

# Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model

SHAO-HUA YANG,<sup>1</sup> EVELYN PEREZ,<sup>1,2</sup> JASON CUTRIGHT,<sup>3</sup> RAN LIU,<sup>1</sup> ZHEN HE,<sup>3</sup> ARTHUR L. DAY,<sup>3</sup> AND JAMES W. SIMPKINS<sup>1</sup>

<sup>1</sup>Department of Pharmacology and Neuroscience, Health Science Center at Fort Worth, University of North Texas, Fort Worth, Texas 76107; and <sup>2</sup>Department of Pharmacodynamic, College of Pharmacy and <sup>3</sup>Department of Neurosurgery, College of Medicine, University of Florida, Gainesville, Florida 32610

Received 22 June 2001; accepted in final form 6 September 2001

**Yang, Shao-Hua, Evelyn Perez, Jason Cutright, Ran Liu, Zhen He, Arthur L. Day, and James W. Simpkins.**

Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. *J Appl Physiol* 92: 195–201, 2002.—Increasing evidence has demonstrated striking sex differences in the outcome of neurological injury. Whereas estrogens contribute to these differences by attenuating neurotoxicity and ischemia-reperfusion injury, the effects of testosterone are unclear. The present study was undertaken to determine the effects of testosterone on neuronal injury in both a cell-culture model and a rodent ischemia-reperfusion model. Glutamate-induced HT-22 cell-death model was used to evaluate the effects of testosterone on cell survival. Testosterone was shown to significantly increase the toxicity of glutamate at a 10  $\mu$ M concentration, whereas 17 $\beta$ -estradiol significantly attenuated the toxicity at the same concentration. In a rodent stroke model, ischemia-reperfusion injury was induced by temporal middle cerebral artery occlusion (MCAO) for 1 h and reperfusion for 24 h. To avoid the stress-related testosterone reduction, male rats were castrated and testosterone was replaced by testosterone pellet implantation. Testosterone pellets were removed at 1, 2, 4, or 6 h before MCAO to determine the duration of acute testosterone depletion effects on infarct volume. Ischemic lesion volume was significantly decreased from 239.6  $\pm$  25.9 mm<sup>3</sup> in control to 122.5  $\pm$  28.6 mm<sup>3</sup> when testosterone pellets were removed at 6 h before MCAO. Reduction of lesion volume was associated with amelioration of the hyperemia during reperfusion. Our in vitro and in vivo studies suggest that sex differences in response to brain injury are partly due to the consequence of damaging effects of testosterone.

androgen; stroke; neuroprotection

GONADAL STEROID HORMONES such as androgens and estrogens may affect various target tissues throughout the body, including central nervous system. Clinical evidence has demonstrated striking sex differences in the incidence and outcome of stroke (27), which precipitated the studies of the potential impact of gonadal steroid hormones in disturbances of the central ner-

vous system. A major focus in basic and clinical research in the last decade has been related to the activities of estrogens. Although the impact of postmenopausal estrogen-replacement therapy on stroke prevention and stroke severity remains inconsistent (7, 26), data from experimental studies in laboratory animals suggest that estrogens may have neuroprotective properties (3, 12, 33, 42), which have led to a growing appreciation of the positive impact of estrogens on the central nervous system. In contrast, effects of androgens on the central nervous system are much less studied.

Testosterone has been shown to be a survival factor for axotomized motoneurons and promotes motor axon regeneration (21, 22). Recently, several in vitro studies suggested that testosterone possessed neuroprotective effects on cerebellar granule neuron (1, 2). In view of the proposed neuroprotective effects both of estrogens and androgens, effects of sex difference on the outcome of stroke (3, 27, 44) could not be explained by sex hormones. We have previously reported that chronic testosterone replacement increased while chronic castration and chronic 17 $\beta$ -estradiol treatment decreased ischemic damage related to middle cerebral artery occlusion (MCAO) in male rats (19) and that the decrease of ischemic lesion volume with chronic 17 $\beta$ -estradiol treatment was associated with a marked reduction of testosterone level in intact males (19). In the present study, effects of acute testosterone depletion on ischemic stroke were evaluated. Our objective was twofold. First, direct effects of testosterone on neuronal survival were evaluated in a HT-22 cell-culture model using glutamate insult. Second, effects of acute testosterone depletion on ischemic lesion volume from MCAO were assessed in male rats. Our strategy was to compare the ischemic lesion volume from MCAO between testosterone depletion animals and animals with physiological level testosterone. Sustained physiological testosterone levels were obtained by castration and steroid pellet replacement technique, which our

Address for reprint requests and other correspondence: J. W. Simpkins, Dept. of Pharmacology and Neuroscience, Health Science Center at Fort Worth, Univ. of North Texas, 3500 Camp Bowie Blvd., Fort Worth, TX 76107 (E-mail: jsimpkin@hsc.unt.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

laboratory has previously reported (19). Acute testosterone depletion was achieved by pellets withdrawn 2 days after castration and testosterone pellet implantation, whereas sham withdrawal was used to maintain physiological testosterone levels in control. By using this strategy, the effects of timed depletion of testosterone before ischemic insult on the lesion volume and regional cerebral blood flow (CBF) from temporary MCAO were assessed in male rats.

## MATERIALS AND METHODS

**Cell culture and treatment.** HT-22 cells (gift from David Schubert, Salk Institute, San Diego, CA), which are a murine hippocampal cell line, were maintained in DMEM media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and 20  $\mu\text{g/ml}$  gentamycin under standard cell culture conditions (5%  $\text{CO}_2$ , 95% air, 37°C). HT-22 cells (passages 18–25) were seeded into Nunc 96 well plates at a density of 5,000 cells/well.

Testosterone and 17 $\beta$ -estradiol were initially dissolved in absolute ethanol and diluted in DMEM media to the final concentration of 0.01–10  $\mu\text{M}$ . Exposure to testosterone and 17 $\beta$ -estradiol was initiated immediately before the addition of glutamate. Ethanol was used at a final concentration of 0.1% as vehicle control. Glutamate was diluted to a final concentration of 10 mM in culture media, and cells were exposed to glutamate for ~24 h. All cell culture experiments are repeated at least three times.

**Cell viability assay.** Cells were exposed to steroids and glutamate for ~24 h, and then cell viability was determined by calcein AM (Molecular Probes, Eugene, OR), an assay that measures cellular esterase activity and plasma membrane integrity. Wells were rinsed with PBS, after which a 25  $\mu\text{M}$  solution of calcein AM in PBS was added. After incubation at room temperature for 15 min, fluorescence was determined (excitation = 485, emission = 530). Raw data were obtained as relative fluorescence units. All data were then normalized to percentage of cells killed, as calculated by treatment value/control value  $\times$  100.

**Experimental animals.** Male Charles River Sprague-Dawley rats (250 g, Wilmington, MA) were maintained in laboratory acclimatization for 3 days before surgery. All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee and University of Florida Animal Care and Use Committee.

**Testosterone concentration in testosterone-replacement and withdrawal animals.** To determine the effect of testosterone pellet implantation on serum testosterone concentration and the time course of testosterone reduction after pellet withdrawal, bilateral castration was performed under methoxyflurane inhalant anesthesia, and two 15-mm-long testosterone Silastic pellets containing crystalline steroid were implanted subcutaneously immediately thereafter. Blood samples (0.5 ml each time) were taken via jugular vein at 24 ( $n = 4$ ) and 48 h ( $n = 4$ ) after the implantation of steroid pellets under methoxyflurane inhalant anesthesia. Then the pellets were removed and blood samples were taken via jugular vein at 1, 2, 4, 8, 12, and 24 h ( $n = 4$  each time point) after steroid pellet removal. Serum was separated from blood cells by centrifugation and stored frozen ( $-20^\circ\text{C}$ ). Serum testosterone concentrations were determined by using duplicate serum aliquots in a radioimmunoassay (Diagnostic Systems Laboratories, Los Angeles, CA). Animals used for tes-

tosterone assessment were not used for ischemia outcome studies.

**Experiment protocol.** Two days after castration and testosterone pellet implantation, ischemic stroke was induced in animals after testosterone pellet removal or sham removal. Pellets were removed in the testosterone depletion groups at 1 ( $n = 7$ ), 2 ( $n = 5$ ), 4 ( $n = 5$ ), or 6 h ( $n = 5$ ) before ischemia under methoxyflurane inhalant anesthesia. Sham pellet removal was performed in the physiological testosterone level group as control in the same condition as pellet removal at 1 ( $n = 5$ ), 4 ( $n = 5$ ), and 6 h ( $n = 5$ ) before MCAO. Ischemic stroke was induced by MCAO described as before (18, 31). Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5 and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical skin incision. CCA and ECA were permanently cauterized. A 3–0 monofilament suture was introduced into the ICA via ECA lumen and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was ~1.9 cm. The middle cerebral artery was occluded for 1 h, and then the suture was withdrawn for reperfusion. ICA was coagulated, and the skin incision was closed.

Animals in each group were decapitated 24 h after reperfusion. Then the brain was harvested and placed in a metallic brain matrix for tissue slicing (Harvard Apparatus, Holliston, MA). Seven slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Each slice was incubated for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37°C and then fixed in 10% formalin. Stained slices were photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

**Regional CBF measurement and physiological parameters monitor.** In a separate study, MCAO was induced 6 h after pellet ( $n = 6$ ) or sham removal ( $n = 6$ ). Left femoral artery was cannalized and connected to a blood pressure monitor. Arterial blood samples (150  $\mu\text{l}$  each time) were taken before, 30 min during, and 30 min after MCAO, respectively. Physiological parameters were measured by an ISTAT portable clinical analyzer (East Windsor, NJ).

Hydrogen clearance blood flowmeter (Digital UH meters, Unique Medical, Tokyo, Japan) was used for regional CBF measurement. Two Teflon-coated platinum probes were stereotaxically inserted into the core area of ischemia (0.5 mm posterior bregma, 4 mm lateral, and 5 mm depth). Regional CBF was monitored bilaterally during occlusion and within 30 min after reperfusion in testosterone pellet removal and sham removal groups.

**Statistical analysis.** All data are presented as means  $\pm$  SE. Cell death, CBF, ischemic volumes, and physiological parameters in each group were compared by one-way ANOVA followed by Tukey tests. A probability of  $<0.05$  was considered significant.

## RESULTS

**Effect of testosterone and 17 $\beta$ -estradiol on glutamate toxicity.** Ten micromolar testosterone significantly increased glutamate toxicity to  $87.5 \pm 3.7\%$  of cells killed, compared with  $71.9 \pm 6.9\%$  at 0  $\mu\text{M}$  testosterone. Opposite to the deleterious effect of testosterone,

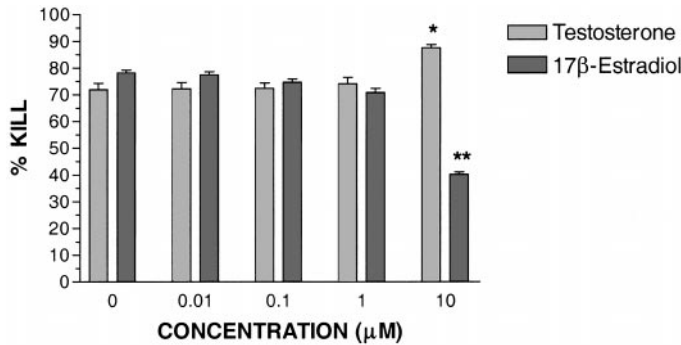


Fig. 1. Effect of testosterone and 17 $\beta$ -estradiol on glutamate toxicity. HT-22 cells were exposed to 10 mM glutamate for ~24 h. Cell viability was determined by calcein assay. All data were normalized of percentage of cells killed (%kill) as calculated by treatment value/control value  $\times$  100. Values are means  $\pm$  SE. \* $P$  < 0.05 vs. 0  $\mu$ M testosterone. \*\* $P$  < 0.05 vs. 0  $\mu$ M 17 $\beta$ -estradiol.

10  $\mu$ M 17 $\beta$ -estradiol ameliorated glutamate toxicity to  $40.3 \pm 3.1\%$  of cells killed, compared with  $78.3 \pm 3.3\%$  at 0  $\mu$ M 17 $\beta$ -estradiol (Fig. 1).

**Testosterone concentrations in testosterone replacement and withdrawal animals.** Subcutaneous implantation of testosterone pellets increased serum testosterone concentrations to  $2.58 \pm 0.47$  and  $1.83 \pm 0.13$  ng/ml at 1 and 2 days after implantation, respectively, both of which are within the reported physiological range of testosterone in male rats (Fig. 2). Serum testosterone concentrations decreased to  $0.24 \pm 0.01$  ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentrations decreased to <0.08 ng/ml, the limits of sensitivity of the assay (Fig. 2).

**Effect of testosterone on ischemic lesion volume.** Ischemic lesion volume was significantly decreased when testosterone pellets were removed at 6 h before MCAO. Lesion volume was  $217.8 \pm 24.69$ ,  $192.6 \pm 13.90$ ,  $151.3 \pm 45.54$ , and  $122.5 \pm 28.62$  mm<sup>3</sup> at 1, 2, 4, and 6 h after pellet removal, respectively, compared with  $239.6 \pm 25.89$  mm<sup>3</sup> in control animals in which physiological testosterone concentrations were maintained (Fig. 3). As no differences in ischemic lesion volume were found between sham pellet removal animals at 1, 4, or 6 h before MCAO, all sham pellet removal animals were pooled together as controls.

**Effect of testosterone on blood pressure, gases, pH, ions, and regional CBF.** Physiological parameters are shown in Table 1. There were no significant differences between testosterone and testosterone-depletion groups for any parameters measured.

Regional CBF decreased to  $8.7 \pm 2.1$  and  $7.5 \pm 1.9$  ml $\cdot$ min<sup>-1</sup> $\cdot$ 100 g tissue<sup>-1</sup> during MCAO in the testosterone and testosterone-depletion groups, respectively. Hyperemia was observed during reperfusion in the testosterone group, which showed a CBF of  $82.2 \pm 12.2$  ml $\cdot$ min<sup>-1</sup> $\cdot$ 100 g tissue<sup>-1</sup> compared with the nonischemic side in the testosterone and testosterone-depletion groups ( $P$  < 0.05), which had CBF of  $32.0 \pm 1.7$  and  $46.0 \pm 3.6$  ml $\cdot$ min<sup>-1</sup> $\cdot$ 100 g tissue<sup>-1</sup>, respectively. In the testosterone-depletion group, no significant hyperemia was observed (Fig. 4).

## DISCUSSION

Brain injury by transient global brain ischemia (cardiac arrest) and focal brain ischemia (ischemia stroke) is the leading cause of serious and long-term disability in the US (40). Striking differences in the incidence and outcome of stroke between males and females have been suggested to have resulted from the neuroprotective effects of estrogens (3, 12, 26, 33, 42). In the present study, testosterone was shown to possess deleterious effects on ischemic stroke in a focal ischemia model, whereas acute testosterone depletion exerts neuroprotective effects, which suggests that effects of testosterone could also contribute to these gender differences of stroke.

Experimental focal brain ischemia is one of the models most widely used to test the neuroprotective effects of estrogens in vivo. Protective effects of estrogens have been documented by using MCAO model (3, 12, 33, 42). In male rats, castration has also been reported to decrease ischemia-reperfusion injury in this model (19), whereas another report showed that castration

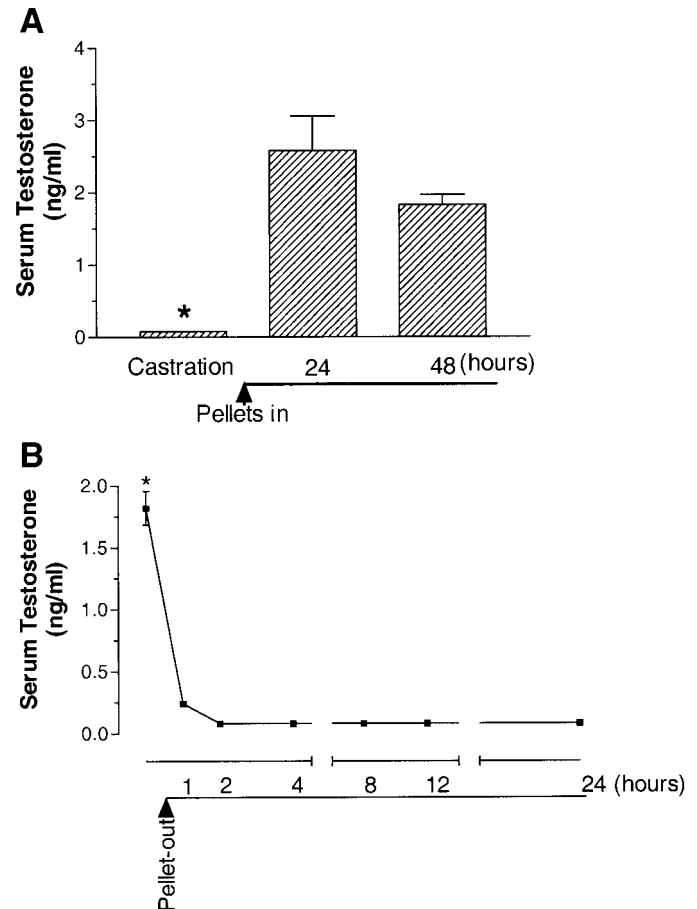


Fig. 2. Testosterone concentrations in testosterone-replacement and -depletion animals. **A:** rats ( $n = 4$ ) were castrated, and two testosterone pellets were implanted subcutaneously. Testosterone concentrations were maintained at  $2.58 \pm 0.47$  and  $1.83 \pm 0.13$  ng/ml at 1 and 2 days after implantation, respectively. **B:** testosterone concentration decreased to  $0.24 \pm 0.01$  ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentration decreased to <0.08 ng/ml. Values are means  $\pm$  SE. \* $P$  < 0.05 vs. pellets in.

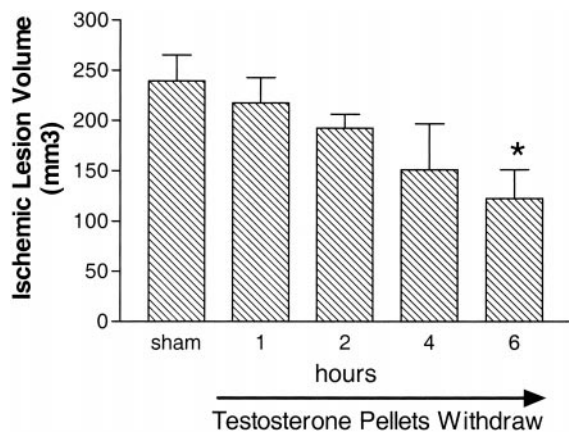


Fig. 3. Effect of testosterone depletion on ischemic lesion volume. Lesion volume was  $217.8 \pm 24.7$ ,  $192.6 \pm 13.9$ ,  $151.3 \pm 45.5$ , and  $122.5 \pm 28.6$  mm<sup>3</sup> at 1 ( $n = 7$ ), 2 ( $n = 5$ ), 4 ( $n = 5$ ), and 6 h ( $n = 5$ ) after pellet removal, respectively, compared with  $239.6 \pm 25.9$  mm<sup>3</sup> in control animals with testosterone ( $n = 15$ ). All values are means  $\pm$  SE. \* $P < 0.05$  vs. sham.

did not affect the ischemia-reperfusion injury by using a similar model (36). Two reasons could attribute to the different result between these studies: 1) difference in the duration of MCAO, which was 1 h in the former study compared with 2 h in the latter study; and 2) wide range of testosterone concentrations in noncastrated animals in the latter study, which ranged from 0.05 to 1.62 ng/ml. The wide range of testosterone concentrations in the intact male animals could have resulted from the different kinds of stress and daily rhythms of testosterone. Testosterone had a daily rhythm in young male rats, with daily troughs as low as  $\sim 0.5$  ng/ml and peaks as high as 2.0 ng/ml (32). In the present study, castration and testosterone-replacement techniques were used to evaluate the effects of acute testosterone depletion on ischemia-reperfusion injury. This technique produces a sustained physiologically relevant testosterone level and avoids the influence of stress and daily rhythms in testosterone levels. Testosterone levels decline rapidly in response to both physical and psychological stress (14), and testosterone levels are reduced in stroke patients (10, 16). Testosterone levels have been shown to be inversely associ-

ated with stroke severity and 6-mo mortality, whereas estradiol levels were not reduced in stroke patients (20). We have also shown that testosterone levels decrease significantly after MCAO (Fig. 5). Physiological consequences of this response are still unclear. It has been shown that adrenomedullary activation may be influenced by the stress-induced decline in testosterone (15). Testosterone receptor blockade using flutamide appeared to ameliorate the depressed adrenal function in males after trauma and severe hemorrhagic shock (5). Stress-induced testosterone reduction could positively influence stroke outcome through adrenomedullary activation. In the present study, acute depletion of testosterone significantly decreased the ischemic lesion volume, which suggests that stress-related testosterone reduction could be a protective response.

Interestingly, acute depletion of testosterone before ischemic insult caused a time-dependent improvement in MCAO outcome. One of the reasons for the time-dependent effects of testosterone depletion could have resulted from the delayed degradation of testosterone in the brain. Our previous study suggested that plasma testosterone was a primary determinant of the size of ischemic lesions following MCAO in male rats (19). The half-life of serum testosterone is very short, and serum testosterone decreased to an undetectable level within 2 h after pellet removal. Testosterone is highly hydrophobic and is cleared much more slowly from lipid-rich tissue, such as brain tissue, than from blood. So central nervous system effects of testosterone can persist after androgen depletion (13). Delayed effects of testosterone depletion also suggest that the effects could be mediated through a transcriptional mechanism, which could take 4 to 6 h to terminate.

Our data show that there was a similar CBF reduction in both the testosterone and testosterone-depletion groups during MCAO. Hyperemia was shown clearly in the ischemic side within 30 min after reperfusion compared with the contralateral side in the testosterone group but not in the testosterone-depletion group. This suggests that the deleterious effects of testosterone could be CBF related. Reactive hyperemia and delayed hyporemia have been found during reper-

Table 1. *Physiological parameters in rats subjected to transient MCAO*

	Testosterone			Testosterone Depletion		
	Before MCAO	During MCAO	After MCAO	Before MCAO	During MCAO	After MCAO
MABP, mmHg	87.0 $\pm$ 2.5	77.8 $\pm$ 2.5	87.0 $\pm$ 5.4	84.3 $\pm$ 1.9	82.0 $\pm$ 5.6	80.4 $\pm$ 2.7
Pco <sub>2</sub> , Torr	48.8 $\pm$ 2.9	46.9 $\pm$ 1.3	45.3 $\pm$ 1.3	56.1 $\pm$ 2.3	47.6 $\pm$ 4.8	42.1 $\pm$ 3.9
PO <sub>2</sub> , Torr	78.2 $\pm$ 7.2	73.0 $\pm$ 3.1	86.0 $\pm$ 4.3	67.2 $\pm$ 4.1	71.0 $\pm$ 7.2	80.6 $\pm$ 8.3
HCO <sub>3</sub> , mmol/l	26.6 $\pm$ 0.4	25.8 $\pm$ 0.2	25.0 $\pm$ 0.3	27.0 $\pm$ 0.7	26.2 $\pm$ 1.1	25.2 $\pm$ 0.9
SO <sub>2</sub> , %	93.2 $\pm$ 2.3	93.2 $\pm$ 0.9	95.7 $\pm$ 0.8	88.8 $\pm$ 2.9	90.8 $\pm$ 3.7	94.2 $\pm$ 1.8
pH	7.35 $\pm$ 0.02	7.35 $\pm$ 0.01	7.35 $\pm$ 0.01	7.29 $\pm$ 0.02	7.36 $\pm$ 0.03	7.39 $\pm$ 0.03
Hb, g/dl	14.4 $\pm$ 0.2	14.2 $\pm$ 0.5	14.6 $\pm$ 0.5	14.5 $\pm$ 0.2	14.0 $\pm$ 0.0	14.4 $\pm$ 0.2
Na <sup>+</sup> , mmol/l	141.4 $\pm$ 0.9	140.0 $\pm$ 0.9	140.2 $\pm$ 1.6	141.2 $\pm$ 1.4	138.0 $\pm$ 1.1	139.8 $\pm$ 1.5
K <sup>+</sup> , mmol/l	4.5 $\pm$ 0.1	5.3 $\pm$ 0.2	5.0 $\pm$ 0.3	4.8 $\pm$ 0.1	5.8 $\pm$ 0.2	5.1 $\pm$ 0.2

Values are means  $\pm$  SE ( $n = 6$  for each group). MCAO, middle cerebral artery occlusion; MABP, mean arterial blood pressure. There were no significant differences between treatment groups at any sampling time.

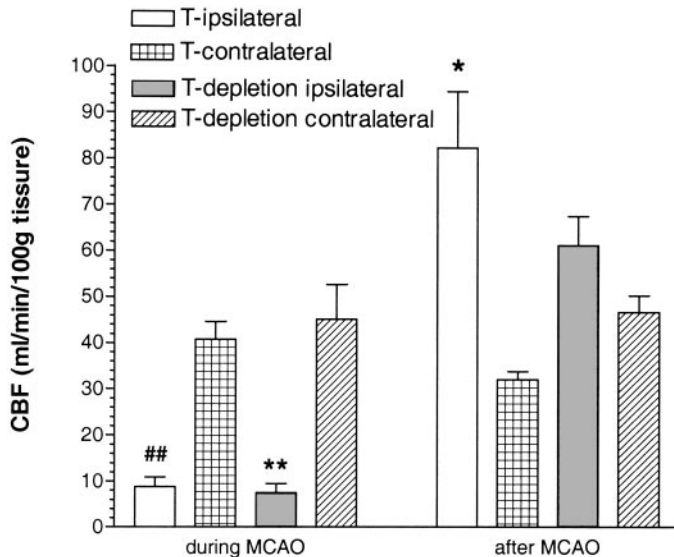


Fig. 4. Effect of testosterone on regional cerebral blood flow (CBF). T-ipsilateral, ischemic side in testosterone group; T-contralateral, contralateral side in testosterone group; T-depletion ipsilateral, ipsilateral side in testosterone-depletion group; T-depletion contralateral, contralateral side in testosterone-depletion group; MCAO, middle cerebral artery occlusion. Values are means  $\pm$  SE. \* $P$  < 0.05 vs. T-contralateral and T-depletion contralateral. \*\* $P$  < 0.01 vs. T-depletion contralateral. \*\*\* $P$  < 0.01 vs. T-contralateral during MCAO.

fusion, and both are thought to be harmful to ischemic tissue (34, 37). Ischemic edema and blood-brain barrier disruption have been found to be exacerbated after acute reperfusion, which is related to the sudden surge reperfusion with hyperemia (23, 41). Gradual blood flow restoration could significantly reduce the exacerbation of ischemic edema and blood-brain barrier opening (17). As such, the damaging effects of testosterone could have partially resulted from reactive hyperemia during reperfusion.

The mechanism of testosterone's effect on CBF is unclear. Testosterone has been shown to be vasoactive in the peripheral artery system. Treatment with testosterone causes a vasorelaxant response in rabbit coronary arteries (43). Other studies (29, 39) also indicated that testosterone infusion into coronary arteries in men with coronary artery disease induced vasodilation and that intravenous administration of testosterone reduced exercise-induced ischemic response in men with coronary artery disease. Testosterone's effect on vascular tone could be because of aromatization of testosterone to estradiol, as aromatase has been identified in the arterial wall (11). However,  $17\beta$ -estradiol inhibits  $Ca^{2+}$  entry, whereas testosterone causes coronary relaxation by inhibiting other mechanisms in addition to  $Ca^{2+}$  entry (8). Furthermore, testosterone has been shown to exacerbate, whereas estrogen decreases, the vulnerability of lateral striatal artery to chemical hypoxia (25). The direct mechanism of testosterone action on arteries should also be taken into account.

Consistent with our in vivo study, testosterone was shown to exacerbate glutamate toxicity in an in vitro

model. Toxic insults by glutamate in neuronal cell culture mimic a key component of ischemic brain injury. Microdialysis studies have shown that there is a severalfold increase in extracellular glutamate during global ischemia, beginning within 1–2 min (6, 24). There is a similar rise during focal ischemia, beginning within 2 min of MCAO (38). Furthermore, glutamate can cause both apoptosis and necrosis (28). In HT-22 cells, glutamate competes with cystine for uptake, leading to a reduction in glutathione, accumulation of reactive oxygen species, and ultimately cell death (35). The present study shows that testosterone treatment exacerbates glutamate toxicity to HT-22 cells, whereas  $17\beta$ -estradiol treatment decreases the cells' susceptibility to glutamate toxicity, which provides us in vitro evidence to support our in vivo study. Although the deleterious effects of testosterone are only present at the micromolar level in vitro, which is thousands of times higher than peak physiological levels in reproductive males, physiological levels of testosterone exert damaging effects on ischemia-reperfusion injury in vivo.

It has been shown that in vivo treatment of postnatal rats with testosterone rendered cerebellar granule neurons less vulnerable to oxidative stress-induced apoptosis in vitro, which was associated with increases in catalase activity as well as in the activity of superoxide dismutase (1). However, the decreased susceptibility to oxidative stress induced by the postnatal treatment with testosterone was more likely due to an accelerated maturation with a consequent developmental age-dependent increase in the antioxidant defense (30). Effects of testosterone could be different in mature animals, as was shown with cerebral ischemia in our study. Testosterone treatment in vitro has also been shown to be neuroprotective for cerebellar granule neurons (2). As  $17\beta$ -estradiol is also neuroprotective in cerebellar granule neurons (9), the neuroprotective effects of testosterone could be due to the conversion of testosterone into  $17\beta$ -estradiol by aro-

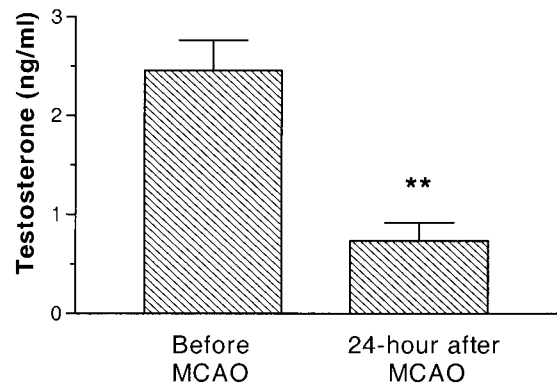


Fig. 5. Effect of ischemia-reperfusion injury on serum testosterone level in normal male rats ( $n = 7$ ). Blood samples were taken before MCAO and 24 h after reperfusion. All blood samples were taken in the morning. Testosterone concentration was reduced to  $0.73 \pm 0.19$  ng/ml at 24 h after reperfusion, compared with  $2.46 \pm 0.31$  ng/ml before MCAO ( $P$  < 0.01). Values are means  $\pm$  SE. \*\* $P$  < 0.01 vs. testosterone concentration before MCAO.

matase. Furthermore, testosterone has been reported to attenuate neuronal death in mice in response to excitotoxins, which were blocked by aromatase (4).

In summary, the present data show that testosterone can increase neuronal toxicity and exacerbate ischemia-reperfusion injury. These results suggest that sex differences in the outcome after stroke may have resulted from both the protective effects of estrogens and the damaging effects of testosterone. Furthermore, acute depletion of testosterone provides neuroprotective effects on ischemia-reperfusion injury, which could be partially related to the amelioration of hyperemia during reperfusion.

This study was supported by National Institute on Aging Grant AG-10485, Apollo BioPharmaceutics, and US Army Grant DAMD 17-19-1-9473.

## REFERENCES

- Ahlbom E, Grandison L, Bonfoco E, Zhivotovsky B, and Ceccatelli S. Androgen treatment of neonatal rats decreases susceptibility of cerebellar granule neurons to oxidative stress in vitro. *Eur J Neurosci* 11: 1285–1291, 1999.
- Ahlbom E, Prins GS, and Ceccatelli S. Testosterone protects cerebellar granule cells from oxidative stress-induced cell death through a receptor mediated mechanism. *Brain Res* 892: 255–262, 2001.
- Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, and Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke* 29: 159–166, 1998.
- Azcoitia I, Sierra A, Veiga S, Honda S, Harada N, and Garcia-Segura LM. Brain aromatase is neuroprotective. *J Neurobiol* 47: 318–329, 2001.
- Ba ZF, Wang P, Koo DJ, Zhou M, Cioffi WG, Bland KI, and Chaudry IH. Testosterone receptor blockade after trauma and hemorrhage attenuates depressed adrenal function. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1841–R1848, 2000.
- Baker AJ, Zornow MH, Scheller MS, Yaksh TL, Skilling SR, Smullin DH, Larson AA, and Kuczenski R. Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. *J Neurochem* 57: 1370–1379, 1991.
- Bushnell CD, Samsa GP, and Goldstein LB. Hormone replacement therapy and ischemic stroke severity in women: a case-control study. *Neurology* 56: 1304–1307, 2001.
- Crews JK and Khalil RA. Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on  $Ca^{2+}$  entry mechanisms of coronary vasoconstriction. *Arterioscler Thromb Vasc Biol* 19: 1034–1040, 1999.
- Dare E, Gotz ME, Zhivotovsky B, Manzo L, and Ceccatelli S. Antioxidants J811 and 17 beta-estradiol protect cerebellar granule cells from methylmercury-induced apoptotic cell death. *J Neurosci Res* 62: 557–565, 2000.
- Dash RJ, Sethi BK, Nalini K, and Singh S. Circulating testosterone in pure motor stroke. *Funct Neurol* 6: 29–34, 1991.
- Diano S, Horvath TL, Mor G, Register T, Adams M, Harada N, and Naftolin F. Aromatase and estrogen receptor immunoreactivity in the coronary arteries of monkeys and human subjects. *Menopause* 6: 21–28, 1999.
- Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, and Wise PM. Estradiol protects against ischemic injury. *J Cereb Blood Flow Metab* 18: 1253–1258, 1998.
- Dyker AG and Lees KR. Duration of neuroprotective treatment for ischemic stroke. *Stroke* 29: 535–542, 1998.
- Elman I and Breier A. Effects of acute metabolic stress on plasma progesterone and testosterone in male subjects: relationship to pituitary-adrenocortical axis activation. *Life Sci* 61: 1705–1712, 1997.
- Elman I, Goldstein DS, Adler CM, Shoaf SE, and Breier A. Inverse relationship between plasma epinephrine and testosterone levels during acute glucoprivation in healthy men. *Life Sci* 68: 1889–1898, 2001.
- Elwan O, Abdallah M, Issa I, Taher Y, and el-Tamawy M. Hormonal changes in cerebral infarction in the young and elderly. *J Neurol Sci* 98: 235–243, 1990.
- Gartshore G, Patterson J, and Macrae IM. Influence of ischemia and reperfusion on the course of brain tissue swelling and blood-brain barrier permeability in a rodent model of transient focal cerebral ischemia. *Exp Neurol* 147: 353–360, 1997.
- Green PS, Yang SH, Nilsson KR, Kumar AS, Covey DF, and Simpkins JW. The nonfeminizing enantiomer of 17 beta-estradiol exerts protective effects in neuronal cultures and a rat model of cerebral ischemia. *Endocrinology* 142: 400–406, 2001.
- Hawk T, Zhang YQ, Rajakumar G, Day AL, and Simpkins JW. Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. *Brain Res* 796: 296–298, 1998.
- Jeppesen LL, Jorgensen HS, Nakayama H, Raaschou HO, Olsen TS, and Winther K. Decreased serum testosterone in men with acute ischemic stroke. *Arterioscler Thromb Vasc Biol* 16: 749–754, 1996.
- Kinderman NB, Harrington CA, Drengler SM, and Jones KJ. Ribosomal RNA transcriptional activation and processing in hamster facial motoneurons: effects of axotomy with or without exposure to testosterone. *J Comp Neurol* 401: 205–216, 1998.
- Kujawa KA, Kinderman NB, and Jones KJ. Testosterone-induced acceleration of recovery from facial paralysis following crush axotomy of the facial nerve in male hamsters. *Exp Neurol* 105: 80–85, 1989.
- Kuroiwa T, Shibutani M, and Okeda R. Nonhyperemic blood flow restoration and brain edema in experimental focal cerebral ischemia. *J Neurosurg* 70: 73–80, 1989.
- Mitani A and Kataoka K. Critical levels of extracellular glutamate mediating gerbil hippocampal delayed neuronal death during hypothermia: brain microdialysis study. *Neuroscience* 42: 661–670, 1991.
- Nishino H, Nakajima K, Kumazaki M, Fukuda A, Muramatsu K, Deshpande SB, Inubushi T, Morikawa S, Borlongan CV, and Sanberg PR. Estrogen protects against while testosterone exacerbates vulnerability of the lateral striatal artery to chemical hypoxia by 3-nitropropionic acid. *Neurosci Res* 30: 303–312, 1998.
- Paganini-Hill A. Estrogen replacement therapy and stroke. *Prog Cardiovasc Dis* 38: 223–242, 1995.
- Paganini-Hill A, Ross RK, and Henderson BE. Postmenopausal oestrogen treatment and stroke: a prospective study. *BMJ* 297: 519–522, 1988.
- Portera-Cailliau C, Price DL, and Martin LJ. Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. *J Comp Neurol* 378: 88–104, 1997.
- Sarrel PM. The differential effects of oestrogens and progestins on vascular tone. *Hum Reprod* 5: 205–209, 1999.
- Scarpa M, Rigo A, Viglino P, Stevanato R, Bracco F, and Battistin L. Age dependence of the level of the enzymes involved in the protection against active oxygen species in the rat brain. *Proc Soc Exp Biol Med* 185: 129–133, 1987.
- Shi J, Bui JD, Yang SH, He Z, Lucas TH, Buckley DL, Blackband SJ, King MA, Day AL, and Simpkins JW. Estrogens decrease reperfusion-associated cortical ischemic damage: an MRI analysis in a transient focal ischemia model. *Stroke* 32: 987–992, 2001.
- Simpkins JW, Kalra PS, and Kalra SP. Alterations in daily rhythms of testosterone and progesterone in old male rats. *Exp Aging Res* 7: 25–32, 1981.
- Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, and Day AL. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg* 87: 724–730, 1997.

34. **Takahashi A, Park HK, Melgar MA, Alcocer L, Pinto J, Lenzi T, Diaz FG, and Rafols JA.** Cerebral cortex blood flow and vascular smooth muscle contractility in a rat model of ischemia: a correlative laser Doppler flowmetric and scanning electron microscopic study. *Acta Neuropathol (Berl)* 93: 354–368, 1997.
35. **Tan S, Wood M, and Maher P.** Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J Neurochem* 71: 95–105, 1998.
36. **Toung TJ, Traystman RJ, and Hurn PD.** Estrogen-mediated neuroprotection after experimental stroke in male rats. *Stroke* 29: 1666–1670, 1998.
37. **Tsuchida R, He QP, Smith ML, and Siesjo BK.** Regional cerebral blood flow during and after 2 hours of middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 17: 1066–1073, 1997.
38. **Wahl F, Obrenovitch TP, Hardy AM, Plotkine M, Boulu R, and Symon L.** Extracellular glutamate during focal cerebral ischaemia in rats: time course and calcium dependency. *J Neurochem* 63: 1003–1011, 1994.
39. **Webb CM, McNeill JG, Hayward CS, de Zeigler D, and Collins P.** Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease. *Circulation* 100: 1690–1696, 1999.
40. **White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, and Krause GS.** Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* 179: 1–33, 2000.
41. **Yang GY and Betz AL.** Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats. *Stroke* 25: 1658–1655, 1994.
42. **Yang SH, Shi J, Day AL, and Simpkins JW.** Estradiol exerts neuroprotective effects when administered after ischemic insult. *Stroke* 31: 745–750, 2000.
43. **Yue P, Chatterjee K, Beale C, Poole-Wilson PA, and Collins P.** Testosterone relaxes rabbit coronary arteries and aorta. *Circulation* 91: 1154–1160, 1995.
44. **Zhang YQ, Shi J, Rajakumar G, Day AL, and Simpkins JW.** Effects of gender and estradiol treatment on focal brain ischemia. *Brain Res* 784: 321–324, 1998.

