basal ganglia. Compared with vehicle-treated controls, the infarct volume in the basal ganglia in rats treated with PBN was 24.2±6.2 mm^3 and in rats treated with thiotriazoline, it was 18.2±4.8 mm^3.PBN and thiotriazoline, caused major reduction of ischemic brain damage when administered in a single dose 60 min after onset of MCAO. This benefit was evident both histologically and neurologically in rats allowed to survive 7 d after the ischemic insult. Animals treated with thiotriazoline had the best neurological recovery, the fewest postoperative cognitive deficits, and the smallest infarct volume.

Key Words: Cerebral ischemia PBN, Thiotriazoline, MCAO, *Search for neuroprotectors in ischemia treatment.*

INTRODUCTION

Recently, clinical trials of several neurodegenerative diseases have increasingly targeted the evaluation of the effectiveness of various antioxidants. Stroke – also referred to as ischemia/reperfusion, a cerebrovascular accident, or brain attack – often has devastating neuropathological, neurophysiological, and biobehavioral consequences that commonly result in permanent disability or death. Ischemic stroke accounts for 70 to 80% of all strokes. The neural damage that results during ischemia is a consequence of a variety of negative factors. To prevent extensive damage, it is most important to restore the blood supply to the ischemic tissue as quickly as possible; however, in doing so, reperfusion with oxygenated blood causes further molecular destruction and neuronal damage and death (Fisher, Schaebitz, 2000). The reduction of blood flow decreases the production of high energy phosphates. The energy failure causes membrane depolarization and uncontrolled release of excitatory aminoacids, such as glutamate, in the extracellular space (excitotoxicity). Glutamate acts on various types of receptors, e.g. NMDA and AMPA, eventually causing calcium overload of neuronal cells (Lafon-Cazal et al 1993). Calcium activates proteolytic enzymes that begin to degrade both intracellular and extracellular structures, and other enzymes, i.e. phospholipase A2 and cyclooxigenase. Factors generally believed to be culpable for ischemia injury include a variety of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxygen-derived reactants of particular interest include the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly toxic hydroxyl radical (•OH). The most damaging RNS include nitric oxide (NO•) and especially the peroxynitrite anion (ONOO). Neuronal nitric oxide synthase (Lipton, Rosenberg, 1994) is also calcium dependent and produces nitric oxide, which is able to react with superoxide generating the highly reactive radical peroxynitrite (Lee et al, 1999). The increased production of free radicals in the setting of cerebral ischemia can arise from induction of nitric oxide synthase (Iadecola, 1996) or cyclooxigenase (Iadecola et al, 1999), autooxidation of catecholamines (Braughler, Hall, 1989); metabolism of free fatty acids, particularly arachidonic acid, released during ischemia (Schmidley, 1990), migration of neutrophils and leukocytes able to generate superoxide anions (Matsuo et al, 1995; Walder et al, 1997).

Multiple in vitro studies have demonstrated that a series of biochemical changes result in the production of free radicals following ischemia. PBN **(**alpha phenyl-N-tret-butyl nitrone) can covalently bind to these radicals and prevent the peroxidation of cellular proteins and fatty acids. The consequence of the trapping of these carbon-centered and oxygen-centered radicals is the termination of the propagation phase of free radical production within the neuron. This interruption of free radical production can decrease the mortality and morbidity seen in strokes. PBN has been shown to be an excellent neuroprotective and anti-inflammatory agent (Floyd et al, 1999; Kotake et al, 1998). PBN has extended the life span of mice (Saito, 1998), improved cognitive performance in rats (Sack, 1996), reversed protein oxidation in aged gerbils and returned them to youthful states (Dubey, 1995). PBN has attenuated hydroxyl radical formation in ischemia-reperfusion injury (Dubey, 1995), blocked nitric oxide synthase, which minimized the exitotoxicity of peroxynitrite radical (Joseph et al, 1995).

The aim of the present study is to provide a comparative estimation of thiotriazoline and PBN neuroprotective activity in the model of MCAO. Thiotriazoline is a biologically active compound from a family of azaheterocyclic substances. It was synthesized at the Department of Pharmaceutical Chemistry in 1986. Principle structures of thiotriazoline and PBN are given in figure 1.

METHODS

The experiment was performed in Wistar rats, m 250-300 gr, bred in the Arboretum of the Institute of Pharmacology and Toxicology of Medical Sciences Academy of Ukraine. Animals were cared for before and at all stages of the experiment in compliance with applicable institutional guidelines and regulations of the bioethics committee of Ukraine.

The 60 rats used in these experiments were randomly divided into six groups (n=10) (four experimental groups and two control groups). For experiment A three groups of animals were used. For experiment B another three groups of animals were used. Rats were fasted overnight before surgery with free access to water. For the operative procedures, the animals received atropine (0.5 mg/kg SC), and anesthesia was induced with 4% halothane. The animals were orally intubated and mechanically ventilated with 0.8% halothane in a mixture of 70% N_2O and 30% O_2 to maintain normal arterial blood gases. All rats were subjected to 90 minutes of the middle cerebral artery (MCA) occlusion by insertion of a silicone-coated, 4-0 nylon monofilament via the external carotid artery as previously described (Schmid-Elsaesser et al, 1998). In brief, the filament was gently advanced until the laser-Doppler flowmeter (LDF) showed a sharp decrease of the ipsilateral local cortical blood flow (LCBF) to 20% of baseline, indicating adequate occlusion of the MCA. SAH was confirmed by autopsy.

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Inventi Impact: Molecular Pharmacology Vol. 1, Issue 3
[E- ISSN 0976–7509, P- ISSN 2229–4171]

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Published on Web 15/01/2011, www.inventi.in

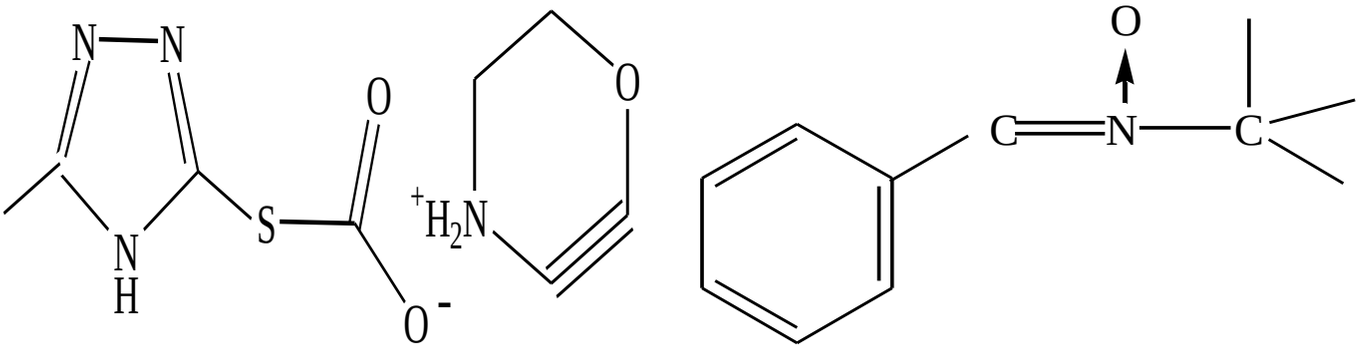

Figure 1. Thitriazoline (morpholinium 3-methyl-1,2,4-triazolil-5-thioacetat) and PBN (n-tret-butyl-alpha-phenylnitrone)

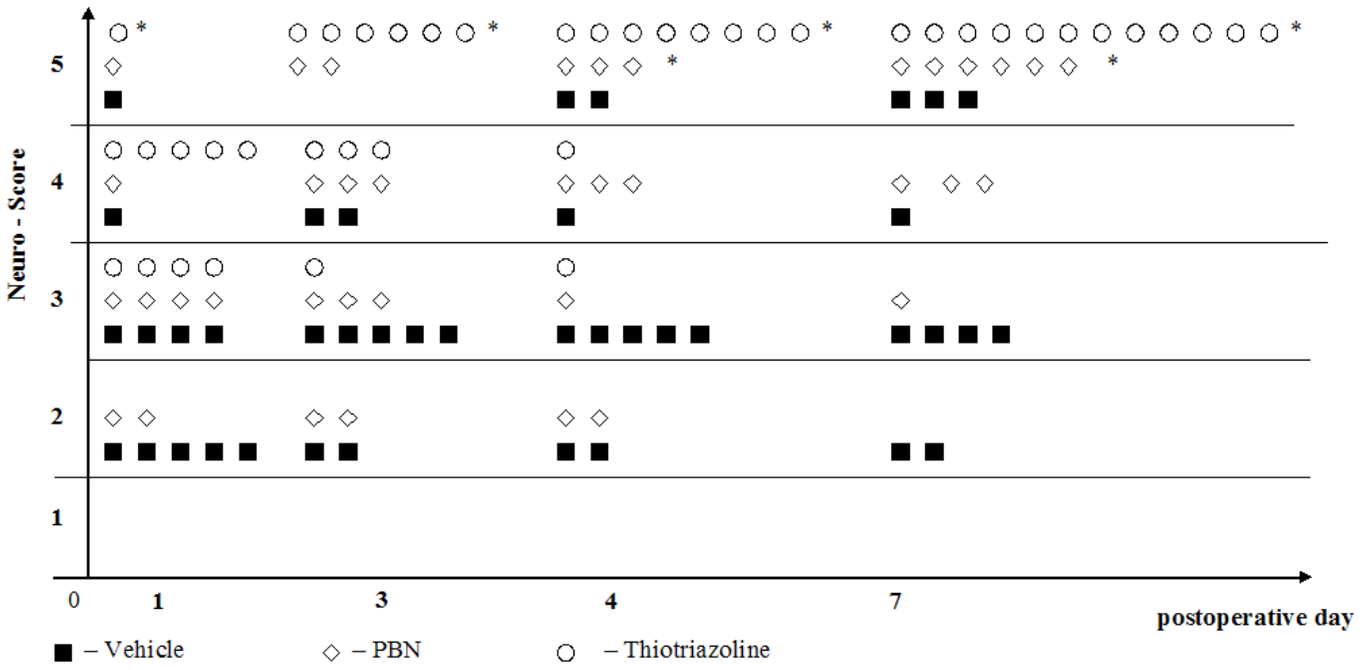

Figure 2. Neurological recovery over a 7-day examination period after focal ischemia. Each symbol depicts the individual neurolo-score of a single animal: 5, no apparent deficit; 4, contralateral forelimb flexion; 3, lowered resistance to lateral push without circling; 2, circling if pulled by tail; 1, spontaneous circling; and 0, no spontaneous activity. The animals were daily examined, but for the sake of clarity, only postoperative days 1, 3, 4, and 7 are presented. Note that animals treated with thiotriazoline had a significantly faster recovery compared with other groups and that no animal in this group had any residual deficit at the end of the observation period. **P*<0.05 vs vehicle-treated controls, by Kruskal-Wallis ANOVA on ranks followed by the Student-Newman-Keuls test

Reperfusion was achieved by withdrawing the filament into the external carotid artery after 90 minutes. Then animals were placed in an O_2-enriched environment (40% O_2 in room air) for overnight recovery. Animals were returned then to their home cages.

Investigated preparations were introduced enteraly 60 min after onset of ischemia at a dose: PBN – 50 mg/kg; "Thiotriazoline", morpholinium 3-methyl-1, 2, 4-triazolil-5-thioacetat (Galichpharm, Ukraine) – 100 mg/kg.

Neurological Examination

In experiment A, postoperatively, each animal underwent a standardized neurological examination designed to evaluate sensorimotor function (Garcia et al., 1995). With the observer blinded to group assignment, this test explored six different functions (spontaneous activity, movement symmetry, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch). The individual performance in each test was rated with a 0-3 point score. Neurological function scores were analyzed for each of the 7 postoperative days. The score that was given to each animal at the completion of the testing was the sum of all six individual scores, 0 being the minimum (worst) and 18 being the maximum (best) score.

Behavior function was evaluated in the Passive Avoidance Conditioned Response (PACR) test (Bures et al, 1991). An automated apparatus consisting of two connected chambers, one lighted, one dark, was used. Rats tend to prefer a dark environment and will immediately enter the darkened chamber. Passive avoidance was performed as a two day task. On day 1 (before MCAO), the training session, the rat was placed in the lighted chamber for ten seconds. The door to the dark chamber was then opened, and latency to enter the dark chamber was measured as a control for visual ability and motor activity. Immediately after the rat entered the dark chamber, a 0.3 mA, 1-sec footshock was delivered. The rat remained in the dark compartment for 10 sec after the shock to allow formation of the association between the

Inventi Impact: Molecular Pharmacology Vol. 1, Issue 3
[E- ISSN 0976–7509, P- ISSN 2229–4171]

Published on Web 15/01/2011, www.inventi.in

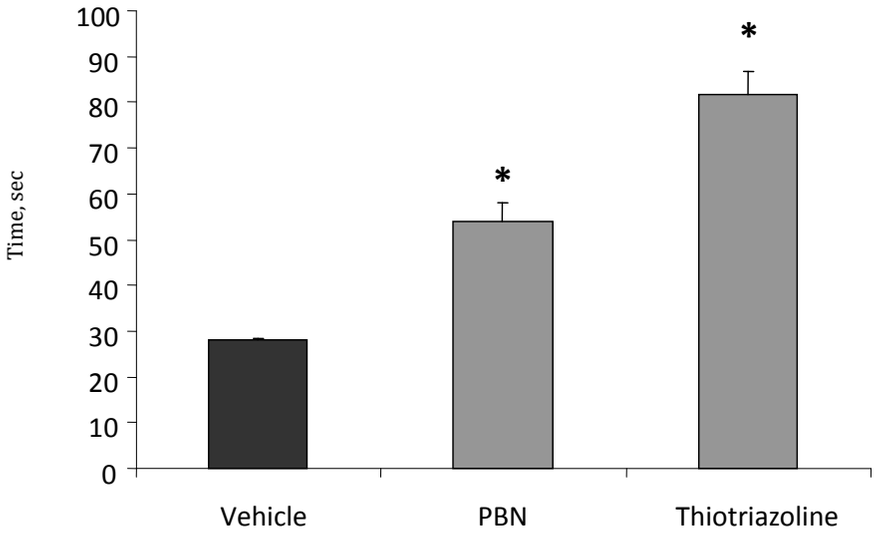

Figure 3. Latency to enter the dark compartment on a 2-day examination period after focal ischemia. *P<0.05 vs vehicle-treated controls

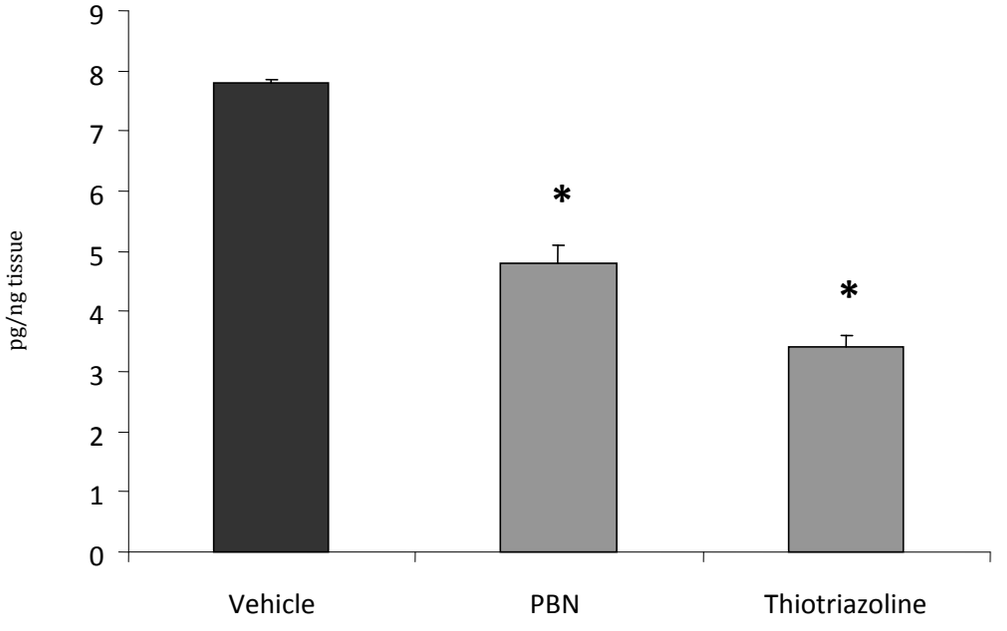

Figure 4. Nitrotyrosine value 3 days after middle cerebral artery occlusion. Nitrotyrosine value (6 pg/ng) was expressed as mean±SD for n=10 in each group. *P<0.05 vs vehicle-treated controls

dark compartment and the footshock. For active avoidance the rat must move into the opposite chamber to avoid receiving a footshock. Latency to enter the non-shocked chamber is the measure of learning. The rat was then returned to the home cage. On day 2 (12 hr after MCAO), the retention test session, each rat was placed in the lighted compartment and the door was opened. Latency to enter the dark compartment was measured. The rat was then returned to its home cage.

Infarct volume measurement: Seven days after transient cerebral ischemia, the rest of rats were again anesthetized and perfused transcardially with isotonic heparinized saline, followed by 2% paraformaldehyde for fixation of tissues. The brain was removed, embedded in paraffin, and cut into 4-µm-thick coronal sections at 400-µm intervals. The brain slices were stained with hematoxylin and eosin. Twenty-four slices from each brain containing the entire infarct were used, and the infarct area on each slice was planimetrically determined. Infarct volume was analyzed with microscope Axioskop (ziess, Germany) and COHU-4922 (COHU Inc., USA). The total infarct volume (I_T) expressed in mm^3 was calculated to be the sum of the area of infarct on each slice (I_n), multiplied by the distance (400 µm) between successive slices (I_T=0.4[I_1+I_2+... I_{24}] mm^3). The volumes of infarcts in the cortex and basal ganglia were determined by measuring the area of infarct in sections obtained 2.0, 3.6, 5.2, 6.8, and 8.4 mm from the frontal pole.

Inventi Impact: Molecular Pharmacology Vol. 1, Issue 3
[E- ISSN 0976–7509, P- ISSN 2229–4171]

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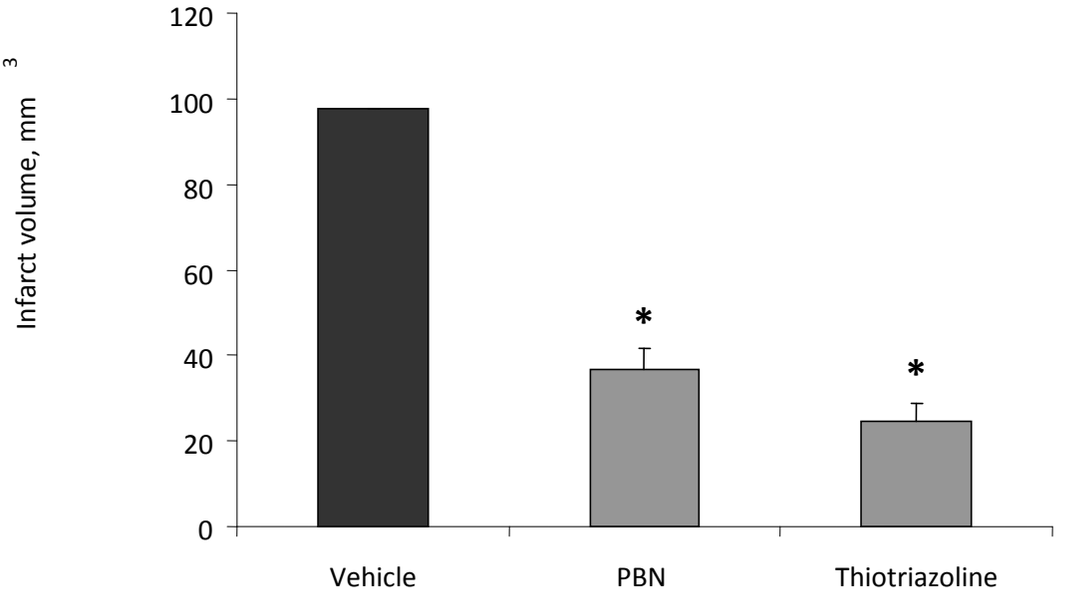

Figure 5. Treatment effects on total infarct volume 7 days after middle cerebral artery occlusion. Infarct volume (mm^3) was expressed as mean±SD for n=10 in each group. **P*<0.05 vs vehicle-treated controls by 1-way ANOVA followed by Dunnett's test

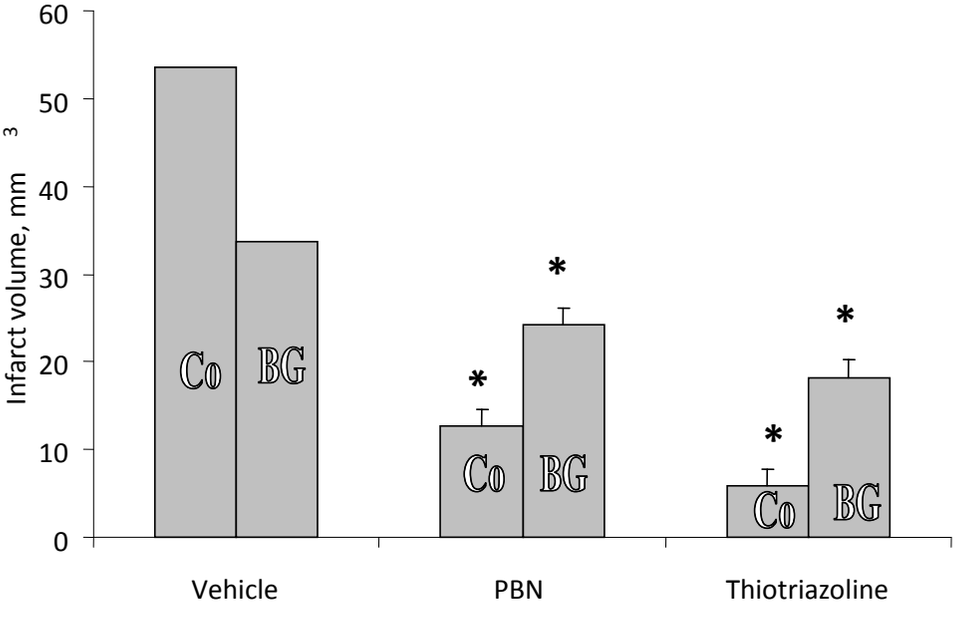

Figure 6. Treatment effects on infarct volume in the cortex (Co) and basal ganglia (BG) 7 days after middle cerebral artery occlusion. Infarct volumes in the cortex and basal ganglia (mm^3) were expressed as mean±SD for n=10 in each group. **P*<0.05 vs vehicle-treated controls, by 1-way ANOVA followed by Dunnett's test

In experiment B, three days after transient cerebral ischemia rats were killed by decapitation and the brains were removed; the striatum was dissected out and stored at –80°C. The tissue sample was acid hydrolyzed in 6 N HCl, and the hydrolyzed striatum protein sample was dissolved in 200 µl of mobile phase, which consisted of 50 mM K_2HPO_4, 50 mg/ml Na EDTA, and 5% methanol, pH 6.3. The sample was injected onto a Prodigy ODS column connected to a glassy carbon electrode maintained at 1V versus an Ag/AgCl reference electrode (BAS Bioanalytical System). Nitrotyrosine values are reported as picograms per nanogram of tissue.

Statistical analyses were performed with the use of STATISTICA 4.0 Statistical Software (Statistica Inc., USA). Neurological function

Inventi Impact: Molecular Pharmacology Vol. 1, Issue 3
[E- ISSN 0976–7509, P- ISSN 2229–4171]
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Published on Web 15/01/2011, www.inventi.in

scores were analyzed with Kruskal-Wallis ANOVA on ranks for each of the 7 postoperative days. When multiple comparisons were indicated, Dunnett's test or the Student-Newman-Keuls test for neurological function scores was applied. Differences were considered significant at the $P<0.05$ level. Results are presented as mean±SD.

Principle of the Nitrotirosine Test

The Nitrotirosine ELISE test kit is a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle. Samples and standards are incubated in microtiter wells coated with antibodies recognizing nitrotirosine. During this incubation nitrotirosine is captured by the solid bound antibody. Unbound material present in the sample is removed by washing. Biotinylated second antibody (tracer) to nitrotirosine is added to the wells. If nitrotirosine was present in the sample, the tracer antibodies will bind to the captured nitrotirosine. Excess tracer is removed by washing. Streptavidin-peroxidase conjugate is applied to the wells; this conjugate reacts specifically with the biotinylated tracer antibody bound into the captured nitrotirosine. Excess streptavidin-peroxidase conjugate is removed by washing and substrate, tetramethylbenzidine is added to the wells. Color develops proportionally to the amount of nitrotirosine present in the sample. The enzyme reaction is stopped by the addition of citric acid and the absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbances versus the corresponding concentrations of defined standard. The nitrotirosine concentration of samples with unknown concentrations, which are run concurrently with the standarts, can be determined from the standard curve.

RESULTS

Neurological function data showed up to 5 point score neurological deficit in 1-7 day. Animals that received PBN and thiotriazoline had significantly ($P<0.05$) less neurological deficits from postoperative days 1 to 7 compared with vehicle-treated controls (Figure 2).

Behavior Function

In the PACR model showed cognitive deficit in rats with middle cerebral artery ischemia. There were statistically significant differences in mean cognitive deficit between animals that received PBN and thiotriazoline compared with vehicle-treated controls ($P<0.05$). Latency to enter the dark compartment in vehicle-treated controls was 28.2 sec, in PBN-treated rats – 53.9 sec; latency to enter the dark compartment in thiotriazoline-treated rats was 81.6 sec (Figure 3).

3-nitrotyrozine in Striatum

Nitrotyrosine value was 7.8 ± 0.6 pg/ng tissue (mean SD) in vehicle-treated controls, 4.8 ± 0.7 pg/ng tissue ($p < 0.05$ compared with vehicle) in animals that received PBN, 3.4 ± 0.6 pg/ng tissue ($p < 0.05$ compared with vehicle) in animals that received thiotriazoline (figure 4).

Infarct Volume

The total infarct volume was 97.6±14.0 mm^3 (mean±SD) in vehicle-treated controls, 36.8±6.7 mm^3 in animals that received PBN, 24.7±14.0 mm^3 in animals that received thiotriazoline. Compared with those in vehicle-treated controls, total infarct volumes were significantly ($P<0.05$) smaller in animals treated with PBN (-56%) and in animals that received thiotriazoline (-63%) (Figure 5).

When infarct volume was determined separately for cortical brain tissue and the basal ganglia, all treatment strategies were significantly ($P<0.05$) effective in limiting cortical infarct volume. The average cortical infarct volume was 53.6±11.2 mm^3 in vehicle-treated controls (mean±SD), 12.6±4.2 mm^3 in animals treated with PBN, 5.8±2.6 mm^3 in animals that received thiotriazoline (Figure 6).

Similarly, PBN and thiotriazoline therapy significantly ($P<0.05$) reduced infarct volume in the basal ganglia. Compared with vehicle-treated controls (33.7±5.3 mm^3, mean±SD), the infarct volume in the basal ganglia in rats treated with PBN was 24.2±6.2 mm^3 and in rats treated with thiotriazoline, it was 18.2±4.8 mm^3 (Figure 6).

DISCUSSION AND CONCLUSION

Several studies demonstrate that ischemic stroke is associated with an increased production of free radicals in animal models (Fisher, Schaebitz, 2000; Iadecola, 1996; Iadecola et al, 1999). Once generated free radicals can react with all the cellular macromolecules leading to lipid peroxidation, DNA and protein oxidation (Belay, 1997; Belenichev et al, 2005). Lipid peroxidation can lead to membrane damage. Damage to proteins, particularly when they are enzymes, can lead to impairment of their function. Finally DNA oxidation can cause the activation of repair enzymes, such as poly(ADP-ribose) polymerase (PARP), that provokes a rapid depletion of intracellular energy leading to cell death. The previous studies examined whether thiatriazoline reduced oxidative stress and improved outcome from experimental cerebral ischemia (Dunaev et al, 2002). The previously summarized findings also certainly indicated that one of the mechanisms by which thiotriazoline reduced neural damage after ischemia involved its ability neutralize free radicals (Belenichev et al, 2002).

The mechanistic basis of the neuroprotective activity of PBN does not appear to rely on its general free radical trapping or antioxidant activity per se, but its activity in mediating the suppression of genes induced by pro-inflammatory cytokines and other mediators associated with enhanced neuroinflammatory processes. Neuroinflammatory processes, induced in part by pro-inflammatory cytokines, yield enhanced reactive oxygen species (ROS) and reactive nitric oxide species (RNS) as well as other components that have neurotoxic properties.

The present study demonstrates that PBN and thiotriazoline treatment provides significant cerebroprotection to rats subjected to transient MCA occlusion. PBN and thiotriazoline caused major reduction of ischemic brain damage when administered in a single dose 60 min before onset of MCAO. This benefit was evident both histologically and neurologically in rats allowed to survive 7 d after the ischemic insult. Animals treated with thiotriazoline had the best neurological recovery, the fewest postoperative cognitive deficits, and the smallest infarct volume. Our findings support the conclusion that thiotriazoline's efficacy in limiting neuronal death and infarct volume that is a consequence of MCAO may well relate to its ability to directly detoxify the devastatingly toxic •OH and ONOO•. It is likely that the thiotriazoline's protective effects in the brain also involve preservation of mitochondrial physiology, stimulation of antioxidative enzymes, as well as other actions.

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Inventi Impact: Molecular Pharmacology Vol. 1, Issue 3
[E- ISSN 0976–7509, P- ISSN 2229–4171]

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Acknowledgement: This study was supported by Dr. Yuriy Kolesnik (Rector of Zaporizhzhia State Medical University); the authors thank Dr. Andriy Abramov for the preparations necessary to conduct these experiments; and Mrs. Svitlana Gorbachova for expert secretarial support.