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# Estrogen attenuates hypoxic–ischemic brain injury in neonatal rats

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**Abstract**

Estrogen is neuroprotective in adult animals. We wished to determine if estrogen protects against brain injury in the newborn. Four-day-old rat pups were treated with subcutaneously implanted pellets containing 0.05 mg (2.4 μg/day) of 17β-estradiol or vehicle, designed to release the estrogen over 21 days. At 7 days old the pups had the right carotid artery ligated followed by 2.5 h of 8% oxygen. Brain damage was evaluated by weight deficit of the right hemisphere at 22 days following hypoxia. Estradiol treatments reduced brain weight loss from −17.4±2.8% S.E.M. in the vehicle group ($n$=32) to −9.3±2.7% in the treated group ($n$=32, $P$<0.05). Brain cortex thiobarbituric acid reacting substances and caspase activities were assessed 24 h after reoxygenation. Estradiol significantly reduced a hypoxia-induced increase in brain thiobarbituric acid reactive substances ($P$<0.05). Levels of caspase-3, -8 and -9 activity increased due to hypoxia–ischemia. Estradiol had no effect on caspase activity. Estradiol reduced brain injury in the neonatal rat.


*Keywords:* Stroke; Neuroprotection; Apoptosis; Thiobarbituric acid reacting substance; Oxygen radical

## 1. Introduction

The incidence of clinical stroke and the severity of stroke related brain damage is less in premenopausal females compared to males or postmenopausal females (Sivenius et al., 1991). In experimental stroke, female animals tend to show more tolerance to ischemic injury of the brain than males (Nakatomi et al., 1979; Hall et al., 1991; Li et al., 1996; Alkayed et al., 1998). The gender-based protection observed in the female has been ascribed to an ovarian factor since ovariectomy was shown to eliminate the protective effect of being female during cerebral ischemia (Alkayed et al., 1998). The purported neuroprotection in premenopausal females is probably caused by higher levels of circulating estrogens. Estrogen is neuroprotective in adult animal models of ischemic stroke, both in vitro and in vivo (for review, see Green and Simpkins, 2000; Hurn and Macrae, 2000; Wise, 2003).

The mechanisms of the neuroprotection seen with estrogen so far remain to be fully understood. Recently, researchers have reported that estrogen attenuates the ischemia-induced activation of caspase-3, and reduces TUNEL positive cells and DNA fragmentation (Jover et al., 2002; Rau et al., 2003). Estrogen can also act as a potent antioxidant and inhibit lipid peroxidation (Behl et al., 1995; Mattson et al., 1995; Green et al., 2001; Gelinas and Martinoli, 2002). Our hypothesis is that estrogen protects against oxygen radicals, which would otherwise initiate the apoptotic cascade through the mitochondrial pathway in the newborn rat.

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 (Ashwal and Pearce, 2001). Therefore, study of the role of neuroprotective agents in the neonatal hypoxic–ischemic rat model may provide important information

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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 (Ashwal and Pearce, 2001). Clinical brain injury is caused by hypoxia or ischemia rather than the combination. However, hypoxia of a clinically relevant severity will cause ischemia by inhibiting heart function. Cellular hypoxia is the end point of both hypoxia and ischemia. The ischemia in the Rice model does not cause damage unless combined with hypoxia (Rice et al., 1981). In addition, the long-term survival of rats from the Rice model allows study of very important late effects (Trescher et al., 1997). 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, 2003a,b,c, 2004). The purpose of the present study was thus to evaluate the effects of estrogen on the neonatal rat hypoxic–ischemic brain injury 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). Because plasma levels of 17β-estradiol are not significantly different between male and female 7-day-old rats and there are no differences in brain damage in 7-day-old rats between males and females in the neonatal rat hypoxic–ischemic brain injury model (Feng et al., 2004), we chose 7-day-old Sprague–Dawley rat pups of either sex, weighing between 12 and 16 g (Harlan Sprague–Dawley, Indianapolis, IN) for our experimental model. The rat pups 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 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, the pups were returned to their dams and some pups were taken for thiobarbituric acid reactive substances testing and caspase-3, -8, and -9 activity assay and other pups were 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. Plasma estradiol assays

To determine plasma levels of estradiol in 1–29-day-old naïve male and female rat pups. 1–4-, 7-, 10-, 15- and 29-day-old naïve male ($n$=3–12 in each group) and female ($n$=3–13 in each group) rat pups were separated and anesthetized with pentobarbital, and the blood was withdrawn from heart. The blood was placed on ice for 20 min and centrifuged at 2000×$g$ for 5 min. Plasma was collected and stored at −20 °C. Plasma estradiol concentrations were measured using a commercially available radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). All samples were assayed in duplicate. The mother rats provided the adult female control.

To determine time course of plasma levels of estradiol in rat pups, 7-day-old rat pups were treated with 50 μg/kg of 17β-estradiol by i.p. injection. The blood was withdrawn prior to or at 0.5, 2, 4, 6, 12, and 24 h after injection ($n$=4–14 in each group).

To determine dose response of plasma levels of estradiol in rat pups treated with 17β-estradiol by i.p. injection, 7-day-old rat pups were treated with 0, 25, 50, 100, and 200 μg/kg of estradiol by i.p. injection ($n$=6–14 in each group). The blood was withdrawn at 2 h after injection.

To determine plasma levels of estradiol in pellet implanted rat pups, rat pups were treated with 0.05 mg (2.4 μg/day) of 17β-estradiol or placebo pellets by subcutaneous implantation. Blood was withdrawn at pre-implantation, or 1, 3, 7, 10, 22, and 25 days after implantation ($n$=6–8 in each group).

### 2.3. Administration of 17β-estradiol by i.p. injection

Pups from each litter were randomly assigned and marked to vehicle or for treatment with 17β-estradiol (Sigma, St. Louis, MO, USA). Four-day-old male and female rat pups, weighing between 8 and 10 g were used this experiment. 17β-Estradiol in doses of 25, 50, or 100 μg/kg was dissolved in 5 μl of sesame oil (Sigma) per gram of body weight and administered by i.p. injection once a day for 5 consecutive days. The vehicle group was given 5 μl of sesame oil per gram of body weight alone. These doses were chosen from our serum estradiol assay results as most promising from previous studies in adult animals (Hurn and Macrae, 2000). On the third injection day, rat pups had the right carotid arteries ligated then were given 17β-estradiol or vehicle i.p. 30 min prior to hypoxia (8% oxygen).

### 2.4. Administration of 17β-estradiol by chronic implant

Four-day-old male and female rat pups, weighing between 8 and 10 g, were separately administered vehicle or 17β-estradiol (0.05 mg/pellet; 2.4 μg/day; 21 days, controlled time release pellets; Innovative Research of America, Sarasota, FL, USA). The pellets were implanted

in the subcutaneous tissue of the dorsal neck, when the pup was anesthetized with isoflurane. Three days after implanting the pellets, the rat pups had the right carotid arteries ligated followed by 2.5 h of hypoxia (8% oxygen). This dose was chosen from our serum estradiol assay results as most promising from previous studies in adult animals (Jover et al., 2002; Shughrue and Merchenthaler, 2003).

### 2.5. Measurement of rectal temperature

To evaluate whether neuroprotection by 17β-estradiol 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 prior to i.p. injection (6 from the vehicle group and 6 given 50 μg/kg of 17β-estradiol) and at 0.25, 0.5, 1, 1.5, 2, 4 and 6 h after injection. Rectal temperature was also measured prior to implanting pellets (6 from the placebo group and 6 given 2.4 μg/day of 17β-estradiol) and at 0.25, 0.5, 1, 2, 4, 6 and 15 h after implantation. Rat pups of this age cool rapidly once they are removed from the nest and the dam (McDonald et al., 1991). To keep the variability of the temperature low, measurements of rectal temperature were made in a 25 °C room 15 min after removal of the pups from the nest (except the 1st post hypoxic measurement which was done immediately). Their temperature at 15 min reflects their maximal thermoregulatory capacity in a uniform cold environment and is much more reproducible than a rapid rectal temperature measurement made quickly after removing the pup from the cage. The cage is not a homogenious environment and has many desparate microenvironments. This technique has allowed us to detect drugs which lower body temperature with small numbers of animals (Feng and LeBlanc, 2002). Rectal temperature and brain temperatures 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).

### 2.6. Gross brain damage grading

Rat pups were anesthetized with pentobarbital and decapitated 22 days after hypoxic exposure. The brains were removed and weighed by an observer blind to the code. 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 (Rice et al., 1981; Palmer et al., 1990). 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; Bona et al., 1997; LeBlanc et al., 2000; Feng et al., 2004). 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 (Andine et al., 1990; Hagberg et al., 1994; Towfighi et al., 1994; Feng et al., 2004). Thus, weighing can assess the degree of brain damage.

### 2.7. Microscopic brain damage grading

A second set of experiments was done 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 with 0.05 mg pellets of 17β-estradiol or vehicle as in the above procedure. Rat pups 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 was 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 to 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.

### 2.8. Determination of lipid peroxidation

A third set of experiments was performed to determine the effect of estradiol on lipid peroxidation. Lipid peroxidations were measured in 17β-estradiol treated, vehicle treated and sham operation groups ($n$=6–7 in each group for each assay). Using the above neonatal hypoxic–ischemic procedure, the rat pups were treated with 50 μg/kg of 17β-estradiol i.p. daily or 0.05 mg (2.4 μg/day) of 17β-estradiol pellets by implantation. At 24 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 and several other aldehydic products of 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 substance values were expressed as picomoles per gram of tissue.

### 2.9. Measurement of caspase-3, -8, and -9 activity

A fourth set of experiments was performed to determine the effect of 17β-estradiol on caspase-3, -8, and -9 activity. Caspase-3, -8, and -9 activity were measured in 17β-estradiol treated, vehicle treated and sham operation groups ($n$=6–16 in each group for each assay). Using the above neonatal hypoxic–ischemic procedure, the rat pups were treated with 50 μg/kg of 17β-estradiol by i.p. or 0.05 mg of 17β-estradiol pellets by implant. Caspase-3, -8, and -9 activity was assayed as we have previously described (Feng and LeBlanc, 2003; Feng et al., 2003b,c). In the newborn rat model of hypoxic–ischemic brain injury, the peak activation of caspase-3 and caspase-9 occurs at 24 h following the hypoxic–ischemic insult (Chen et al., 1998; Feng et al., 2003c). The peak of caspase-8 activation is at 12 h, but the elevation in activity is still present at 24 h (Feng et al., 2003b). Therefore, pups were anesthetized with 50 mg/kg pentobarbital at 24 h after hypoxia. The cortex in both lesioned and unlesioned hemisphere was separately dissected and homogenized. Caspase-3, -8, and -9 activity were measured using commercially available assay kits (Calbiochem, Diego, CA, USA), following the manufacturer's instructions. Caspase-3, -8, and -9 activity was determined colorimetrically using the recognition sequence of the caspase attached to *p*-nitroanilide with a microplate reader at 405 nm. A *p*-nitroanilide calibration curve was established to quantify units of caspase-3, -8, and -9 activity. The protein concentration was determined by the method of Bradford (1976).

### 2.10. Statistical analysis

Data are expressed as mean±S.E.M. and the statistical significance of differences between groups were determined using analysis of variance with the Newman–Keuls test or Student's *t*-test, or Kruskal–Wallis or Mann–Whitney. 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 were not significantly different between 17β-estradiol- and vehicle-treated pups at the times measured. 17β-Estradiol did not effect body temperature in either mode of administration. Body weights of the 17β-estradiol treated groups in both modes of administration were not significantly different from controls prior to injury or at 4, 7, 11, 14, and 22 days after injury. Body weights increased significantly with time in all groups as the pups grew.

### 3.2. Plasma estradiol assays

Plasma levels of 17β-estradiol in 1-, 4-, 7-, 10-, 15-, and 29-day-old male and female naïve rat pups and adult mom rats are shown in Table 1. There were no significant differences between male and female rat pups in any age group but there were significant differences between adult female rats and all of the age groups of rat pups.

The time course of i.p. injection of 50 μg/kg of 17β-estradiol on plasma 17β-estradiol level in 7-day-old rat pups is shown in Fig. 1. At all time points after i.p. injection, the 17β-estradiol levels were significantly higher than baseline level ($n$=4–14 rat pups per time point, $P<0.01$ vs. baseline). The plasma level of 17β-estradiol at 2 h after i.p. injection of 0, 25, 50, 100 and 200 μg/kg of 17β-estradiol in 7-day-old rat pups is shown in Fig. 2. There is a significant relationship between administered dose and plasma level of 17β-estradiol.

Plasma level of 17β-estradiol after 0.05 mg of 17β-estradiol pellet was implanted was significantly greater than that in placebo implanted animals after 1, 3, 10, and 22 days implanted ($n$=6–10 rat pups in each point, Fig. 3).

Table 1
Plasma levels of 17 β-estradiol in 1–29-day-old naïve male and female rat pups (pg/ml)

| Pup age (day) | Male | Female |
|---|---|---|
| 1–4 | 2.91±0.49 ($n$=4) | 3.14±0.69 ($n$=5) |
| 7 | 2.12±0.14 ($n$=12) | 2.08±0.17 ($n$=13) |
| 10 | 2.58±0.2 ($n$=9) | 2.55±0.16 ($n$=10) |
| 15 | 5.16±0.54 ($n$=3) | 6.09±0.45 ($n$=3) |
| 29 | 5.84±0.31($n$=8) | 6.18±0.50 ($n$=12) |
| Adult female rats | | 19.5±4.9 ($n$=5)* |

Data are represented as mean±S.E.M. Animal numbers are shown in parenthesis.

* $P<0.01$ vs. each naïve pup group.

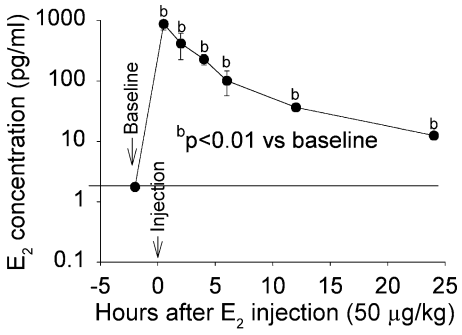


Fig. 1. The time course of i.p. injection of 50 μg/kg of 17β-estradiol (E2) on plasma level of 17β-estradiol in 7-day-old rat pups. Data is presented as mean±S.E.M. At all of the time points, 0.5, 2, 4, 6, 12 and 24 h, after i.p. injection, the 17β-estradiol level is significantly higher than baseline level ($n$=4–14 rat pups per time point, $^{a}P$<0.05 or $^{b}P$<0.01 vs. baseline). Please note that the $x$-axis in this figure is in hours while that of Fig. 3 is in days.

### 3.3. Neuroprotective effects of treatment with 17β-estradiol by i.p. injection

Gross neuropathologic damage was scored 22 days after injury. Left hemisphere weight was not affected by the hypoxia and was not significantly different from vehicle at any dose of 17β-estradiol in the treated pups. The percent reduction in right hemispheric weight is shown in Fig. 4. There were no significant differences between the vehicle treated pups and those treated with any dose of 17β-estradiol by i.p. injection ($P$>0.05).

### 3.4. Neuroprotective effects of treatment with 17β-estradiol by chronic implant

Gross neuropathologic damage was scored 22 days after injury. Left hemisphere weights were 491.8±7.1 mg ($n$=32) in vehicle group and 492.4±5.8 mg ($n$=32) in 17β-estradiol group. 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. 5. 17β-Estradiol significantly reduced the decrease in right hemisphere weight from −17.4±2.8%

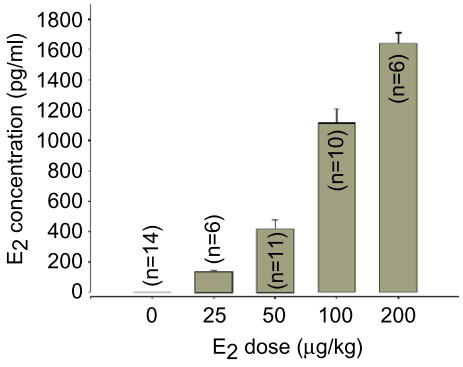


Fig. 2. The plasma level of 17β-estradiol (E2) at 2 h after i.p. injection of 0, 25, 50, 100 and 200 μg/kg of 17β-estradiol in 7-day-old rat pups. There is a significant relation between administration doses and plasma level of 17β-estradiol.

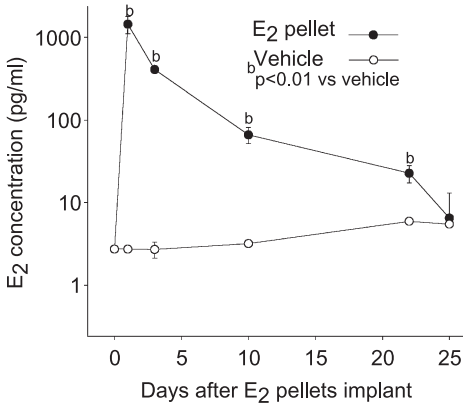


Fig. 3. Plasma 17β-estradiol (E2) levels 1, 3, 10, 22, and 25 days after implantation of slow release pellets containing 0.05 mg (2.4 ug/day for 21 days) of 17β-estradiol or pellets containing vehicle were implanted. There were significant differences between 17β-estradiol and placebo implanted animals after 1, 3, 10, and 22 days. $^{b}P$<0.01 vs. vehicle. Please note that the $x$-axis of this figure is in days while that in Fig. 1 is in hours.

(n=32) in the vehicle group to −9.3±2.7% ($n$=32) in the group receiving the pellets (0.05 mg) of 17β-estradiol ($P$<0.05).

### 3.5. Effect of 17β-estradiol on histopathology

The histopathologic score for the cortex in the 17β-estradiol implanted group was lower than in the vehicle group, $P$<0.05. The histopathologic score for the hippocampus in the 17β-estradiol implanted group was lower, but not statistically significantly lower than in the vehicle group, $P$=0.09. The histopathologic score for the thalamus in the 17β-estradiol implanted group was lower but not statistically significantly lower than in the vehicle group, $P$=0.06.

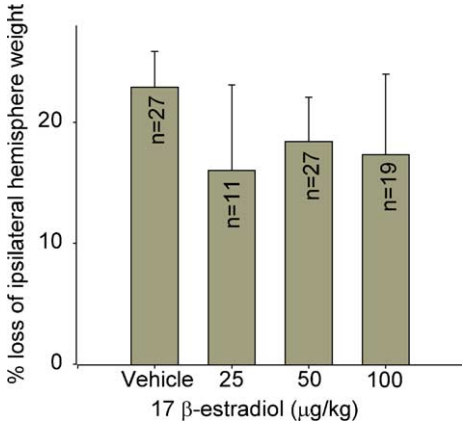


Fig. 4. The dose–response for percentage reduction in right cerebral hemisphere weight measured using the left hemisphere weight as standard. 17β-Estradiol (25–100 μg/kg) was administered by i.p. once a day for 5 days. On the third injection day, rat pups had the right carotid arteries ligated then were given 17β-estradiol or vehicle i.p. 30 min prior to hypoxia. Brain injury was evaluated 22 days later. Data are presented as mean±S.E.M. Treatment with 25–100 μg/kg of 17β-estradiol by i.p did not decrease the percentage reduction in right hemisphere weight compared to vehicle ($P$>0.05).

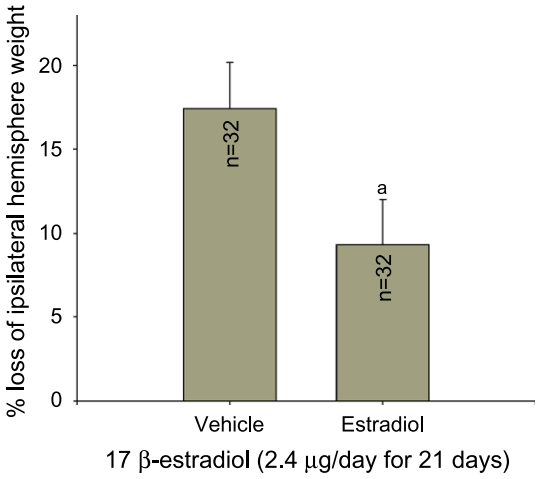


Fig. 5. The effect of chronic implant with 0.05 mg pellets (2.4 μg/day) of 17β-estradiol for 21 days on percentage reduction in right cerebral hemisphere weight measured using the left hemisphere weight as standard. At 3 days after implanting the pellets, the rat pups had the right carotid arteries ligated followed by 2.5 h of hypoxia. Brain injury was evaluated by a blind observer 22 days after hypoxia. Data are presented as mean±S.E.M. Chronic implantation of pellets of 17β-estradiol significant decreased the percentage reduction in right hemisphere weight compared to vehicle ($^{a}P<0.05$ vs. vehicle).

17β-Estradiol by chronic implant significantly improved the outcome in the cortex. Similar, but not statistically significant trends, are seen in the hippocampus and thalamus (Fig. 6).

### 3.6. Effect of 17β-estradiol on thiobarbituric acid reactive substances

The concentrations of thiobarbituric acid reactive substances in left hemisphere were not effected by the hypoxia and there were no significant differences among the sham, vehicle, and 17β-estradiol treated groups. The concentrations of thiobarbituric acid reastive substances in right hemisphere were significantly higher in vehicle group (126.8±15.2 pmol/g, $n$=6) than in sham group (80.9±9.7 pmol/g, $n$=7, $P<0.01$). Implanted pellets of 17β-estradiol significantly reduced a hypoxia-induced increase in brain thiobarbituric acid reastive substances compared with vehicle group ($P<0.05$). 17β-Estradiol by i.p. injection did not change thiobarbituric acid reactive substances (Fig. 7).

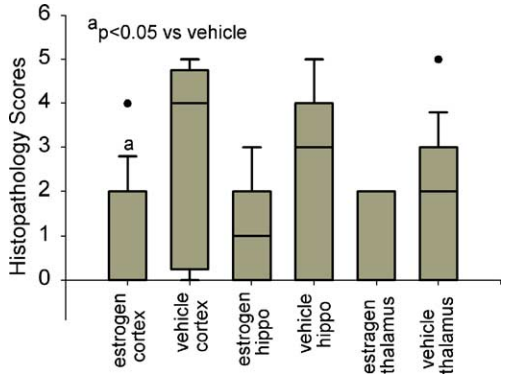


Fig. 6. Box plot of the histopathology score for cortex, hippocampus (hippo), and thalamus for the estrogen treated and vehicle treated groups. The line is the median. The median for the estrogen treated cortex is 0 as is the median for the estrogen treated thalamus. The box is 25 and 75 percentile. The error bars are 90 and 10 percentile. Pups were randomised to treatment with 0.05 mg slow release pellets (2.4 μg/day) of 17β-estradiol or vehicle treated pellets. At 3 days after implanting the pellets, rat pups had right carotid arteries ligated followed by 2.5 h of hypoxia. Brains were perfusion fixed 3 days after reoxygenation. A score of 1 means 1–5% of the neurons were damaged and a score of 4 means that 51–75% of the neurons were damaged.

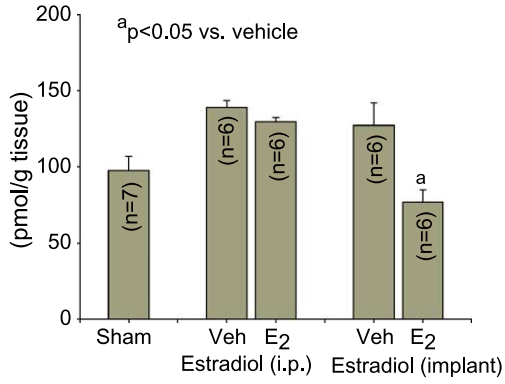


Fig. 7. Effect of 17β-estradiol (E2) on brain thiobarbituric acid reactive substance. The concentrations of thiobarbituric acid reactive substance in right hemisphere were significantly higher in vehicle (veh) group than in the sham group. Implanted pellets of 17β-estradiol significantly reduced a hypoxia-induced increase in brain thiobarbituric acid reactive substance ($^{a}P<0.05$) compared with vehicle group. 17β-Estradiol given by i.p. injection had no significant effect on right brain thiobarbituric acid reactive substance.

### 3.7. Effect of 17β-estradiol on caspase-3, -8, and -9 activity

The caspase-3, -8, and -9 activity is shown in Fig. 8A,B. Hypoxia–ischemia resulted in a significant increase in caspase-3, -8, and -9 activity in the right cortex at 24 h after hypoxia compared with sham group ($P<0.05$). 17β-Estradiol either administered by i.p. injection (A), or by implant pellet (B) did not eliminate the increase in casepase-3, -8, and -9 activity at 24 h after hypoxic exposure ($P>0.05$).

## 4. Discussion

The present study demonstrates for the first time that 17β-estradiol significantly reduces hypoxic ischemic brain injury in the focal ischemia model of the neonatal rat pup. Administration of 0.05 mg of 17β-estradiol by slow release pellets rather than 50 μg/kg of 17β-estradiol by i.p. injection daily was necessary to produce statistically significant protection. Implanted pellets at the dose used in this study produce both higher peak levels of 17β-estradiol (66% higher with implanted pellets, than with i.p. injection) and the implanted pellets produce a much more

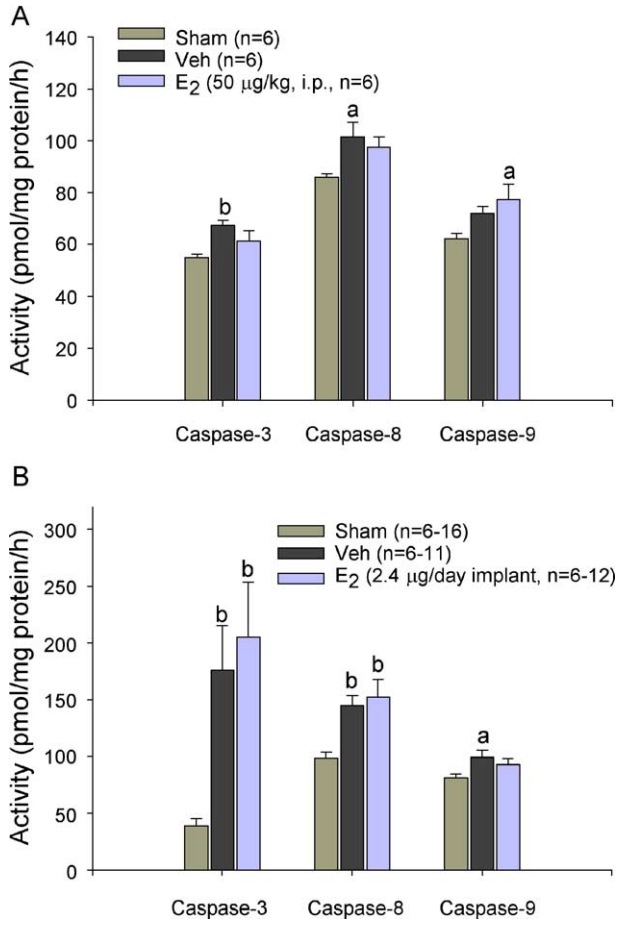


Fig. 8. The effects of 17β-estradiol (E2) on brain caspase-3, -8, and -9 activity. Rat pups were treated with 17β-estradiol (50 μg/kg) or vehicle (Veh) administered by i.p. injection once a day for 5 days or implanted pellets of 0.05 mg of 17β-estradiol or vehicle designed to last for 21 days. At 3 days into treatment, rat pups had the right carotid arteries ligated followed by 2.5 h of hypoxia. Caspase-3, -8 and -9 activity in the cortex as pmol/h/mg protein for the ipsilateral hemispheres for sham pups and pups treated with vehicle or 17β-estradiol by i.p. injection, or by implanted pellets were assessed 24 h after reoxygenation. Hypoxia results in an increase in caspase activity at 24 h after hypoxic exposure ($^{a}P<0.05$, $^{b}P<0.01$ vs. sham). Neither 17β-estradiol administration by i.p. injection (A) nor by implanted pellets (B) decreased the rise of casepase-3, -8, and -9 activity compared to vehicle ($P>0.05$).

sustained blood level. At 12 h after the dose, half way to the next dose, the blood levels in i.p. treated rat pups had fallen from 880 to 36 pg/ml. The implanted pups would still be close to their peak level of 1460 pg/ml at 12 h and would not fall to 36 pg/ml till approximately 15 days after implantation. The implanted estradiol pellets also significantly reduce a hypoxia-induced increase in brain thiobarbituric acid reactive substances concentrations compared with the vehicle group, but i.p. injection did not. This suggests that in the newborn rat oxygen radicals are important in the neuroprotective efficacy of 17β-estradiol. 17β-Estradiol does not reduce the increase in caspase-3, -8, and -9 activity at 24 h after hypoxic exposure in the neonatal rat hypoxic–ischemia model suggesting that caspase dependent apoptosis plays little role in the neuroprotective effect of estrogen in the newborn rat.

Our results demonstrate that chronic implants of 17β-estradiol produce neuroprotection in the rat pup hypoxia–ischemia model and confirm that 17β-estradiol is able to reduce ischemic infarct size. In studies by others in adult animals neuroprotection was seen in both ovariectomized adult female and intact male adult rats and in both transient and permanent cerebral ischemia models (Alkayed et al., 1998; Dubal et al., 1998; Toung et al., 1998; Rusa et al., 1999; Simpkins et al., 1997; Zhang et al., 1998; Wang et al., 1999; Rau et al., 2003) and in gerbils global ischemia models (Jover et al., 2002; Shughrue and Merchenthaler, 2003).

Estrogen treatment seems to be of low toxicity. Even with 0.05 mg (2.4 μg/day) pellet for 21 days, the pups growth was not adversely affected in this model. We did not investigate the effect of estrogen on sexual development where low doses are likely to have dramatic effects.

Our results show that plasma levels of estradiol in 1–29-day-old naïve pups were 2–6 pg/ml which was 1/7 to 1/3 of the levels in adult female rats. Our results are similar to that of Presl et al. (1967), who showed the normal level of serum estrogen in 10-day-old female rat pups is ¼ of that in pro-oestrus and oestrus adult female rats. The plasma level of estradiol in ovariectomized female mice were 4–5 pg/ml (Horsburgh et al., 2002; Yang et al., 2000; Harukuni et al., 2001). Therefore, the neonatal rat hypoxic–ischemic brain injury model may be a useful model for investigating neuroprotective effects of estrogens because normal levels of estrogen are very low.

In the present study, we found that implant of 0.05 mg (2.4 μg/day) of estradiol pellets produced a persistently high level of estradiol in plasma. A similar result has been reported by other (Yang et al., 2000). The effective concentrations for estradiol-mediated neuroprotection reported in adult rats range from low physiologic concentrations, 10 pg/ml (Dubal et al., 1998; Rusa et al., 1999), to high pharmacological concentrations, 550 to 3500 pg/ml (Simpkins et al., 1997; Yang et al., 2000; Horsburgh et al., 2002). The plasma levels of estradiol, which produced neuroprotective effects in the newborn rat, were in the high pharmacological range, and even the 12 h period with levels which would be physiologic for adult rats as seen with daily i.p. injection eliminated the neuroprotective effect.

Some adult animal studies also report that estrogen must be delivered chronically beginning before ischemia, rather than as a single injection or subcutaneous implant at ischemic onset, to be effective (Dubal et al., 1998; Rusa et al., 1999). 17β-Estradiol exerts complex effects in the brain, and has many possible mechanisms for neuroprotection. Estrogen can act through estrogen receptor-dependent gene activation or deactivation. Estrogen can act through receptor-independent mechanisms (for review, see Wise, 2003). The fact that estrogen needs to be provided at supraphysiologic doses to produce neuro-

protection in the newborn rat would argue for a receptor independent mechanism of action. Estrogen can also act as a potent antioxidant and inhibit lipid peroxidation (Mattson et al., 1995; Behl et al., 1997; Green et al., 2001; Gelinas and Martinoli, 2002). Estrogen can also improve perfusion (Pelligrino et al., 1998; Hurn and Macrae, 2000); reduce inflammation (Liao et al., 2002); inhibit excitotoxicity (Singer et al., 1996; Regan and Guo, 1997; Weaver et al., 1997; Shy et al., 2000) as well as reduce apoptotic effects (Dubal et al., 1998; Jover et al., 2002; Rau et al., 2003).

In this study, although we did not directly explore all potential mechanisms of action of 17β-estradiol, we did look at the roll of oxyradicals and the apoptotic caspase cascade. Estradiol reduced a hypoxia-induced increase in brain thiobarbituric acid reactive substances. The same dosage efficacy pattern was seen for reducing thiobarbituric acid reactive substances as for neuroprotection. Thiobarbituric acid reactive substances primarily reflect production of lipid peroxides, which are broken down during the assay to yield malondialdehyde (Hageman et al., 1992). Lipid peroxide formation must be initiated by hydroxyl radical or its by-products or by peroxynitrite (Chan, 1996). The formation of 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 pup 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, 2003a). 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 (Inan et al., 1995; Feng et al., 2000; Zhao et al., 2001). In the present study, hypoxic–ischemic rat pups showed elevations in thiobarbituric acid reactive substances concentrations in the brain. Treatment with chronic implants of 17β-estradiol eliminated the increase in thiobarbituric acid reactive substances. Similar results showing estradiol inhibited lipid peroxidation have been reported by other observers (Subbiah et al., 1993; Goodman et al., 1996; Behl et al., 1997; Gridley et al., 1997). Estradiol's inhibition of lipid peroxidation may be one important mechanism for its neuroprotective effect.

Caspases are cysteine proteases that mediate apoptosis by proteolysis of specific substrates. Caspase-3 has been identified as a key executioner of apoptosis. Caspase-3 is in turn activated by the initiator caspases, caspase-8 and caspase-9 (Depraetere and Golstein, 1998). Estrogen had no effect on either the initiator caspases, caspase-8 and caspase-9, or on caspase-3, the principal effector caspase. We and others have reported that caspase-3, -8, and -9 activity was increased in neonatal rat hypoxic–ischemic brain injury model (Zhu et al., 2000; Blomgren et al., 2001; Northington et al., 2001; Wang et al., 2001; Feng and LeBlanc, 2003; Feng et al., 2003b,c). Also, reduced caspase activity has been shown to be associated with neuroprotection (Cheng et al., 1998; Endres et al., 1998; LeBlanc et al., 2000; Puka-Sundvall et al., 2000; Feng and LeBlanc, 2003; Feng et al., 2003b,c).

Estradiol has been thought to be an inhibitor of the programmed cell death cascade (Jover et al., 2002; Linford and Dorsa, 2002). Rau et al. (2003) reported that both attenuation of DNA fragmentation and reduced infarct volume are preceded by an estradiol-mediated reduction in the relative amount of the caspase-3-mediated spectrin breakdown product (SBDP120) at 4 h but not at 8 or 16 h after middle cerebral artery occlusion. We and others (Cheng et al., 1998; Wang et al., 2001; Feng and LeBlanc, 2003; Feng et al., 2003b,c) observed that caspase-3 and caspase-9 are activated relatively late after injury, peaking at 24 h after injury in neonatal rat cerebral hypoxia–ischemia. The present data show that estradiol did not suppress caspase-3, -8 and -9 activity at 24 h after hypoxia. This difference in findings may be due to animal age or other variation in the protocols.

In conclusion, our findings indicate that 17β-estradiol has neuroprotective properties in the neonatal rat hypoxia–ischemia model. It is important to appreciate that the dose and duration of estrogen administered impacts the efficacy of its neuroprotective actions. The results also support the view that the suppression of lipid peroxidation after hypoxia–ischemia by 17β-estradiol is one potential mechanism of this neuroprotection.

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