Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 484 (2004) 65–74
www.elsevier.com/locate/ejphar

Treatment with tamoxifen reduces hypoxic–ischemic brain injury in neonatal rats

Yangzheng Feng[a], Jonathan D. Fratkins[b], Michael H. LeBlanc[a,*]

[a] Department of Pediatrics, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA
[b] Department of Pathology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA

Received 28 August 2003; received in revised form 27 October 2003; accepted 31 October 2003

Abstract

Tamoxifen, an estrogen receptor modulator, is neuroprotective in adult rats. Does tamoxifen reduce brain injury in the rat pup? Seven-day-old rat pups had the right carotid artery permanently ligated followed by 2.5 h of hypoxia (8% oxygen). Tamoxifen (10 mg/kg) or vehicle was given i.p. 5 min prior to hypoxia, or 5 min after reoxygenation, with a second dose given 6 h after the first. Brain damage was evaluated by weight deficit of the right hemisphere 22 days following hypoxia and gross and microscopic morphology. Tamoxifen pre-treatment reduced brain weight loss from 21.5 ± 4.0% in vehicle pups ($n = 27$) to 2.6 ± 2.5% in the treated pups ($n = 22$, $P < 0.05$). Treatment 5 min after reoxygenation reduced brain weight loss from 27.5 ± 4.0% in vehicle pups ($n = 42$) to 12.0 ± 3.9% in the treated pups ($n = 30$, $P < 0.05$). Tamoxifen reduces brain injury in the neonatal rat.

Keywords: Tamoxifen; Neuroprotection; Oxygen radical; Thiobarbituric acid reactive substance; Rat, neonatal

1. Introduction

Hypoxia–ischemia causes brain damage, activating a cascade of biochemical events (Strijbos et al., 1996). When oxygenation fails, ATP formation drops and excessive calcium influx occurs through voltage-sensitive calcium channels leading to glutamate release with activation of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate acid (AMPA) glutamate receptors. At the core of the mechanism is a vicious cycle in which glutamate receptor stimulation causes activation of tetrodotoxin-sensitive Na^+ channels, leading to further glutamate release and further NMDA and AMPA receptor stimulation. The output of the cycle is an enduring production of nitric oxide (NO) and free radicals from neuronal and glial sources, and is responsible for delayed neuronal death (Strijbos et al., 1996; Mishra and Delivoria-Papadopoulos, 1999). Therefore, most studies in seeking therapeutic targets in hypoxic–ischemic brain damage have focused on antagonists of NMDA or AMPA glutamate receptors, inhibitors of nitric oxide synthase (NOS) and free radicals and antiapoptotic pathways. Research evidence accumulated during recent years suggests that antagonists of NMDA or AMPA glutamate receptors, inhibitors of NOS and free radicals scavengers are neuroprotective in hypoxic–ischemic brain injury (for review, see Volpe, 2001).

Over the last few years, increasing evidence has indicated that 17β-estrogen is a neuroprotective agent in adult animal models (for review, see Dhandapani and Brann, 2002). However, there are only a few reports concerning the actions and effects of selective estrogen receptor modulators in the brain. Selective estrogen receptor modulators are steroidal or nonsteroidal compounds that can exhibit either estrogen-like agonistic or estrogen-antagonistic effects. Tamoxifen, a nonsteroidal selective estrogen receptor modulator, is widely used for the treatment and prevention of estrogen-dependent breast cancer (Butta et al., 1992; Jordan, 1993, 1997). Tamoxifen, which binds competitively to estradiol receptors to form distinct estrogen–ligand complexes (DeGregorio and Wiebe, 1996), is an effective estrogen antagonist in breast tissue, but an estrogen receptor agonist in bone, liver and uterus (Love et al., 1991; Jordan, 1993). Recently, tamoxifen has shown to be an effective inhibitor of Ca^+/calmodulin (Osuka et al., 2001; Lam, 1984; Lopes et al., 1990) and neuronal NOS

* Corresponding author. Tel.: +1-601-984-5260; fax: +1-601-815-3666.
E-mail address: mleblanc@ped.umsmed.edu (M.H. LeBlanc).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.10.048

(Renodon et al., 1997) and to reduce the production of nitrotyrosine in transient focal cerebral ischemia in adult rats (Osuka et al., 2001). Tamoxifen is an effective inhibitor of swelling-activated anion release (Kirk and Kirk, 1994) and excitatory amino acids release during ischemia (Seki et al., 1999). Tamoxifen also reduced ischemia-evoked excitatory amino acid release in a cortical superfusion model (Phillis et al., 1998) and protected glial cells against glutamate toxicity (Shy et al., 2000). Furthermore, tamoxifen inhibited both metal ion-dependent enzymatic and non-enzymatic lipid peroxidation in a number of model membranes (Thangaraju et al., 1994; Wiseman et al., 1990, 1992, 1993; Kimelberg et al., 2002), and suppressed 1-methyl-4-phenylpyridine induced hydroxyl radical formation (Obata and Kubota, 2001). More recently, tamoxifen have been shown to be markedly neuroprotective in adult rat models of reversible or permanent middle cerebral artery occlusion (Kimelberg et al., 2000, 2001; Mehta et al., 2003; for review, see Dhandapani and Brann, 2002). Considering these reports, our hypothesis is that tamoxifen has neuroprotective effects in the neonatal rat hypoxic–ischemic brain injury model.

Hypoxic–ischemic brain injury is a serious cause of death and disability in human newborns. The developmental stage of the brain of the 7-day-old rat pup resembles that of newborn humans (Palmer et al., 1990). The Rice–Vannucci–Brierley hypoxic–ischemic rat pup model (Rice et al., 1981) may best match the injury caused by birth asphyxia in full-term human infants (for review, see Ashwal and Pearce, 2001). Therefore, study of the role of neuroprotective agents in the neonatal hypoxic–ischemic rat model may provide important information pertinent to the development of treatment for perinatal hypoxic–ischemic brain damage. The neonatal rat hypoxic–ischemic model (Rice et al., 1981) has been well characterized and extensively used to assess synthetic neuroprotective agents (for review, see Ashwal and Pearce, 2001). We have used this hypoxic–ischemic model to evaluate the neuroprotective potency of several drugs (LeBlanc et al., 2000; Feng and LeBlanc, 2002; Feng et al., 2002, 2003). The purpose of the present study was thus to evaluate the effects of tamoxifen, on neuroprotection and lipid peroxidation by using the neonatal rat hypoxic–ischemic model. This has not previously been tested.

2. Materials and methods

2.1. Animal protocol

This protocol was approved by our institutional committee on animal use. Rats were cared for in accordance with National Institutes of Health guidelines. The neonatal rat hypoxic–ischemic procedure was performed as described by Rice et al. (1981). In preliminary results, we found plasma levels of 17β-estradiol in untreated 7-day-old male and female pups to be 1.8 ± 0.1 ($n=6$) and 1.7 ± 0.1 ($n=8$) pg/ml, respectively. These levels are not significantly different between male and female rat pups and are much lower than in adult female rats (19.5 ± 4.9, $n=5$, $P<0.01$). This result is similar to reports by others (for review, see Hurn and Mhairi Macrae, 2000). In preliminary results, we also found there are no differences in brain damage between 7-day-old male (50.0%, $n=20$) and female (45%, $n=20$, $P>0.05$) rats in neonatal rat hypoxic–ischemic brain injury model. Seven-day-old Sprague–Dawley rat pups of either sex, weighing between 12 and 17 g, were used (Harlan Sprague Dawley, Indianapolis, IN). They were anesthetized with isoflurane (4% induction, 2% maintenance). The right common carotid artery was exposed, isolated and permanently doubly ligated. After surgery, the rat pups were returned to their dams for 2–3 h recovery. Hypoxic exposure was achieved by placing the rat pups for 2.5 h in 1.5-l sealed jars immersed 5.5 cm deep in a 37 °C water bath and subjected to a warmed, humidified mixture of 8% oxygen/92% nitrogen bubbled through 37 °C water and delivered at 4 l/min for 2.5 h. This results in a jar temperature of 33 °C immediately above the pups. After this hypoxic exposure, some pups were taken for thiobarbituric acid reactive substances testing and other pups were returned to their dams and allowed to recover and grow for 22 days for estimating brain injury. Pups were weighed prior to injury and again at 4, 7, 11, 14 and 22 days after injury.

2.2. Drug treatment

Pups from each litter were randomly assigned and marked to a control group or for treatment with tamoxifen citrate salt (Sigma, St. Louis, MO). Tamoxifen in doses of 2.5, 5, 10 or 20 mg/kg was dissolved in 0.67 μl of dimethyl sulfoxide per gram of body weight and administered i.p. 5 min prior to hypoxia or 5 min or 1 h after reoxygenation and then again (second dose) at 6 h after the first injection. The control group was given 0.67 μl of dimethyl sulfoxide per gram of body weight alone. These doses were chosen as most promising from previous studies in adult rats (Kimelberg et al., 2000, 2001). Biegon et al. (1996) reported that maximum levels of tamoxifen in brain were recorded 5–60 min post-injection by tail vein and the $t_{1/2}$ was 4 h. We chose to inject a second dose of tamoxifen at 6 h after the first injection to maintain brain concentration. Dimethyl sulfoxide, at slightly higher doses, has neuroprotective effects acting as an oxyradical inhibitor and an excitatory amino acid inhibitor (Lu and Mattson, 2001; Greiner et al., 2000; Sugita et al., 1993). Thus it is important that the controls received an equal dose of solvent.

2.3. Measurement of rectal temperature

To evaluate whether neuroprotection by tamoxifen was dependent on systemic hypothermia, rectal temperature was

measured with a 36-gauge flexible thermocouple (Omega Engineering, Stamford, CT). This was done in a subset of pups (six from the control group and six given 10 mg/kg of tamoxifen at 5 min after hypoxia exposure) prior to placing the pups in the jar and at 0.25, 0.5, 1, 2, 4, 6 and 24 h after removal. Measurements of rectal temperature were made in a 25 °C room, 15 min after removal of the pups from the nest. Rat pups of this age cool rapidly once they are removed from the nest and the dam (McDonald et al., 1991). Rectal temperature and brain temperature are almost identical and are tightly correlated (Yager et al., 1993). Since decreased body temperature both during and after the hypoxia can effect the outcome, it is essential that both the treated and control animals maintain similar temperatures (Feng and LeBlanc, 2002; Yager et al., 1993).

2.4. Gross brain damage grading

Rat pups were anesthetized with pentobarbital and decapitated 22 days after hypoxic exposure. The brains were removed, scored and weighed by an observer blind to the code. Brains were scored normal, mild, moderate or severe by the method of Palmer et al. (1990). "Normal" or "1" meant no reduction in the size of the right hemisphere, "mild" or "2" meant visible reduction in right hemisphere size, "moderate" or "3" meant large reduction in hemisphere size with a visible infarct in the right parietal area, and "severe" or "4" meant near total destruction of the hemisphere (Palmer et al., 1990). After removing the cerebellum and brainstem, the brain was divided into two hemispheres and weighed. Results are presented as the percent loss of hemispheric weight of the right side relative to the left [(left − right)/left × 100]. This hypoxic–ischemic model results in brain damage only on the ipsilateral side (Palmer et al., 1990; Rice et al., 1981). The loss of hemispheric weight can be used as a measure of brain damage in this model, since enough time elapsed to allow resorption of the dead tissue (Hagberg et al., 1994; LeBlanc et al., 2000; Bona et al., 1997). Also, the brain of the newborn rat grows rapidly and the dead tissue does not contribute to this growth.

2.5. Microscopic brain damage grading

To verify that the gross changes were a reflection of the expected histopathologic changes, microscopic examination of the tissues was carried out in a group of rat pups treated prior to hypoxia with 10 mg/kg of tamoxifen or vehicle. Fourteen rat pups were treated 5 min prior to hypoxia and 6 h after hypoxia with 10 mg/kg tamoxifen i.p. or the equivalent volume of vehicle. They were anesthetized with pentobarbital 3 days after injury. Their brains were perfusion fixed by cardiac puncture. They were flushed with saline then fixed with 10% buffered formalin. After removal, the brains were stored in 10% buffered formalin. Sections were then embedded with paraffin. Five-micron coronal sections were cut in the parietal region aiming for the equivalent of Bregma − 4.3 to − 4.5 mm (Kruger et al., 1995) in the adult rat and then stained with hemotoxylin and eosin. Cerebral cortex, hippocampus and thalamus were scored by an observer blind to the treatment group of the animal from 0 to 5 by the method of Cataltepe et al. (1995), where "0" is normal, "1" is 1–5% of neurons damaged, "2" is 6–25% of neurons damaged, "3" is 26–50% of neurons damaged, "4" is 51–75% of neurons damaged, "5" is >75% of neurons damaged. Damaged neurons for scores of 1–3 usually were shrunken cells with pyknotic nuclei and eosinophillic cytoplasm replacing the healthy neurons in patchy areas of the brain. Damaged neurons for scores of 4 and 5 usually showed loss of tissue with partially replacement by inflammatory cells and connective tissue.

2.6. Determination of lipid peroxidation

A second set of experiments was performed to determine the effect of tamoxifen on lipid peroxidation. The same neonatal hypoxia–ischemia procedure was performed using a 2.5-h period of hypoxia. Pups were randomly assigned to a sham group ($n=8$), pre-treatment with vehicle ($n=25$) or pre-treatment with tamoxifen at a dose of 10 mg/kg ($n=26$). At 1, 3 and 6 h after reoxygenation, the pups were decapitated, brains were removed, and each side of the cerebral cortex was frozen at − 80 °C. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances levels as described by Buege and Aust (1978). Though the thiobarbituric acid assay is not specific for malondialdehyde determination and several other aldehydic products and cellular molecules can react with thiobarbituric acid, this is one of the reliable methods used to determine lipid peroxidation. In brief, brain samples were homogenized in four volumes of ice-cold buffer (5 mM butylated hydroxytoluene, 3 mM EDTA and 20 mM Tris–HCl) and centrifuged at 2000 × g for 20 min at 4 °C. A portion of this homogenate (0.2 ml) was added to a tube containing 0.4 ml of 5% trichloracetic acid with 0.5% thiobarbituric acid. This was spun at 2000 × g for 20 min to remove the precipitate, and the supernatant was heated in a water bath at 95 °C for 30 min. Thiobarbituric acid reactive substances were measured using the absorption spectra between 400 and 585 nm. The peak at 532 nm relative to background was measured. The assays were tested using a standard of 1,1,3,3-tetramethoxypropane. Brain thiobarbituric acid reactive substances values were expressed as picomoles per gram of tissue.

2.7. Statistics

Categorical variables were analyzed with the χ^2 test. Other variables are presented as mean ± S.E.M. and the statistical significance of differences between groups were determined using analysis of variance with the Student–Newman–Keuls test. Repeated measures analysis of variance was used for rectal temperature and body weight. Differences were considered significant at $P<0.05$.

3. Results

3.1. Temperature and body weight

Rectal temperatures obtained prior to placing the pups in the jar containing 8% O_2, and at 0.25, 0.5, 1, 2, 4, 6 and 24 h after hypoxia were not significantly different between 10 mg/kg tamoxifen- and vehicle-treated pups at any times (Fig. 1). The pups had a temperature of 33 °C prior to the hypoxic exposure. This rises to 35 °C in the heated hypoxic jars and returns to 33 °C after returning the pups to their dams. The pups' temperatures were measured in a 25 °C room, 15 min after removal from the nest and dam. Pups' temperatures measured immediately after removal from the nest would be warmer. Tamoxifen does not effect body temperature. Body weights of the tamoxifen-treated groups were not significantly different from controls prior to injury or at 4, 7, 11, 14 or 22 days after injury. Body weights increased significantly with time in all groups as the pups grew. No pups died during the 22-day recovery period.

3.2. Neuroprotective effects of pre-treatment with tamoxifen

The pup's brain damage scores are shown in Fig. 2 (1 is normal, 4 is severe). Pre-treatment with tamoxifen significantly improved the brain scores from 2.33 ± 0.22 S.E.M., $n=27$ in the vehicle group to 1.66 ± 0.17 ($n=29$) with 5 mg/kg ($P<0.05$) and to 1.41 ± 0.13 ($n=22$) with 10 mg/kg ($P<0.05$). The dose of 2.5 mg/kg of tamoxifen produced a similar effect [1.67 ± 0.13 ($n=22$)], but this was not quite statistically significant. The dose of 20 mg/kg did not affect the brain damage score [2.13 ± 0.31, ($n=15$), $P>0.05$].

Left hemisphere weights were 501.5 ± 7.4 mg ($n=27$), 494.8 ± 9.8 mg ($n=16$), 493.0 ± 7.6 mg ($n=29$), 509.5 ± 7.5 mg ($n=22$) and 495.6 ± 7.8 mg ($n=15$) in the 0 (vehicle), 2.5, 5, 10 and 20 mg/kg tamoxifen pre-treatment groups, respectively. The left hemisphere was unaffected by either the hypoxia–ischemia procedure (Palmer et al., 1990) or the treatment. The percent reduction in right hemispheric weight is shown in Fig. 3. Tamoxifen significantly reduced the decrease in right hemisphere weight from 21.5 ± 4.0% ($n=27$) in the vehicle group to 2.6 ± 2.6% ($n=22$) in the group receiving 10 mg/kg of tamoxifen ($P<0.05$). The doses of 2.5 and 5 mg/kg of tamoxifen produced a similar effect (reduced the decrease in weight to 11.3 ± 4.1% in the group receiving 2.5 mg/kg of tamoxifen, $n=16$; and to 10.8 ± 3.9% in the group receiv-

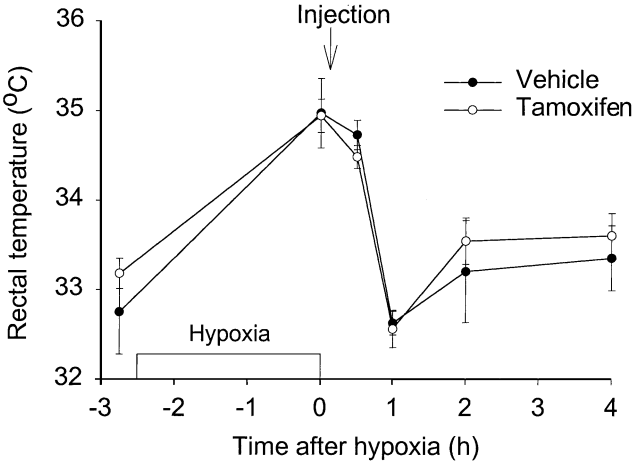

Fig. 1. Effect of tamoxifen on rectal temperatures (mean ± S.E.M.) in the hypoxic–ischemic rat pups. Administration of tamoxifen (10 mg/kg) or vehicle occurred i.p. at 5 min after hypoxia. There were no significant differences between groups during the various times.

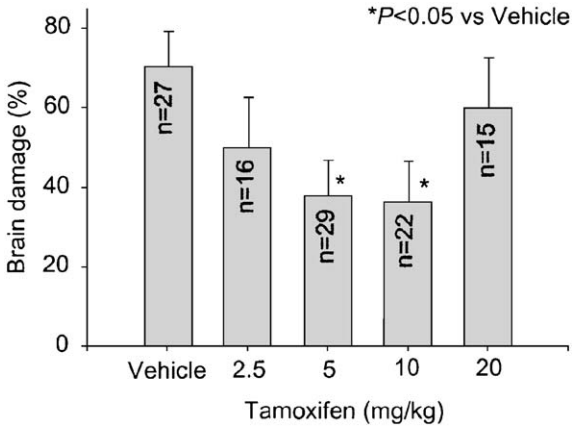

Fig. 2. The effect of different doses (2.5–20 mg/kg i.p.) of tamoxifen administered at 5 min prior to hypoxia and 6 h later on the degree of gross brain damage. Brain damage was scored by a blind observer 22 days after hypoxia–ischemia. Data are presented as mean ± S.E.M. Pre-treatment with 5 or 10 mg/kg of tamoxifen improved the brain scores compared to vehicle (*$P<0.05$). Pre-treatment with 2.5 mg/kg of tamoxifen also tended to reduce brain damage score, but this reduction was not statistically significant ($P>0.05$).

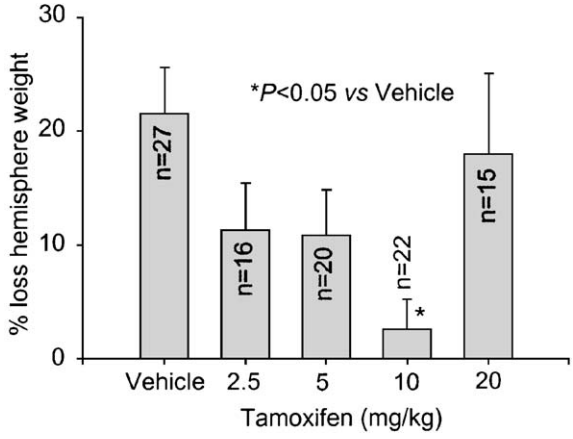

Fig. 3. The dose–response for percentage reduction in right cerebral hemisphere weight measured using the left hemisphere weight as the standard. Treatment was given 5 min prior to hypoxia and 6 h later. Injury was evaluated 22 days later. Data are presented as mean ± S.E.M. Pre-treatment with 10 mg/kg of tamoxifen decreased the percentage reduction in right hemisphere weight compared to vehicle (*$P<0.05$). Pre-treatment with 2.5 or 5 mg/kg of tamoxifen also tended to reduce weight loss in the right hemisphere, but these reductions were not statistically significant ($P>0.05$).

ing 5 mg/kg of tamoxifen, $n=29$), but these were not quite statistically significant ($P>0.05$). Pre-treatment with 20 mg/kg of tamoxifen did not affect the reduction in right hemispheric weight ($17.9 \pm 7.0\%$, $n=15$) compared to the vehicle ($P>0.05$).

The histopathologic score (Fig. 4) for the cortex at 3 days after injury of rat pups pretreated 5 min prior to hypoxia with 10 mg/kg of tamoxifen i.p. was 2.1 ± 0.7 ($n=7$), while that in the vehicle-treated group was 4.1 ± 0.6, $n=7$, $P<0.05$. The histopathologic score for the hippocampus was 1.4 ± 0.5 in the tamoxifen group and 3.6 ± 0.3 in the vehicle group, $P<0.01$. The histopathologic score for the thalamus was 0.4 ± 0.5 in the tamoxifen group and 3.3 ± 0.7 in the vehicle group, $P<0.01$. In all three tissues, tamoxifen significantly improved the outcome.

3.3. Neuroprotective effects of post-treatment with tamoxifen

The effect of time of treatment on brain damage score is shown in Fig. 5. At 5 min after reoxygenation, treatment with tamoxifen improved the brain scores from 2.52 ± 0.18 ($n=42$) in the vehicle group to 1.90 ± 0.18 ($n=30$) with 10 mg/kg and to 2.21 ± 0.23 ($n=29$) with 5 mg/kg of tamoxifen, but these changes were not quite statistically significant ($P=0.08$). There was no difference in brain score between 10 mg/kg of tamoxifen- (1.95 ± 0.25, $n=21$) and vehicle-treated pups (1.86 ± 0.16, $n=29$) when treated at 1 h after reoxygenation (Fig. 5).

Left hemisphere weights were 504.1 ± 9.4 mg ($n=42$), 517.8 ± 10.4 mg ($n=29$), 502.2 ± 8.1 mg ($n=30$), 504.5 ± 8.1 mg ($n=21$) and 502.5 ± 8.1 mg ($n=29$) in the post-5-min treatment with 0 (vehicle), 5 and 10 mg/kg, and in the post-1-h treatment with 0 (vehicle) and 10 mg/kg of tamoxifen groups, respectively. Thus, the left hemisphere was unaffected by the procedure. The percent

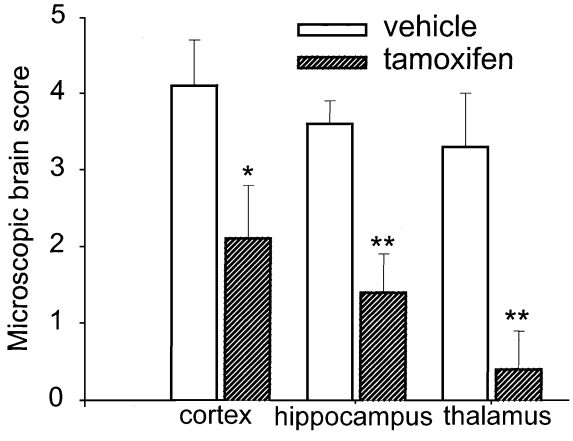

Fig. 4. The effect of 10 mg/kg of tamoxifen administered i.p. 5 min before hypoxia and again 6 h later on microscopic brain damage score. Data are presented as mean ± S.E.M. Tamoxifen improved microscopic brain damage score in the cortex, hippocampus and thalamus. *$P<0.05$, **$P<0.01$ vs. vehicle.

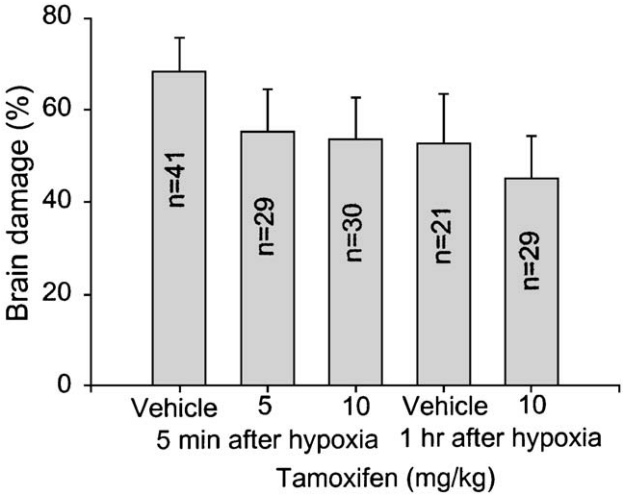

Fig. 5. The effect of tamoxifen administered at 5 min or 1 h after hypoxia and again 6 h later on the degree of gross brain damage. Brain damage was scored by a blind observer 22 days after hypoxia–ischemia. Data are presented as mean ± S.E.M. Post-treatment at 5 min reoxygenation with 5 or 10 mg/kg of tamoxifen tended to reduce brain damage score, but these reductions were not quite statistically significant ($P=0.08$). Post-treatment at 1 h after reoxygenation with 10 mg/kg of tamoxifen was ineffective.

reduction in right hemispheric weight is shown in Fig. 6. At 5 min after reoxygenation treatment with tamoxifen significantly reduced the decrease in right hemisphere weight from $27.5 \pm 4.1\%$ in the vehicle group ($n=41$) to $12.0 \pm 3.9\%$ in the group receiving 10 mg/kg of tamoxifen ($n=30$, $P<0.05$), but not in 5 mg/kg of tamoxifen-treated group ($22.0 \pm 4.3\%$, $n=29$, $P>0.05$). Post-treatment at 1 h after reoxygenation with 10 mg/kg of tamoxifen did not affect the reduction in right hemispheric weight

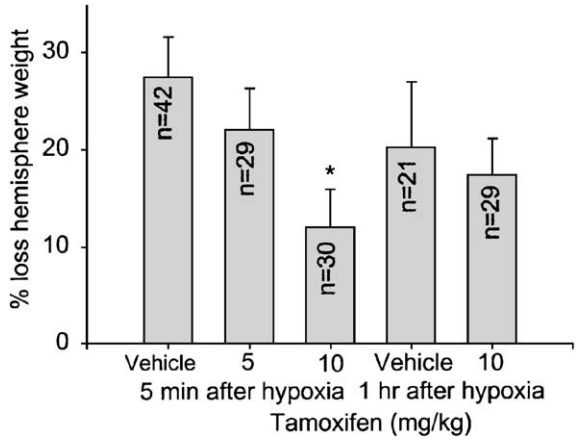

Fig. 6. The time-course for percentage reduction in right cerebral hemisphere weight measured using the left hemisphere weight as the standard. Treatment was given 5 min or 1 h after hypoxia and repeated 6 h later, and injury was evaluated 22 days later. Data are presented as mean ± S.E.M. Post-treatment at 5 min after reoxygenation with 10 mg/kg of tamoxifen significantly reduce weight loss in the right hemisphere (*$P<0.05$). Post-treatment at 5 min after reoxygenation with 5 mg/kg of tamoxifen or at 1 h after reoxygenation with 10 mg/kg of tamoxifen was ineffective ($P>0.05$).

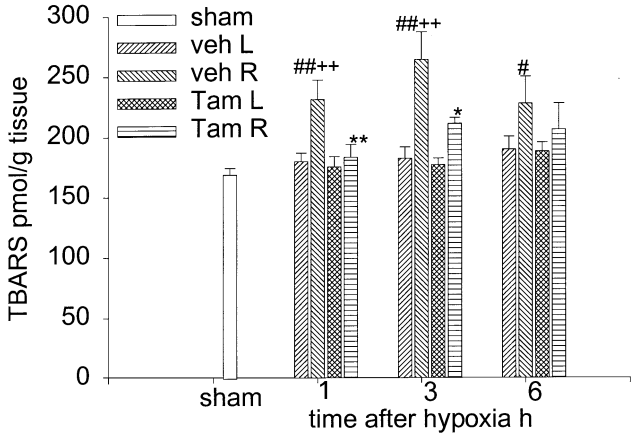

Fig. 7. The effects of tamoxifen on brain thiobarbituric acid reactive substances (TBARS). Treatment with tamoxifen (10 mg/kg) was given 5 min prior to hypoxia and brain thiobarbituric acid reactive substances were assessed 1, 3 and 6 h after reoxygenation. Data are expressed as the mean ± S.E.M. from eight to nine pups in each group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with sham; $^{*}P<0.05$, $^{**}P<0.01$ compared with right (R) hemisphere cortex in the vehicle-treated group; $^{++}P<0.01$ compared with left (L) hemisphere cortex.

(17.4 ± 3.7%, $n=21$) compared with the vehicle (20.2 ± 6.7%, $n=29$, $P>0.05$, Fig. 6).

3.4. Effect of tamoxifen on thiobarbituric acid reactive substances

The concentrations of thiobarbituric acid reactive substances in the left hemisphere cortex were not affected by the hypoxia and there were no significant differences among the sham-, vehicle- and tamoxifen-treated groups. The concentrations of thiobarbituric acid reactive substances in the right hemisphere cortex were significantly higher in the vehicle group at 1 h (230.9 ± 16.1 pmol/g, $n=8$, $P<0.01$), 3 h (263.7 ± 22.9 pmol/g, $n=8$, $P<0.01$) and 6 h (227.5 ± 22.3 pmol/g, $n=9$, $P<0.05$) after reoxygenation than in the sham group (168.6 ± 5.5 pmol/g, $n=8$). The concentrations of thiobarbituric acid reactive substances in the right hemisphere were also significantly higher in the vehicle group at 1 and 3 h after reoxygenation than in the left hemisphere ($P<0.01$). Pre-treatment with tamoxifen (10 mg/kg) eliminated the increase in thiobarbituric acid reactive substances in the right hemisphere at 1 h (183.2 ± 10.7 pmol/g, $n=9$, $P<0.01$) and significantly reduced the increase seen at 3 h (210.9 ± 5.1 pmol/g, $n=9$, $P<0.05$) after reoxygenation relative to the vehicle group (Fig. 7).

4. Discussion

The present study demonstrates for the first time that pre- or post-treatment with tamoxifen significantly reduces the degree of brain injury in the focal ischemia model of the neonatal rat pup. Tamoxifen has been shown to be neuroprotective in the adult rat focal cerebral ischemia model (Kimelberg et al., 2000, 2001; Mehta et al., 2003). Delayed neuronal injury sometimes requires a prolonged period to develop (Trescher et al., 1997). Three weeks following the insult, hemispheric hypotrophy is present with a resultant weight difference between the injured and the non-injured hemisphere. There is a high degree of correspondence between the weight deficit of the injured hemisphere and the histologically evaluated loss of brain tissue (Hagberg et al., 1994; Andine et al., 1990; Towfighi et al., 1994). Thus, pre-treatment with tamoxifen is effective at dose of 5 or 10 mg/kg in the rat pup and post-treatment with tamoxifen at 5 min after hypoxia is effective at dose of 10 mg/kg in the rat pup. Kimelberg et al. (2000) reported that treatment with 5 mg/kg of tamoxifen by intravenous infusion before ischemia or at 1 h after reperfusion was strongly neuroprotective in the adult rat model of reversible middle cerebral artery occlusion, but not when given at 2 or 4 h after reperfusion. They also reported that pre-treatment with 20 mg/kg of tamoxifen by i.v. and maintained with 20 mg/kg of tamoxifen by i.p. every 12 h for 3 days was neuroprotective in a model of permanent middle cerebral artery occlusion (Kimelberg et al., 2001). Mehta et al. (2003) reported that treatment with 0.8 and 2.4 mg/kg/day of tamoxifen by implanting sustained release pellets for 1 week exerts neuroprotective effect in the rat middle cerebral artery occlusion model, but not a dose of 0.1 mg/kg/day. However, unlike in the adult rat middle cerebral artery occlusion model, pre-treatment with 2.5 mg/kg of tamoxifen, or post-treatment at 5 min after reoxygenation with 5 mg/kg of tamoxifen tended to reduce brain injury in newborn rat hypoxic–ischemic model, but these reductions were not statistically significant. Pre-treatment with a dose of 20 mg/kg or post-treated at 1 h reoxygenation with 10 mg/kg of tamoxifen showed no neuroprotective effect in our study. The reason for this difference is unclear but may relate to differences in animal age, animal model or other variation in the protocols.

Neuroprotective effects of tamoxifen have been studied in adult ovariectomized rats (Kimelberg et al., 2001; Mehta et al., 2003) and it has been suggested that tamoxifen may be neuroprotective in post-menopause in women (Mehta et al., 2003). Our preliminary data showed that normal serum estrogen level in newborn is lower than that of adults. The normal level of serum estrogen in 10-day-old female rat pups is less than 1/4 of that in pro-oestrus and oestrus adult female rats (Presl et al., 1967). Therefore, we speculate that the neonatal rat hypoxic–ischemic brain injury model will provide a useful model for investigating neuroprotective effects of tamoxifen or estrogen. In our present study, the neuroprotective effects of tamoxifen in newborn rats were observed. Our finding is important as it suggests that tamoxifen may be neuroprotective in human newborn hypoxic–ischemic brain damage.

An inverted U-shaped dose–response curve was observed for the neuroprotective effect of tamoxifen (Figs. 2 and 3). This type dose–response curve has been seen with other neuroprotective agents (Hagberg et al., 1994; Cai et al., 2002; Feng and LeBlanc, 2002; Sullivan et al., 2000). The dose of 20 mg/kg of tamoxifen showed no neuroprotective effect in this model. High concentrations or high doses of tamoxifen have been reported to activate caspases and trigger apoptotic cell death (Hashimoto et al., 1997; Mandlekar and Kong, 2001). Low concentrations (5×10^{-7} M) of tamoxifen block 3-nitropropionic acid-induced intracellular Ca^{2+} increase and cell damage but high concentrations (5×10^{-4} M) of tamoxifen induce cell damage (Mogami et al., 2002). The results of the current study show the therapeutic dose range for the neuroprotective effects of tamoxifen was narrow and the therapeutic time window of tamoxifen in the neonatal rat hypoxic–ischemic brain injury model was before, or immediately after hypoxia.

As noted in the introduction, tamoxifen exerts complex effects in the brain, and has many possible mechanisms for neuroprotection.

In this study, we did not directly explore other potential mechanisms of action of tamoxifen, apart from showing that tamoxifen reduced a hypoxia-induced increase in brain thiobarbituric acid reactive substances. Thiobarbituric acid reactive substances primarily reflect production of lipid peroxides, which are broken down during the assay to yield malonadialdehyde (Hageman et al., 1992). Lipid peroxide formation must be initiated by hydroxyl radical or its by-products or by peroxynitrite (Chan, 1996). The formation thiobarbituric acid reactive substances is one of the oldest and most frequently used tests for measurement of lipid peroxidation (Shinnhuber and Yu, 1957). Thiobarbituric acid reactive substances have been detected in rat and piglet brains during hypoxia–ischemia and the levels of thiobarbituric acid reactive substances increased significantly with hypoxia–ischemia (Inan et al., 1995; Feng et al., 2000; Kimelberg et al., 2002). Agents that inhibit lipid peroxidation have been shown to reduce a hypoxia–ischemia induced increase in brain thiobarbituric acid reactive substances and to be neuroprotective in animal models of cerebral hypoxia–ischemia (Zhao et al., 2001; Inan et al., 1995; Feng et al., 2000). In the present study, hypoxic–ischemic rat pups showed 1.5-fold elevations in thiobarbituric acid reactive substances concentrations in the brain at 1 and 3 h after reoxygenation. Pre-treatment with 10 mg/kg of tamoxifen eliminated the increase in thiobarbituric acid reactive substances at 1 and 3 h after reoxygenation. Similar results showing tamoxifen inhibited lipid peroxidation have been reported by other observers (Thangaraju et al., 1994; Wiseman et al., 1990, 1992, 1993; Kimelberg et al., 2002; Obata and Kubota, 2001). Tamoxifen, being a highly lipophilic compound, can physically partition into the cell membrane, and the lipophilic moiety of the molecule interacts with the saturated, monounsaturated and polyunsaturated residues of phospholipids (Obata and Kubota, 2001). Tamoxifen has been shown to prevent lipid peroxidation in both animal and human systems (Thangaraju et al., 1994; Wiseman et al., 1992) and has a protective effect on MPP^{+}-induced hydroxyl radical generation in the extracellular space of the striatum (Obata and Kubota, 2001). Tamoxifen's inhibition of lipid peroxidation may be one important mechanism for its neuroprotective effect.

Tamoxifen may act by inhibiting NOS. NO is enzymatically formed from the terminal guanidino-nitrogen of L-arginine by NOS. NO is an important neural messenger molecule in the central nervous system. Under brain hypoxia–ischemia, NO, through the production of peroxynitrite, can cause oxidative DNA damage and single-stranded DNA breaks and initiate the apoptotic cascade through stimulation of poly(-ADP–ribose) polymerase (Samdani et al., 1997). A number of studies have reported an increase in NO in the hypoxic–ischemic neonatal rat brain (Hamada et al., 1994; Tsuji et al., 2000; Higuchi et al., 1998, LeBlanc et al., 2000; Feng et al., 2002). The NOS inhibitors, N^{G}-nitro-L-arginine (NOARG), N^{G}-nitro-L-arginine methyl ester (L-NAME), aminoguanidine and agmatine, reduce the increase in NO metabolites in the hypoxic–ischemic brain and have shown neuroprotective effects on neonatal hypoxic–ischemic brain damage (Hamada et al., 1994; Tsuji et al., 2000; Higuchi et al., 1998; Feng et al., 2002). Tamoxifen is an effective inhibitor of nitrotyrosine production and inhibited neuronal NOS in transient focal cerebral ischemia (Renodon et al., 1997; Osuka et al., 2001). Tamoxifen also inhibited calmodulin (Lam, 1984; Lopes et al., 1990; Massom et al., 1990). Calmodulin antagonists have been shown to be neuroprotective in the adult rat middle cerebral artery occlusion model (Kuroda et al., 1997; Sato et al., 1999). More recently, Mogami et al. (2002) have reported that tamoxifen blocks 3-nitropropionic acid-induced increase in intracellular Ca^{2+} and cell damage in cultured rat cerebral endothelial cells.

Tamoxifen may act by reducing excitatory amino acid release. Glutamate has been implicated in neuronal death after both focal and transient global ischemia and glutamate receptor blockers have been shown to be highly neuroprotective in animal models of cerebral hypoxia–ischemia (Olney et al., 1989; Cai et al., 2002). Tamoxifen is a highly effective inhibitor of swelling activated amino acid release (Kirk and Kirk, 1994), and has been reported to act as a chloride channel blocker, reducing glutamate and aspartate release from the ischemic cerebral cortex (Phillis et al., 1998). Tamoxifen also has shown a protective action on glutamate toxicity in glial cells (Shy et al., 2000). Therefore, a mechanism responsible for the neuroprotective effects of tamoxifen may involve selective blockade of the NMDA receptor.

Neuroprotective effect of tamoxifen may be via the estrogen receptor. Over the last few years, increasing evidence has indicated that estrogen is a neuroprotective agent in vitro and in vivo (for review, see Hurn and Mhairi Macrae, 2000; Dhandapani and Brann, 2002). Tamoxifen has estrogen-like neuroprotective effects in the regulation of glucose transporter and insulin like growth factor-1 (IGF1) expression in the primate cerebral cortex (Cheng et al., 2001). Tamoxifen has estrogen-like neuroprotective effects on glutamate receptor levels in the rat cerebral cortex (Cyr et al., 2001a,b), and on intracellular Ca^{2+} concentration in cultured rat cerebral endothelial cells (Mogami et al., 2002). Tamoxifen has been shown to act as a modulator of estrogen responsive genes (Pearce et al., 2003). However, there is a report that tamoxifen abolishes estrogen's neuroprotective effect upon methamphetamine neurotoxicity on the nigrostriatal dopaminergic system (Gao and Dluzen, 2001). The mechanisms of action of tamoxifen's neuroprotection are therefore unclear. The theoretical beneficial effects of tamoxifen caused by activation of estrogen receptors needs further investigation.

In conclusion, our findings indicate that tamoxifen has neuroprotective properties. Treatment with tamoxifen reduces brain injury in the neonatal rat hypoxia–ischemia model. The results also support the view that the suppression of lipid peroxidation after hypoxia–ischemia by tamoxifen is one potential mechanism of this neuroprotection.

References

Andine, P., Thordstein, M., Kjellmer, I., Nordborg, C., Thiringer, K., Wennberg, E., Hagberg, H., 1990. Evaluation of brain damage in rat model of neonatal hypoxic–ischemia. J. Neurosci. Methods 35, 253–260.

Ashwal, S., Pearce, W.J., 2001. Animal models of neonatal stroke. Curr. Opin. Pediatr. 13, 506–516.

Biegon, A., Brewster, M., Degani, H., Pop, E., Somjen, D., Kaye, A.M., 1996. A permanently charged tamoxifen derivative displays anticancer activity and improved tissue selectivity in rodents. Cancer Res. 56, 4328–4331.

Bona, E., Johansson, B.B., Hagberg, H., 1997. Sensorimotor function and neuropathology five and six weeks after hypoxia–ischemia in seven-day-old rats. Pediatr. Res. 42, 678–683.

Buege, J.S., Aust, S.D., 1978. Microsomol lipid peroxidation. Methods Enzymol. 52, 302–310.

Butta, A., MacLennan, K., Flanders, K.C., Sacks, N.P.M., Smith, I., McKinna, A., Dowsett, M., Wakefield, L.M., Sporn, M.B., Baum, M., Colletta, A.A., 1992. Induction of transforming growth factor β1 in human breast cancer in vivo following tamoxifen treatment. Cancer Res. 52, 4261–4264.

Cai, Z., Lin, S., Rhodes, P.G., 2002. Neuroprotective effects of *N*-acetylaspartylglutamate in a neonatal rat model of hypoxia–ischemia. Eur. J. Pharmacol. 437, 139–145.

Cataltepe, O.V.R., Heitjan, D.F., Towfighi, J., 1995. Effect of status epilepticus on hypoxic–ischemic brain damage in the immature rat. Pediatr. Res. 38, 251–257.

Chan, P.H., 1996. Role of oxidants in ischemic brain damage. Stroke 27, 1124–1129.

Cheng, C.M., Cohen, M., Wang, J., Bondy, C.A., 2001. Estrogen augments glucose transporter and IGF1 expression in primate cerebral cortex. FASEB J. 15, 907–915.

Cyr, M., Thibault, C., Morissette, M., Landry, M., Di Paolo, T., 2001a. Estrogen-like activity of tamoxifen and raloxifene on NMDA receptor binding and expression of its subunits in rat brain. Neuropsychopharmacology 25, 242–257.

Cyr, M., Morissette, M., Landry, M., Di paolo, T., 2001b. Estrogenic activity of tamoxifen and raloxifene on rat brain AMPA receptors. NeuroReport 12, 535–539.

DeGregorio, M.W., Wiebe, V.J., 1996. Tamoxifen and Breast Cancer. Yale University Press, New Haven.

Dhandapani, K.M., Brann, D.W., 2002. Protective effects of estrogen and selective estrogen receptor modulators in the brain. Biol. Reprod. 67, 1379–1385.

Feng, Y., LeBlanc, M.H., 2002. Drug-induced hypothermia begun 5 min after injury with a poly(ADP–ribose) polymerase inhibitor reduces hypoxic brain injury in rat pups. Crit. Care Med. 30, 2420–2424.

Feng, Y., LeBlanc, M.H., LeBlanc, E.B., Parker, C.C., Fratkin, J.D., Qian, X.B., Patel, D.M., Huang, M., Smith, E.E., Vig, P.J.S., 2000. Desmethyl tirilazad improves neurologic function after hypoxic ischemic brain injury in piglets. Crit. Care Med. 28, 1431–1438.

Feng, Y., Piletz, J.E., LeBlanc, M.H., 2002. Agmatine suppresses nitric oxide production and attenuates hypoxic–ischemic brain injury in neonatal rats. Pediatr. Res. 52, 606–611.

Feng, Y., Shi, W., Huang, M., LeBlanc, M.H., 2003. Oxypurinol administration fails to prevent hypoxic–ischemic brain injury in neonatal rats. Brain Res. Bull. 59, 453–457.

Gao, X., Dluzen, D.E., 2001. Tamoxifen abolishes estrogen's neuroprotective effect upon methamphetamine neurotoxicity of the nigrostriatal dopaminergic system. Neuroscience 103, 385–394.

Greiner, C., Schmidinger, A., Hulsmann, S., Moskopp, D., Wolfer, J., Kohling, R., Speckmann, E.J., Wassmann, H., 2000. Acute protective effect of nimodipine and dimethyl sulfoxide against hypoxic and ischemic damage in brain slices. Brain Res. 887, 316–322.

Hagberg, H., Gilland, E., Diemer, N.H., Andiné, P., 1994. Hypoxia–ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. Biol. Neonate 66, 205–213.

Hageman, J.J., Bast, A., Vermeulen, N.P.E., 1992. Monitoring of oxidative free radical damage in vivo: analytical aspects. Chem.-Biol. Interact. 82, 243–293.

Hamada, Y., Hayakawa, T., Hattori, H., Mikawa, H., 1994. Inhibitor of nitric oxide synthesis reduces hypoxic–ischemic brain damage in the neonatal rat. Pediatr. Res. 35, 10–14.

Hashimoto, M., Inoue, S., Muramatsu, M., Masliah, E., 1997. Estrogen stimulate tamoxifen-induced neuronal cell apoptosis in vitro: a possible nongenomic action. Biochem. Biophys. Res. Commun. 240, 464–470.

Higuchi, Y., Hattori, H., Kume, T., Tsuji, M., Adaike, A., Furusho, K., 1998. Increase in nitric oxide in the hypoxic–ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. Eur. J. Pharmacol. 342, 47–49.

Hurn, P.D., Mhairi Macrae, I., 2000. Estrogen as a neuroprotectant in stroke. J. Cereb. Blood Flow Metab. 20, 631–652.

Inan, C., Kilic, I., Kilinc, K., Kalayci, O., Kotiloglu, E., 1995. The effect of high dose antenatal vitamin E on hypoxia-induced changes in newborn rats. Pediatr. Res. 38, 685–689.

Jordan, V.C., 1993. A current view of tamoxifen for the treatment and prevention of breast cancer. Br. J. Pharmacol. 110, 507–517.

Jordan, V.C., 1997. Tamoxifen treatment for breast cancer concept to gold standard. Oncology (Huntington) 11 (Suppl. 1), 7–13.

Kimelberg, H.K., Feustel, P.J., Jin, Y., Paquette, J., Boulos, A., Keller Jr., R.W., Tranmer, B.I., 2000. Acute treatment with tamoxifen reduces ischemic damage following middle cerebral artery occlusion. NeuroReport 11, 2675–2679.

Kimelberg, H.K., Jin, Y., Feustel, P.J., 2001. Tamoxifen is an effective

neuroprotectant in permanent as well as transient focal ischemia. Abstr.-Soc. Neurosci. 27 (Program No. 437.10).

Kimelberg, H.K., Samoilenko, S.G., Levko, A.V., Konev, S.V., Mongin, A.A., 2002. Tamoxifen inhibits $Fe2^{+}$/ascorbate-induced lipid peroxidation in brain synaptosomes: possible implications for neuroprotection in vivo. Abstr.-Soc. Neurosci. 27 (Program No. 201.19).

Kirk, J., Kirk, K., 1994. Inhibition of volume-activated I- and taurine efflux from HeLa cells by P-glycoprotein blockers correlates with calmodulin inhibition. J. Biol. Chem. 269, 29389–29394.

Kruger, L., Saporta, S., Swanson, L.W., 1995. Photographic Atlas of the Rat Brain. Cambridge Univ. Press, Cambridge. Plates 21 to 23.

Kuroda, S., Nakai, A., Kristian, T., Siesjo, B.K., 1997. The calmodulin antagonist trifluoperazine in transient focal brain ischemia in rats. Anti-ischemic effect and therapeutic window. Stroke 28, 2539–2544.

Lam, H.Y., 1984. Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem. Biophys. Res. Commun. 118, 27–32.

LeBlanc, M.H., Feng, Y., Fratkin, J.D., 2000. *N*-tosyl-L-phenylalanyl-chloromethylketone reduces hypoxic–ischemic brain injury in rat pups. Eur. J. Pharmacol. 390, 249–256.

Lopes, M.C.F., Vale, M.G.P., Carvalho, A.P., 1990. $Ca2^{+}$-dependent binding of tamoxifen to calmodulin isolated from bovine brain. Cancer Res. 50, 2753–2758.

Love, R.R., Wiebe, D.A., Newcomb, P.A., Cameron, L., Leventhal, H., Jordan, V.C., Feyxi, J., DeMets, D.L., 1991. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann. Intern. Med. 115, 860–864.

Lu, C., Mattson, M.P., 2001. Dimethyl sulfoxide suppresses NMDA- and AMPA-induced ion currents and calcium influx and protects against excitotoxic death in hippocampal neurons. Exp. Neurol. 170, 180–185.

Mandlekar, S., Kong, A.N., 2001. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6, 469–477.

Massom, L., Lee, H., Jarrett, H.W., 1990. Trifluoperazine binding to porcine brain calmodulin and skeletal muscle troponin C. Biochemistry 29, 671–681.

McDonald, J.W., Chen, C.K., Trescher, W.H., Johnston, M.V., 1991. The severity of excitotoxic brain injury is dependent on brain temperature in the immature rat. Neurosci. Lett. 126, 83–86.

Mehta, S.H., Dhandapani, K.M., De Sevilla, L.M., Webb, R.C., Mahesh, V.B., Brann, D.W., 2003. Tamoxifen, a selective estrogen receptor modulator, reduces ischemic damage caused by middle cerebral artery occlusion in the ovariectomized female rat. Neuroendocrinology 77, 44–50.

Mishra, O.P., Delivoria-Papadopoulos, M., 1999. Cellular mechanisms of hypoxic injury in the developing brain. Brain Res. Bull. 48, 233–238.

Mogami, M., Hida, H., Hayashi, Y., Kohri, K., Kodama, Y., Jung, C.G., Nitoo, N., 2002. Estrogen blocks 3-nitropropionic acid-induced $[Ca^{2+}]_i$ increase and cell damage in cultured rat erebral endothelial cells. Brain Res. 956, 116–125.

Obata, T., Kubota, S., 2001. Protective effect of tamoxifen on 1-methyl-4-phenylpyridine-induced hydroxyl radical generation in the rat striatum. Neurosci. Lett. 308, 87–90.

Olney, J.W., Ikonomidou, C., Mosinger, J.L., Friedrich, G., 1989. MK-801 prevents hypoxic–ischemic neuronal degeneration in infant rat brain. J. Neurosci. 9, 1701–1704.

Osuka, K., Feustel, P.J., Mongin, A.A., Tranmer, B.I., Kimelberg, H.K., 2001. Tamoxifen inhibits nitrotyrosine formation after reversible middle cerebral artery occlusion in the rat. J. Neurochem. 76, 1842–1850.

Palmer, C., Vannucci, R.C., Towfighi, J., 1990. Reduction of perinatal hypoxic–ischemic brain damage with allopurinol. Pediatr. Res. 27, 332–336.

Pearce, S.T., Liu, H., Jordan, V.C., 2003. Modulation of estrogen receptor α function and stability by tamoxifen and a critical amino acid (Asp-538) in Helix 12. J. Biol. Chem. 278, 7630–7638.

Phillis, J.W., Song, D., O'Regan, M.H., 1998. Tamoxifen, a chloride channel blocker, reduces glutamate and aspartate release from the ischemic cerebral cortex. Brain Res. 780, 352–355.

Presl, J., Horsky, J., Herzmann, J., Mikulas, I., Henzl, M., 1967. Fluorimetric estimation of oestrogen in the blood of infant female rats. J. Endocrinol. 38, 201–202.

Renodon, A., Boucher, J.L., Sari, M.A., Delaforge, M., Quazzani, J., Mansuy, D., 1997. Strong inhibition of neuronal nitric oxide synthase by the calmodulin antagonist and anti-estrogen drug tamoxifen. Biochem. Pharmacol. 54, 1109–1114.

Rice, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann. Neurol. 9, 131–141.

Samdani, A.F., Dawson, T.M., Dawson, V.L., 1997. Nitric oxide synthase in models of focal ischemia. Stroke 28, 1283–1288.

Sato, T., Morishima, Y., Sugimura, M., Uchida, T., Shirasaki, Y., 1999. DY-9760e, a novel calmodulin antagonist reduces brain damage induced by transient focal cerebral ischemia. Eur. J. Pharmacol. 370, 117–123.

Seki, Y., Feustel, P.J., Keller Jr., R.W., Tranmer, B.I., Kimelberg, H.K., 1999. Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. Stroke 30, 433–440.

Shinnhuber, R.O., Yu, T.C., 1957. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products: II. The quantitative determination of malonaldehyde. Food Technol. 12, 9–12.

Shy, H., Malaiyandi, L., Timiras, P.S., 2000. Protective action of 17β-estradiol and tamoxifen on glutamate toxicity in glial cells. Int. J. Dev. Neurosci. 18, 289–297.

Strijbos, P.J.L.M., Leach, M.J., Garthwaite, J., 1996. Vicious cycle involving Na^{+} channels, glutamate release, and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. J. Neurosci. 16, 5004–5013.

Sugita, Y., Kondo, T., Kanazawa, A., Itou, T., Mizuna, Y., 1993. Protective effect of FPF 1070 (cerebrolysin) on delayed neuronal death in the gerbil—detection of hydroxy radicals with salicylic acid. No To Shinkei 45, 325–331.

Sullivan, P.G., Rabchevsky, A.G., Hicks, R.R., Gibson, T.R., Fletcher-Turner, A., Scheff, S.W., 2000. Dose–response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats. Neuroscience 101, 289–295.

Thangaraju, M., Vijayalakshmi, T., Sachdanandam, P., 1994. Effect of tamoxifen on lipid peroxide and antioxidative system in postmenopausal women with breast cancer. Cancer 74, 78–82.

Towfighi, J., Housman, C., Vannycci, R.C., Heitjan, D.F., 1994. Effect of unilateral perinatal hypoxic–ischemic brain damage on the gross development of opposite cerebral hemisphere. Biol. Neonate 65, 108–118.

Trescher, W.H., Ishiwa, S., Johnston, M.V., 1997. Brief post-hypoxic–ischemic hypothermia markedly delays neonatal brain injury. Brain Develop. 19, 326–338.

Tsuji, M., Higuchi, Y., Shiraishi, K., Kume, T., Akaike, A., Hattori, H., 2000. Protective effect of aminoguanidine on hypoxic–ischemic brain damage and temporal profile of brain nitric oxide in neonatal rat. Pediatr. Res. 47, 79–83.

Wiseman, H., Laughton, M.J., Arnstein, H.R.V., Cannon, M., Halliwell, B., 1990. The antioxidant action of tamoxifen and its metabolites: Inhibition of lipid peroxidation. FEBS Lett. 263, 192–194.

Wiseman, H., Cannon, M., Arnstein, H.R.V., Barlow, D.J., 1992. The structural mimicry of membrane sterols by tamoxifen: evidence from cholesterol coefficients and molecular-modelling for its action as a membrane anti-oxidant and an anti-cancer agent. Biochim. Biophys. Acta 1138, 197–202.

Wiseman, H., Cannon, M., Arnstein, H.R.V., Halliwell, B., 1993. Enhancement by tamoxifen of the membrane antioxidant action of the yeast membrane sterol ergosterol: relevance to the antiyeast and anticancer action of tamoxifen. Biochim. Biophys. Acta 1181, 201–206.

Volpe, J.J., 2001. Neurology of the Newborn, 4th ed. W.B. Saunders, Philadelphia, PA.

Yager, J., Towfighi, J., Vannucci, R.C., 1993. Influence of mild hypothermia on hypoxic–ischemic brain damage in the imature rat. Pediatr. Res. 34, 525–529.

Zhao, Z., Cheng, M., Maples, K.R., Ma, J.Y., Buchan, A.M., 2001. NXY-059, a novel free radical trapping compound, reduces cortical infarction after permanent focal cerebral ischemia in the rat. Brain Res. 909, 46–50.