Endocrine, *vol. 21, no. 1, 17–26, June 2003* 0969–711X/03/21:17–26/$20.00

Estrogen and Selective Estrogen Receptor Modulators

Neuroprotection in the Women's Health Initiative Era

Stephanie J. Murphy,[1] Louise D. McCullough,[2]
Marguerite T. Littleton-Kearney,[1] and Patricia D. Hurn[1]

Departments of [1]Anesthesiology and Critical Care Medicine and [2]Neurology, Johns Hopkins Medical Institutions, Baltimore, MD

Estrogen has been comprehensively studied as a neuroprotective agent in women, animals, and a variety of in vitro models of neural injury and degeneration. Most data suggest that estrogen can benefit the ischemic brain and reduce cell death. However, recent data from the Women's Health Initiative have raised concerns about the utility and safety of chronic estrogen use in women. While estrogen is a potent and reproducible neuroprotectant in animals and in vitro, its current administration in women has had unanticipated and paradoxical effects. Nonetheless, estrogen's diverse actions make it an ideal prototype for developing new neuroprotectants such as selective estrogen receptor modulators (SERMs). SERMs represent a class of drugs with mixed estrogen agonistic and antagonistic activity. Experimental and clinical data suggest a neuroprotective role for SERMs in normal and injured brain. The discrepancy among observational studies, preclinical data, and clinical trials emphasizes the need for further study of the mechanisms leading to the increased incidence of stroke observed in postmenopausal women. Research is still needed to optimize combined or estrogen alone hormone replacement therapy options as well as the prevention/management of cerebrovascular/central nervous system disorders. This review critiques estrogen and SERMs' neuroprotective potential in experimental and clinical studies of stroke and cerebrovascular disease.

Key Words: Cerebral ischemia; estrogen; hormone replacement therapy; neuroprotection; selective estrogen receptor modulators; stroke.

Introduction

Estrogen has been extensively studied as a neuroprotective agent in women, animals, and a variety of in vitro models of neural injury and degeneration. Most data suggest that estrogen can benefit the brain confronted with an ischemic challenge and reduce cell death. However, recent data from the Women's Health Initiative (WHI) have brought many fundamental issues to light about the utility and safety of chronic estrogen use in women. The WHI, one of the largest preventative studies of its kind, was initiated in 1991 with the overall goal of identifying major causes of death and disability in postmenopausal women through prevention/intervention protocols and risk factor identification. Hormone replacement therapy (HRT) is one intervention under examination in WHI clinical trials *(1)*. Two parallel HRT trials were originally designed. In one arm, women with a prior hysterectomy were randomized to placebo or unopposed estrogen. The other trial examined women with an intact uterus who were randomized to placebo or estrogen plus progestin therapy in acknowledgment of the increased risk of endometrial cancer with unopposed estrogen therapy. The primary outcome of these trials was incidence of coronary heart disease (CHD), while the primary adverse outcome was invasive breast cancer. Secondary endpoints included the effect of HRT on stroke, pulmonary embolism, endometrial cancer, colorectal cancer, hip fracture, and death *(1)*. The combined estrogen plus progestin HRT trial, which was to have continued until 2005, was terminated in July 2002 based on recommendations by the WHI Data and Safety Monitoring Board (DSMB). The DSMB found that overall risks from use of combined HRT outweighed the benefits. In addition to an increased risk of breast cancer, other adverse effects included an increased risk of stroke, with eight more strokes per year for every 10,000 women in the combined HRT group *(1)*. Other outcomes also suggested an overall negative effect on health including increases in cardiovascular events and pulmonary embolism. The treatment arm for unopposed estrogen continues.

These recent results emphasize that there are unanticipated and paradoxical effects of estrogen as it is currently administered in women. In light of the WHI, estrogen's neuroprotective properties and potential benefit in central nervous system (CNS) ischemic injury must be reassessed. Because the largest burden for stroke is in postmenopausal women, there is great and continued interest in HRT as a means of preventing or treating cerebrovascular disease.

Received February 4, 2003; Revised February 4, 2003; Accepted February 11, 2003.

Author to whom all correspondence and reprint requests should be addressed: Stephanie J. Murphy, Oregon Health & Science University–West Campus, Department of Anesthesiology & Peri-Operative Medicine, 20000 N.W. Walker Road, Mail Code: OGI, Beaverton, OR 97006. E-mail: murphyst@ohsu.edu

Furthermore, there is increasing interest in selective estrogen receptor modulators (SERMs) as a safe HRT option and a potential neuroprotective modality. This review critiques estrogen and SERMs' neuroprotective potential in experimental and clinical studies of stroke and cerebrovascular disease. Because less is known about SERMs and their effects in the brain, a broader discussion including other CNS disorders is presented.

Estrogen

Clinical Studies

Premenopausal women have a lower incidence of stroke and other vascular diseases than age-matched men and postmenopausal women, a finding attributed to higher estrogen levels in younger women. Over the past 30 yr, observational studies have found lower risks of CHD and stroke in women taking postmenopausal estrogens, suggesting that estrogen is vasoprotective (for a review see ref. *2*). Observational studies for stroke prevention are not as clearly positive but most describe no increased risk or small benefits in the prevention of fatal strokes *(3)*.

The Heart and Estrogen-Progestin Replacement Study (HERS) was the first randomized, blinded trial on the effect of HRT (0.625 mg of estrogen + 2.5 mg of medroxyprogesterone acetate (MPA), daily) on coronary disease progression. After 4 yr of HRT therapy, HERS found no reduction in risk for coronary events, stroke, or transient ischemic attack (TIA) but did observe a threefold increase in venous thromboembolism *(4)*. An important observation in HERS was that patients receiving HRT sustained an early *increased* risk of cardiovascular events that was offset by a lower event rate in subsequent years. It was presumed that this was owing to an early prothrombotic risk, followed later by protection, and that prolonged follow-up would demonstrate an overall beneficial effect for HRT. However, the release of the 6.8-yr follow-up on the HERS cohort (HERS II) in 2002 *(5)* showed no benefit of prolonged HRT treatment on cardiovascular or cerebrovascular endpoints.

The HERS study was designed to investigate the effects of HRT on coronary disease progression with stroke and TIA as secondary endpoints. By contrast, the Women's Estrogen for Stroke Trial (WEST) was the first randomized trial designed to examine stroke recurrence as the primary endpoint *(6)*. The WEST found no benefit on total stroke incidence and a surprising *increase* in fatal stroke among women who were assigned to unopposed estradiol therapy. This trial, like the HERS, evaluated secondary prevention and enrolled older women with preexisting cerebrovascular disease (TIA or stroke 90 d prior to randomization). The unexpected findings of these three trials are not easily aligned with earlier epidemiologic data suggesting that estrogen would be protective.

As discussed later in this review, estrogen is a potent and reproducible neuroprotectant in animals and in vitro. Thus, it is not immediately obvious why HRT was ineffective and potentially deleterious in humans. One factor is that both the HERS and WEST included women with known vascular disease and who began treatment at an advanced age well beyond menopause. The WHI data are believed to partly address this question because the WHI evaluates effects in healthy postmenopausal women. Although the WHI was oriented toward primary prevention, 7.7% of women participants had documented vascular disease. Furthermore, many women were enrolled despite relative contraindications to HRT, such as smoking, previous stroke, or venous thromboembolism *(1)*. It is believed that such subjects more likely represent the "average patient" considering HRT than if such variables were excluded. Earlier observational trials may have preselected a healthier, lower-risk group of women who may have been able to derive benefit from HRT.

Although combined estrogen/progestin compounds are the most commonly prescribed hormone regimen in the United States, it is not known if progestins interact with estrogen and diminish its neuroprotective effects. Experimental data suggest that progesterone increases subcortical damage after vascular occlusion in animals *(7)* and can reverse the beneficial effect seen on atherosclerotic plaque formation in nonhuman primates *(8,9)*. However, recent clinical results argue against this hypothesis. The Estrogen Replacement and Atherosclerosis trial utilized estrogen with or without a progestin and found no benefit in coronary disease progression as measured angiographically in either treatment group *(10)*. Results from the oEStrogen in the Prevention of ReInfarction Trial (ESPRIT) *(11)* demonstrate that estradiol valerate did not reduce risk of recurrent myocardial infarction. Furthermore, the WEST did not demonstrate a beneficial effect of 17β-estradiol for secondary prevention of stroke and ischemic injury. In summary, the total evidence for the clinical benefit of HRT as an ischemic neuroprotectant is arguably quite small (see ref. *12* for a review). However, in animals and cell models, estrogen is a well-recognized anti-ischemic hormone.

Experimental Ischemic Brain Injury

Gender is an important factor in cerebral ischemic pathophysiology, and several studies have demonstrated that female animals sustain smaller injury after ischemia than males *(13–22)*. In normal, cycling female rats, variable postischemic changes in cortical infarction, neutrophil accumulation, and antioxidant enzyme activities, which were inversely correlated with circulating estrogen levels, have also been demonstrated *(23)*. In female rats, this gender-specific effect may be further influenced by the estrous cycle stage, with lesser infarct volumes in proestrous (high endogenous estradiol levels) animals compared with metestrous animals (low endogenous estradiol levels) *(24)*. Sex differences in stroke sensitivity can be abolished by ovariectomy *(13,22,25–29)* or by declining estrogen levels during reproductive senescence *(30)*.

The effects of exogenous 17β-estradiol treatment or replacement on cerebral injury size have been comprehensively studied in experimental stroke and neuroprotection models (Table 1). Almost universally, the steroid reduces brain injury after an ischemic, glutamatergic, prooxidant, or proapoptotic insult (for reviews, see refs. *17* and *31–33*). However, several factors can be extracted from the plethora of animal data. In both permanent and transient focal cerebral ischemia models, estrogen appears to have a neuroprotective effect in estrogen-deficient rodents (males, ovariectomized [OVX] females, and reproductively senescent females). However, the therapeutic range of "neuroprotective" steroid doses is not large; there are few studies of long-term estrogen exposure, so the effect of treatment duration is not clear. The effective dose and duration may differ between sexes, suggesting that the mechanisms of protection are not necessarily identical in male and female animals. Most studies have evaluated 17β-estradiol, and there are few data with the less potent estrogens such as estriol. A single study has demonstrated a deleterious effect of estrogen in a rat model of transient forebrain ischemia *(34)*, but no mechanism of injury was tested. Therefore, we know little about what distinguishes the neuroprotectant estrogen from a proinjury estrogen.

Almost all of our understanding of estrogen's neuroprotection originates in rodent data. Few data are available in higher-order, gyroencephalic animals such as cat or nonhuman primates. Finally, most estrogens are vasoactive and have potent effects on endothelium and vascular smooth muscle cells of brain blood vessels, as well as neurons and glia within brain parenchyma. For example, 17β-estradiol can increase cerebral blood flow during and after vascular occlusion and ameliorate postischemic hyperemia *(35–37)*. Therefore, it will be important in future research to evaluate systematically and quantitatively vaso- vs neuroprotection if we are to understand fully estrogen's action in the brain.

It must also be emphasized that estrogen's mechanisms of protection for the brain and cerebral vasculature are quite complex. There is evidence implicating—and refuting—the importance of nuclear hormone receptor signaling mechanisms to gender differences and to the anti-ischemic activity of 17β-estradiol *(28,38–40)*. However, it is also quite clear that rapid receptor-mediated and receptor-independent intracellular signaling is relevant in neuroprotection, not involving gene transcription *(41,42)*. These actions involve putative membrane estrogen receptors (ERs), kinase cascades, and intracellular signaling that activate ion channels, neurotransmitter receptors, and enzymes such as nitric oxide synthase (NOS). Such mechanisms may be critical to estrogen's protection in experimental stroke. Finally, many estrogens, such as 2-hydroxy estrone, 2-hydroxy estradiol, phenolic estrone, and 17β-estradiol, have potent, concentration-dependent lipid antioxidant activity *(43,44)*.

In summary, it is likely that the estrogens act at multiple sites in injured brain and utilize receptor-dependent, receptor-independent, and non-cell type–specific signaling processes. For example, if estrogen targets postischemic blood vessels (promoting microcirculation) *and* protects neurons (amplifying gene transcription or antioxidant activity), then the net benefit to tissue should be large. As a unifying hypothesis, estrogen's very breadth of actions as a multifunctional molecule makes it an ideal prototype for developing neuroprotectants. A new pharmacology involving SERMs may harness this prototype and expand estrogen's neuroprotective potential.

Selective Estrogen Receptor Modulators

SERMs represent a class of drugs with mixed estrogen agonistic and antagonistic activity in different tissues. An ideal SERM would theoretically function as an antagonist in breast and uterus and as an agonist in bone, brain, and the cardiovascular system. Of the SERMs available today (Table 2), none can be considered "ideal." Only a few SERMs are currently approved for the following clinical uses: (1) treatment of breast cancer (tamoxifen, toremifene), (2) treatment of hormone receptor–positive metastatic breast cancer with disease progression after antiestrogen therapy (fulvestrant), (3) prevention and treatment of postmenopausal osteoporosis (raloxifene), and (4) treatment of infertility in anovulatory women (clomiphene). The effects and mechanism(s) of action of SERMs in the brain are currently under investigation, but few studies have addressed the potential neuroprotective benefits of SERMs following brain injury, neurodegeneration, or aging. This section reviews what is known about the effects of SERMs in the brain and in CNS/cerebrovascular disorders.

Clinical Studies

Several ongoing clinical studies are attempting to evaluate CNS and cerebrovascular effects of SERMs in women. These trials have focused exclusively on two of the most widely used SERMs: tamoxifen and raloxifene. Only one study has examined the effects of SERMs on aging brain chemistry. A study utilizing proton magnetic resonance spectroscopy compared *myo*-inositol levels, a marker of glial metabolism, in elderly women taking either tamoxifen or HRT *(45)*. Cerebral *myo*-inositol levels normally increase with age *(46)*. Women in both treatment groups exhibited lower brain *myo*-inositol levels in all regions compared with the control group, suggesting that tamoxifen has effects similar to estrogen in the brain and may positively modulate aging *(45)*.

Thus far, there are no prospective, placebo-controlled data on the effects of tamoxifen on cognitive function or prevention of dementia in healthy women *(47–49)*. One prospective breast cancer study showed no effect of tamoxifen on female patients' self-reported cognitive problems *(50)*. A retrospective study in young women with breast cancer reported no differences in cognitive tests between tamoxifen and controls *(51)*. However, women using tamoxifen in this

Table 1
Effect of Exogenous Estrogen Treatment in Rodent Experimental Stroke Models[a]

Stroke model	Rodent species	Estrogen dose	Estrogen effect on ischemic injury	Reference
Transient focal ischemia (MCAO – intraluminal filament); 2-h ischemia + 22-h reperfusion	Wistar male rats	iv estrogen (1 mg/kg of Premarin) at initiation of reperfusion	Reduced total hemispheric and striatal infarct size	*36*
Transient focal ischemia (MCAO – intraluminal filament); 1-h ischemia + 6 to 23-h reperfusion	Sprague-Dawley OVX female rats	Preischemic treatment (100 μg/kg of 17β-estradiol) 2 h before ischemia	Selectively protected cortical tissue from ischemic damage	*95*
Transient focal ischemia (MCAO – intraluminal filament); 2-h ischemia + 22-h reperfusion	Wistar RSF rats	Preischemic treatment via sc implant (25 μg of 17β-estradiol) for 1 wk	Reduced cortical and striatal infarct size	*30*
Transient focal ischemia (MCAO + bilateral common carotid artery occlusion); 3-h ischemia + 72-h reperfusion	Wistar OVX rats	(1) iv 17β-estradiol (0.1 or 1.0 mg/kg) 30 min before ischemia (2) Preischemic treatment via sc implant (20 or 200 μg of 17β-estradiol) for 1 wk	Ischemic volume not altered by acute or chronic treatment	*96*
Transient focal ischemia (MCAO – intraluminal filament); 2-h ischemia + 22-h reperfusion	Wistar OVX female rats	Preischemic treatment via sc implant (0, 25, 100 μg of 17β-estradiol) for 7–16 d; iv saline or Premarin (1 mg/kg) immediately before ischemia	Cortical and striatal injury reduced by chronic estrogen replacement (25 μg); no effect with acute single-injection estrogen exposure	27
Transient focal ischemia (MCAO – intraluminal filament); 2-h ischemia + 22-h reperfusion	(1) Wistar intact male rats	(1) Preischemic treatment via sc implant (25 or 100 μg of 17β-estradiol) for 7–10 d; iv saline or Premarin (1 mg/kg) 30 min before ischemia	(1) Reducion of cortical and striatal injury by acute and chronic treatment	*97*
	(2) Wistar castrated male rats	(2) Preischemic treatment via sc implant (25 or 100 μg of 17β-estradiol) for 7–16 d	(2) Reduced cortical and striatal injury	
Transient focal ischemia (MCAO – intraluminal filament); 40-min ischemia + 23-h, 20-min reperfusion	Charles River intact and castrated male rats	Preischemic treatment via sc Silastic pellet for 1 wk	Reduced ischemic area	*98*
Transient focal ischemia (MCAO – intraluminal filament); 40-min ischemia + 23-h, 20-min reperfusion	Sprague-Dawley OVX female rats	iv estrogen (1 mg/kg of 17β-estradiol) at initiation of reperfusion	Reduced ischemic area	*22*
Transient focal ischemia (MCAO – intraluminal filament); 40-min ischemia + 6-h, 24-h, or 1 wk reperfusion	CD OVX female rats	Preischemic treatment via sc Silastic pellet 24 h before MCAO, or iv 17β-estradiol via brain-targeted chemical delivery at 30 and 90 min after MCAO onset	Reduced ischemic area	*28*
Permanent focal ischemia (MCAO – intraluminal filament) for 24 h	Sprague-Dawley OVX young (3–4 mo) and middle-aged (9–12 mo) female rats	Preischemic treatment via sc (0, 180, or 1000 μg/mL of 17β-estradiol in oil) Silastic capsule for 1 wk	Decreased infarct volume	*99*

Stroke model	Rodent species	Estrogen dose	Estrogen effect on ischemic injury	Reference
Permanent focal ischemia (MCAO – bipolar electrical coagulation) for 4 h	Sprague-Dawley male rats	Local microinjection of 17β-estradiol (0.5 μ*M* in 250 nL) into insular cortex or caudate putamen 10 or 30 min before ischemia	Reduced infarct size	*100*
Permanent focal ischemia (MCAO – bipolar electrical coagulation) for 4 h	Sprague-Dawley male rats	iv injection of 17β-estradiol (1×10^{-2} mg/kg) 30 min before, immediately before, or 30 min after MCAO	Reduced infarct size	*101*
Permanent focal ischemia (MCAO – photothrombotic) for 3 d	SHR OVX female rats	sc estradiol valerate (200 μg/kg) in sesame oil weekly for 3 wk prior to ischemia	Reduced infarct volume	*26*
Permanent focal ischemia (MCAO – intraluminal filament) for 24 h	Sprague-Dawley OVX young (3–4 mo) and middle-aged (9–11 mo) female rats	Preischemic treatment (1 mg/mL of 17β-estradiol in oil) via sc Silastic capsule for 1 wk	Decreased infarct volume	*33*
Permanent focal ischemia (MCAO – electrocoagulation) for 2 d	NMRI male mice	17β-estradiol (0.3–30 mg/kg) and 2-OH-estradiol (0.003–30 mg/kg) subcutaneously for 24 h and intraperitoneally for 3 h before MCAO	Reduced brain infarct area	*102*
Permanent focal ischemia (MCAO – intraluminal filament) for 24 h	Sprague-Dawley OVX female rats	Preischemic treatment (0, 180, or 1000 μg/mL of 17β-estradiol in oil) via sc Silastic capsule for 1 wk or at onset of ischemia	Cortical infarct volume decreased by chronic estradiol replacement; no effect when given at onset of ischemia	*25*
Transient global ischemia (4-VO); 20-min ischemia + 96 h reperfusion	Sprague-Dawley OVX female rats rats	Preischemic treatment via sc implant (4 mg/mL of 17β-estradiol, two 5-mm pellets) 24 h before ischemia	Significantly higher live cell counts than intact or OVX female	*103*
Transient forebrain ischemia (4-VO); 5- or 10-min ischemia + 1-wk recovery	Wistar OVX female rats	Preischemic treatment via sc implant (25 μg of 17β-estradiol) for 1 wk	Significant relationship between cell loss and estradiol level; no protective effect	*34*
Transient forebrain ischemia (right common carotid artery occlusion + hemorrhagic hypotension); 30-min ischemia + 72-h reperfusion	Sprague-Dawley OVX female rats	ip 17β-estradiol (0.1 mg/[kg·d] for 2 wk before ischemia	Reduced brain damage	*29*
Transient forebrain ischemia (right common carotid artery occlusion + hemorrhagic hypotension); 30-min ischemia + 72-h reperfusion	Sprague-Dawley OVX female rats	17β-estradiol (0.1, 0.5, or 5.0 mg/[kg·d]) up to 2 wk before ischemia	Hippocampal and striatal neuronal damage reduced by low doses of estrogen	*104*
Transient global ischemia (bilateral common carotid artery occlusion); 17-min ischemia + 72-h reperfusion	C57Bl/6J OVX female mice	Preischemic treatment via sc implant (0.025 mg of 17β-estradiol) for 2 wk	Significant reduction in neuronal damage in caudate putamen	*105*

(continued)

Table 1 (Continued)

Stroke model	Rodent species	Estrogen dose	Estrogen effect on ischemic injury	Reference
Transient global ischemia (bilateral carotid artery occlusion); 5-min ischemia + 7-d reperfusion	Male Mongolian gerbils	Preischemic treatment via sc implant (0.36 mg/pellet of 17β-estradiol) for 2 wk	Significant neuroprotection against ischemia-induced neuronal death	*106*
Transient forebrain ischemia (bilateral carotid artery occlusion); 3-min ischemia + 7-d reperfusion	Male Mongolian gerbils	17β-estradiol either intracerebroventricularly (3, 10, or 30 μg) or intraperitoneally (4 mg/kg) 1 h before ischemia	Significantly prevented damage in hippocampal CA1 pyramidal cells with highest icv dose and ip dose	*107,108*
Transient forebrain ischemia; 3-min ischemia + 7-d reperfusion	Male gerbils	17β-estradiol via osmotic minipump into left ventricle (0.05 or 0.25 μg/d) for 7 d; infusion begun 2 h before ischemia	Prevented neuronal loss at early stages after ischemia with higher dose	*109*

[a] 4-VO, four-vessel occlusion; RSF, reproductively senescent female.

Table 2
Selective Estrogen Receptor Modulators[a]

Structural classification	Name
Triphenylethylenes (first-generation SERMs)	Clomiphene
	Droloxifene
	GW5638
	Idoxifene
	MDL 103,323
	Miproxifene phosphate (TAT-59)
	Ospemifene
	Tamoxifen
	Toremifene (chlorotamoxifen)
Benzothiophenes (second-generation SERMs)	Arzoxifene (LY353381)
	LY117018
	Raloxifene
Naphthalenes (third-generation SERMs)	Trioxifene
	Lasofoxifene
Benzopyrans (fourth-generation SERMs)	Acolbifene
	EM-652 (SCH 57068)
	EM-800
	Levormeloxifene
Steroidals	EM-139
	Fulvestrant
	ICI 164,384
	ICI 182,780
	Tibolone
Phytoestrogens	
Coumestans	Coumestrol
Isoflavones	Daidzein
	Genestein
Lignans	Enterodiol
	Enterolactone
Mycoestrogens	
Resorcylic acid lactones	Zearalenone
	Zearalonol

[a] From refs. *60* and *110–114*.

study report more memory problems *(51)*. In elderly nursing home residents, women receiving tamoxifen have better cognitive skills for daily decision making, are less likely to exhibit signs of Alzheimer's disease, but are more likely to be diagnosed with depression *(52)*. This latter finding is in contrast to studies showing that in relatively younger women, tamoxifen had no effect on the risk of developing depression *(53,54)*. Currently, the effects of tamoxifen and raloxifene on cognitive function and age-related memory changes are under evaluation as a cohort within the Study of Tamoxifen and Raloxifene (STAR), an ongoing breast cancer prevention trial *(47,48)*. The same cognitive testing protocols as those used in the Women's Health Initiative Memory Study are used in this STAR sub-study *(47)*.

The effects of raloxifene on cognition and mood are presently under active investigation. One small trial in postmenopausal women found that raloxifene had no effect on cognition and mood compared to placebo after 1 yr of treatment *(55)*. Another small study evaluating quality of life in healthy postmenopausal women demonstrated that raloxifene-treated subjects achieve improved anxiety scores, no increase in depression, and no decrease in memory or concentration compared with the placebo or estrogen-treated groups *(56)*. A more recent and larger trial (Multiple Outcomes of Raloxifene Evaluation, or MORE) demonstrated that 3 yr of raloxifene treatment in postmenopausal women with osteoporosis does affect overall cognitive scores *(49)*. Some of the individual test results suggested that raloxifene may have some limited beneficial effects on attention and verbal memory *(49)*. Another study utilizing functional magnetic resonance imaging demonstrated that daily raloxifene affects brain activation patterns in regions involved in cognitive function during visual encoding in postmenopausal women *(57)*. Further studies of SERMs on cognitive outcomes are clearly indicated.

With respect to the cerebrovascular effects of SERMs in women, only raloxifene has been explored. Secondary analysis of data from the MORE trial included evaluation of stroke and TIAs in osteoporotic postmenopausal women *(58)*. In this investigation, chronic raloxifene therapy (4 yr) significantly reduced stroke risk in women designated as high risk for cardiovascular events *(59)*, but the effect was not robust in the overall study cohort *(58)*.

Animal Studies: Basal Effects in Brain

Several SERMs (raloxifene, azoxifene, and tamoxifen) have been shown to cross the blood-brain barrier in quantities sufficient to produce pharmacologic effects *(57,60, 61)*. Neurotrophic effects have been observed. Raloxifene induces neurite outgrowth in PC13 cells that express ERs *(62)*. Chronic administration of tamoxifen in OVX rats increases synaptic density in the hippocampus *(63)*. However, LY117018 does not produce increases in hippocampal dendritic spine density comparable to estrogen nor does LY117018 impede estrogen's actions in CA1 neurons *(64,65)*.

SERMs may modulate regional neurotransmitter receptor binding and density. In OVX rat brain, both tamoxifen and raloxifene increase hippocampal *N*-methyl-D-aspartate (NMDA) receptors and decrease cortical and striatal NMDA and amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor binding sites *(66–68)*. Raloxifene also increases 5-hydroxytryptamine 2A (5-HT_{2A}) receptor density and expression *(69,70)*. Similar receptor effects in the same brain regions have also been observed with 17β-estradiol *(66, 68,70)*. However, tamoxifen antagonized estrogen-induced forebrain and dorsal raphe nucleus 5-HT_{2A} receptor gene expression and binding site densities *(71)* as well as striatal dopaminergic receptor supersensitivity *(72,73)*. Overall, these studies would suggest that tamoxifen and raloxifene act as estrogen agonists and/or antagonists in several brain regions.

It is thought that estrogen's effects on mood and cognition may be mediated through alterations in serotoninergic and cholinergic neurotransmission. Both raloxifene and arzoxifene have effects similar to those of estrogen on tryptophan hydroxylase and serotonin reuptake transporter gene expression in macaque serotonin neurons *(74)*. Raloxifene and tamoxifen also have effects on a known estrogen-responsive gene product, choline acetyltransferase (ChAT) in OVX rat brain *(75,76)*. Like estradiol, raloxifene increases hippocampal ChAT activity without changing hypothalamic levels *(76)*, and tamoxifen enhances ChAT mRNA expression in basal forebrain *(75)*. Although the mechanisms(s) underlying these effects of SERMs are unclear, these studies would suggest that SERMs may have modulatory actions similar to those of estrogen on serotoninergic and cholinergic neurotransmission. Such effects could be useful in treatment of cognitive decline, Alzheimer's disease and mood disorders.

SERMs, like estrogen, can affect dopaminergic neurotransmission as well. Hypothalamic concentrations of both dopamine (DA) and norepinephrine in female rats are altered by chronic tamoxifen administration *(77)*. In OVX mice, tamoxifen increases caudate nucleus DA release to levels comparable with those observed in estradiol-treated animals *(78)*. Both tamoxifen and estradiol reduce cortical and hypothalamic DA levels in immature female rabbits, but only estradiol decreases striatal DA levels *(79)*. Interestingly, tamoxifen, but not estradiol, was shown to increase striatal DA binding in this animal model *(79)*. These studies indicate that tamoxifen differentially alters DA metabolism and release in various brain regions. Such effects could have clinical implications for diseases such as Parkinson's.

SERMs can also modify gene transcription and protein expression in the brain. For example, tamoxifen antagonizes estrogen-induced increases in progesterone receptor (PR) expression in OVX rat medial preoptic nucleus *(75)*, OVX rat hypothalamus-preoptic area *(80)*, and OVX mouse hypothalamus *(81)*. However, tamoxifen alone in OVX mice does not induce hypothalamic PR *(81)*. Raloxifene has been shown to upregulate hypothalamic ER mRNA *(82)*.

Experimental Ischemic Brain Injury/Disease

Few studies address the potential neuroprotective effects of SERMs following experimental stroke or brain injury. One recent study suggests that chronic preischemic treatment with azoxifene (10 mg/kg orally) is neuroprotective in the caudoputamen in an OVX rat focal stroke model *(83)*. The neuroprotective mechanism for azoxifen in this model does not seem to be linked to preservation of end-ischemic regional cerebral blood flow *(83)*. Most laboratory inquiries have focused on tamoxifen, which appears to be neuroprotective in ischemic brain. In male *(84)* and OVX rats *(85)*, preischemic administration of tamoxifen reduces infarct size in both transient and permanent middle cerebral artery occlusion (MCAO), respectively. Tamoxifen is equally effective when given 1 h into reperfusion after 2 h of MCAO *(84)*. While tamoxifen's protective effects appear to be independent of cerebral blood flow changes *(84)*, the mechanism of neuroprotection may be owing to decreased nitrotyrosine formation via inhibition of calcium/calmodulin-dependent neuronal NOS production *(86)*. Another potential neuroprotective mechanism is suggested by a global (four-vessel occlusion) ischemia study done in rats that examined amino acid release using a cortical cup technique *(87)*. It was thought that tamoxifen reduces ischemia-evoked amino acid efflux from cerebral cortex by inhibiting chloride channels, thereby preventing cell swelling *(87)*.

SERMs may also be beneficial in nonischemic brain injuries and diseases. Glutamate-induced toxicity can lead to cell loss in trauma and chronic neurodegenerative diseases as well as in stroke *(88)*. Tamoxifen has been shown to protect glial cells from glutamate toxicity and to stimulate cell differentiation *(89)*. In primary cultured neurons, tamoxifen inhibits glutamate-induced mitochondrial depolarization *(90)*. In a dose-dependent manner, tamoxifen suppresses extracellular hydroxyl radical generation via DA efflux induced by 1-methyl-4-phenylpyridine in rat striatum *(91,92)*. Raloxifene has also been shown to be neuroprotective in a mouse model of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine neurotoxicity *(93,94)*. The mechanism of action is thought to be mediated via an intracellular steroid receptor *(93)*. These findings would indicate a neuroprotective role for SERMs.

Conclusion

The discrepancy among observational studies, preclinical data, and large randomized clinical trials emphasizes the need for further study of the mechanisms leading to the increased incidence of stroke observed in postmenopausal women. The duration of the estrogen-deficient state is clearly an important issue. Animal studies suggest that the timing of administration is critical and may be one possible explanation for the lack of benefit seen in clinical trials. Most of the clinical literature addressing the issue of female sex hormones and cerebrovascular/CNS disorders has focused on combined HRT in postmenopausal women. This emphasis on combined hormone scenarios makes it difficult to separate out the individual as well as interactive roles of estrogen and progestins. Progestin type, formulation, and route and timing of administration are important factors to consider when determining whether progestins alone increase susceptibility or protection in cerebrovascular disease or ischemic brain injury. The role of MPA in the suspended combined HRT WHI trial has yet to be elucidated. Progestins that might be detrimental, neutral, or beneficial when solely administered might prove to be antagonistic, neutral, or synergistic when combined with estrogen. Furthermore, interest in SERMs as an alternative form of HRT has been growing in an effort to maximize estrogen's benefits and minimize its disadvantages. The widening gap between clinical trial results and experimental laboratory–based data would suggest that our understanding of the cerebral ischemic pathophysiology and of estrogen's role as a cerebroprotectant is incomplete. Continuing and future studies are still needed to optimize combined or estrogen-alone HRT options as well as the prevention/management of cerebrovascular/CNS disorders.

Acknowledgments

This work was supported by the American Stroke Association; Hazel K. Goddess Fund for Stroke Research in Women; and National Institutes of Health grants RR00163, NS33668, NR04943, and NR03521.

References

1. Writing Group for the Women's Health Initiative Investigators. (2002). *JAMA* **288(3),** 321–333.
2. Langer, R. D. (2002). *Am. J. Cardiol.* **89(Suppl. 12),** 36E–46E.
3. Paganini-Hill, A. (2001). *Maturitas* **38,** 243–261.
4. Hulley, S., Grady, D., Bush, T., et al. (1998). *JAMA* **280(7),** 605–613.
5. Grady, D., Herrington, D. Bittner, V., et al. (2002). *JAMA* **288(1),** 49–57.
6. Viscoli, C. M., Brass, L. M., Kernan, W. N., Sarrel, P. M., Suissa, S., and Horowitz, R. I. (2001). *N. Engl. J. Med.* **345,** 1243–1249.
7. Murphy, S. J., Traystman, R. J., and Hurn, P. D. (2000). *Stroke* **31,** 1173–1178.
8. Adams, M. R., Register, T. C., Golden, D. L., Wagner, J. D., and Williams, J. K. (1997). *Arterioscler. Thromb. Vasc. Biol.* **17,** 217–221.
9. Williams, J. K., Honore, E. K., Washburn, S. A., and Clarkson, T. B. (1994). *J. Am. Coll. Cardiol.* **24,** 1757–1761.
10. Herrington, D. M., Reboussin, D. M., and Brosnihan, B. (2000). *N. Engl. J. Med.* **343,** 522–529.
11. Cherry, N., Gilmour, K., Hannaford, P., et al. (2002). *Lancet* **360(9350),** 2001–2008.
12. Nelson, H. D., Humphrey, L. L., Nygren, P., Teutsch, S. M., and Allan, J. D. (2002). *JAMA* **288,** 872–881.
13. Alkayed, N. J., Harukuni, I., Kimes, A. S., London, E. D., Traystman, R. J., and Hurn, P. D. (1998). *Stroke* **29,** 159–166.
14. Carswell, H. V. O., Anderson, N. H., Clark, J. S., et al. (1999). *Hypertension* **33,** 681–685.
15. Hall, E. D., Pazara, K. E., and Linseman, K. L. (1991). *J. Cereb. Blood Flow Metab.* **11,** 292–298.

16. Hurn, P. D., Aardelt, A. A., Alkayed, N. J., et al. (2002). In: *Pharmacology of cerebral ischemia 2002*. Krieglstein, J. (ed.). Medpharm Scientific: Stuttgart, Germany.
17. Hurn, P. D. and Macrae, I. M. (2000). *J. Cereb. Blood Flow Metab.* **20,** 631–652.
18. Li, K., Futrell, N., Tovar, J. S., Wang, L. C., Wang, D. Z., and Schultz, L. R. (1996). *Stroke* **27,** 498–503.
19. Payan, H. M. and Conard, J. R. (1977). *Stroke* **8,** 194–196.
20. Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W., Bottner, M., and Rosewell, K. L. (2001). *Brain Res. Rev.* **37,** 313–319.
21. Yamori, Y., Horie, R., Sato, M., and Ohta, K. (1976). *Jpn. Heart J.* **17(3),** 404–406.
22. Zhang, Y. Q., Shi, J., Rajakumar, G., Day, A. L., and Simpkins, J. W. (1998). *Brain Res.* **784,** 321–324.
23. Liao, S. L., Chen, W. Y., Kuo, J. S., and Chen, C. J. (2001). *Neurosci. Lett.* **297,** 159–162.
24. Carswell, H. V. O., Dominiczak, A. F., and Macrae, I. M. (2000). *Am. J. Physiol. Heart Circ. Physiol.* **278,** H290–H294.
25. Dubal, D. B., Kashon, M. L., Pettigrew, L. C., et al. (1998). *J. Cereb. Blood Flow Metab.* **18,** 1253–1258.
26. Fukuda, K., Yao, H., Ibayashi, S., Nakahara, T., Uchimura, H., and Fujishima, M. (2000). *Stroke* **31,** 155–160.
27. Rusa, R., Alkayed, N. J., Crain, B. J., et al. (1999). *Stroke* **30,** 1665–1670.
28. Simpkins, J. W., Rajakumar, G., Zhang, Y. Q., et al. (1997). *J. Neurosurg.* **87(5),** 724–730.
29. Wang, Q., Santizo, R., Baughman, V. L., and Pelligrino, D. A. (1999). *Stroke* **30,** 630– 637.
30. Alkayed, N. J., Murphy, S. J., Traystman, R. J., and Hurn, P. D. (2000). *Stroke* **31,** 161–168.
31. Dhandapani, K. M. and Brann, D. W. (2002). *Biol. Reprod.* **67,** 1379–1385.
32. Green, P. S. and Simpkins, J. W. (2000). *Int. J. Dev. Neurosci*. **18,** 347–358.
33. Wise, P. M. and Dubal, D. B. (2000). *Biol. Reprod.* **63,** 982–985.
34. Harukuni, I., Hurn, P. D., and Crain, B. J. (2001). *Brain Res.* **900,** 137–142.
35. Hurn, P. D., Littleton-Kearney, M. T., Kirsch, J. R., Dharmaragan, A. M., and Traystman, R. J. (1995). *J. Cereb. Blood Flow Metab.* **15(4),** 666–672.
36. McCullough, L. D., Alkayed, N. J., Traystman, R. J., Williams, M. J., and Hurn, P. D. (2001). *Stroke* **32,** 796–802.
37. Watanabe, Y., Littleton-Kearney, M. T., Traystman, R. J., and Hurn, P. D. (2001). *Am. J. Physiol. Heart Circ. Physiol.* **281(1),** H155–H160.
38. Dubal, D. B., Zhu, H., Yu, J., et al. (2001). *Proc. Natl. Acad. Sci. USA* **98(4),** 1952–1957.
39. Sampei, K., Goto, S., Alkayed, N. J., et al. (2000). *Stroke* **31 (3),** 738–743.
40. Sawada, M., Alkayed, N. J., Goto, S., et al. (2000). *J. Cereb. Blood Flow Metab.* **20(1),** 112–118.
41. Falkenstein, E., Tillman, H. C., Christ, M., Feuring, M., and Wehling, M. (2000). *Pharmacol. Rev.* **52(4),** 513–556.
42. Linford, N., Wade, C., and Dorsa, D. (2000). *J. Neurocytol.* **29,** 367–374.
43. Kume-Kick, J. and Rice, M. E. (1998). *Brain Res.* **803,** 105–113.
44. Mooradian, A. D. (1993). *J. Steroid Biochem. Mol. Biol.* **45,** 509–511.
45. Ernst, T., Chang, L., Cooray, D., et al. (2002). *J. Natl. Cancer Inst.* **94(8),** 592–597.
46. Chang, L., Ernst, T., Poland, R. E., and Jenden, D. J. (1996). *Life Sci.* **58,** 2049–2056.
47. Ganz, P. A., Castellon, S. A., and Silverman, D. H. S. (2002). *J. Natl. Cancer Inst.* **94(8),** 547–549.
48. Resnick, S. M. and Maki, P. M. (2001). *Ann. NY Acad. Sci*. **949,** 203–214.
49. Yaffe, K., Krueger, K., Sarkar, S., et al. (2001). *N. Engl. J. Med.* **344(16),** 1207–1213.
50. Schagen, S. B., van Dam, F. S., Muller, M. J., Boogerd, W., Lindeboom, J., and Bruning, P. F. (1999). *Cancer* **85,** 640–650.
51. Paganini-Hill, A. and Clark, L. J. (2000). *Breast Cancer Res. Treat.* **64,** 165–176.
52. Breuer, B. and Anderson, R. (2000). *Women Health* **31(1),** 71–85.
53. Day, R., Ganz, P. A., Costantino, J. P., Cronin, W. M., Wickerham, D. L., and Fisher, B. (1999). *J. Clin. Oncol.* **17(9),** 2659–2669.
54. Day, R., Ganz, P. A., and Costantino, J. P. (2001). *J. Natl. Cancer Inst.* **93,** 1615–1623.
55. Nickelsen, T., Lufkin, E. G., Riggs, B. L., Cox, D. A., and Crook, T. H. (1999). *Psychoneuroendocrinology* **24,** 115–128.
56. Strickler, R., Stovall, D. W., Merritt, D., Shen, W., Wong, M., and Silfen, S. L. (2000). *Obstet. Gynecol.* **96(3),** 359–365.
57. Neele, S. J. M., Rombouts, S., Bierlaagh, M. A., Barkhof, F., Scheltens, P., and Netelenbos, J. C. (2001). *J. Clin. Endocrinol. Metab.* **86(3),** 1422–1424.
58. Barrett-Connor, E., Grady, D., Sashegyi, A., et al. (2002). *JAMA* **287,** 847–857.
59. Barrett-Connor, E., Grady, D., Sashegyi, A., et al. (2002). American Heart Scientific Sessions, abstract ID 100292.
60. Littleton-Kearney, M. T., Ostrowski, N. L., Cox, D. A., Rossberg, M. I., and Hurn, P. D. (2002). *CNS Drug Rev.* **8(3),** 309–330.
61. Zhou, W., Koldzic-Zivanovic, N., Clarke, C. H., et al. (2002). *Neuroendocrinology* **75,** 24–33.
62. Nilsen, J., Mor, G., and Naftolin, F. (1998). *Menopause* **5,** 211–216.
63. Silva, I., Mello, L. E., Freymuller, E., Haidar, M. A., and Baracat, E. C. (2000). *Neurosci. Lett.* **291,** 183–186.
64. McEwen, B. (2002). *Recent Prog. Horm. Res.* **57,** 357–384.
65. McEwen, B. S., Alves, S. E., Bullock, K., and Weiland, N. G. (1997). *Neurology* **48,** S8– S15.
66. Cyr, M., Ghribi, O., Thibault, C., Morissette, M., Landry, M., and Di Paolo, T. (2001). *Brain Res. Rev.* **37,** 153–161.
67. Cyr, M., Morissette, M., Landry, M., and Di Paolo, T. (2001). *Neuroreport* **12,** 535–539.
68. Cyr, M., Thibault, C., Morissette, M., Landry, M., and Di Paolo, T. (2001). *Neuropyschopharmacology* **25,** 242–257.
69. Cyr, M., Bosse, R., and Di Paolo, T. (1998). *Neuroscience* **83,** 826–836.
70. Cyr, M., Landry, M., and Di Paolo, T. (2000). *Neuropyschopharmacology* **23,** 69–78.
71. Sumner, B. E., Grant, K. E., Rosie, R., Hegele-Hartung, C. H., Fritzemeier, K. H., and Fink, G. (1999). *Mol. Brain Res.* **73,** 119–128.
72. Ferretti, C., Blengio, M., Ghi, P., Racca, S., Genazzani, E., and Portaleone, P. (1988). *Life Sci.* **42(24),** 2457–2465.
73. Ferretti, C., Ghi, P., Blengio, M., Gaietta, G., and Genazzani, E. (1989). *Pharmacol. Res.* **21(1),** 93–94.
74. Bethea, C. L., Mirkes, S. J., Su, A., and Michelson, D. (2002). *PNEC* **27,** 431–445.
75. McMillan, P. J., LeMaster, A. M., and Dorsa, D. M. (2002). *Mol. Brain Res.* **103,** 140– 145.
76. Wu, X., Glinn, M. A., Ostrawski, N. L., et al. (1999). *Brain Res.* **847,** 98–104.
77. Baksi, S. N., Redington, T. E., and Hughes, M. J. (1981). *Neuropharmacology* **20,** 1163– 1167.
78. McDermott, J. L., Liu, B., and Dluzen, D. E. (1995). *Brain Res.* **698,** 248–252.
79. Baksi, S. N., Hughes, M. J., and Lights, K. E. (1985). *Neuroscience* **14(4),** 1053–1059.
80. Etgen, A. M. and Shamamian, P. (1986). *Horm. Behav.* **20,** 166–180.
81. McKenna, S. E., Simon, N. G., and Cologer-Clifford, A. (1992). *Horm. Behav.* **26,** 536– 544.
82. Bryant, H. U., Glasebrook, A. L., Yang, N. N., and Sato, M. (1996). *J. Bone Miner. Metab.* **14,** 1–9.

83. Rossberg, M. I., Murphy, S. J., Traystman, R. J., and Hurn, P. D. (2000). *Stroke* **31,** 3041–3046.
84. Kimelberg, H. K., Feustel, P. J., Jin, Y., et al. (2000). *Neuroreport* **11,** 2675–2679.
85. Mehta, S. J., Dhandapani, K. M., Webb, R. C., Mahesh, V. B., and Brann, D. W. (2001). *Soc. Neurosci. Abstr.* **27,** 437.11.
86. Osuka, K., Feustel, P. J., Mongin, A. A., Tranmer, B. I., and Kimelberg, H. K. (2001). *J. Neurochem.* **76,** 1842–1850.
87. Phillis, J. W., Song, D., and O'Regan, M. H. (1998). *Brain Res.* **780,** 352–355.
88. Choi, D. W. (1988). *Neuron* **1,** 623–634.
89. Shy, H., Malaiyandi, L., and Timiras, P. S. (2000). *Int. J. Dev. Neurosci.* **18,** 289– 297.
90. Hoyt, K. R., McLaughlin, B. A., Higgins, D. S. Jr., and Reynolds, I. J. (2000). *J. Pharm. Exp. Ther.* **293,** 480–486.
91. Obata, T. (2002). *Neurochem. Res.* **27(5),** 423–431.
92. Obata, T. and Kubota, S. (2001). *Neurosci. Lett.* **308,** 87–90.
93. Callier, S., Morissette, M., Grandbois, M., Pelaprat, D., and Di Paolo, T. (2001). *Synapse* **41,** 131–138.
94. Grandbois, M., Morissette, M., Callier, S., and DiPaolo, R. (2000). *Neuroreport* **11,** 343– 346.
95. Shi, J., Bui, J. D., Yang, S. H., et al. (2001). *Stroke* **32,** 987–992.
96. Vergouwen, M. D. I., Anderson, R. E., and Meyer, F. B. (2000). *Brain Res.* **878,** 88–97.
97. Toung, T. J. K., Traystman, R. J., and Hurn, P. D. (1998). *Stroke* **29,** 1666–1670.
98. Hawk, T., Zhang, Y. Q., Rajakumar, G., Day, A. L., and Simpkins, J. W. (1998). *Brain Res.* **796(1-2),** 296–298.
99. Dubal, D. B. and Wise, P. M. (2001). *Endocrinology* **142(1),** 43–48.
100. Saleh, T. M., Cribb, A. E., and Connell, B. J. (2001). *Am. J. Physiol. Regul. Integrative Comp. Physiol.* **281,** R2088–R2095.
101. Saleh, T. M., Cribb, A. E., and Connell, B. J. (2001b). *Am. J. Physiol. Regul. Integrative Comp. Physiol.* **281,** R1531–R1539.
102. Culmsee, C., Vedder, H., Ravati, A., et al. (1999). *J. Cereb. Blood Flow Metab.* **19(11),** 1263–1269.
103. He, Z., He, Y. J., Day, A. L., and Simpkins, J. W. (2002). *J. Neurol. Sci.* **193,** 79–87.
104. Pelligrino, D. A., Santizo, R., Baughman, V. L., and Want, Q. (1998). *Neuroreport* **9,** 3285–3291.
105. Horsburgh, K., Macrae, I. M., and Carswell, H. (2002). *J. Cereb. Blood Flow Metab.* **22,** 1189–1195.
106. Jover, T., Tanaka, H., Calderone, A., et al. (2002). *J. Neurosci.* **22(6),** 2115–2124.
107. Chen, J., Adachi, N., Liu, K., and Arai, T. (1998). *Neuroscience* **87(4),** 817–822.
108. Chen, J., Weiren, X., and Jiang, H. (2001). *Anesth. Analg.* **92,** 1520–1523.
109. Sudo, S., Wen, T. C., Desaki, J., et al. (1997). *Neurosci. Res.* **29,** 345–354.
110. Davis, S. R. (2002). In: *Selective estrogen receptor modulators: research and clinical applications*. Manni, A. and Verderame, M. F. (eds.). Humana: Totowa, NJ.
111. Goldstein, J. S. and Sites, C. K. (2002). *Ageing Res. Rev.* **1,** 17–28.
112. Labrie, F., Labrie, C., Belanger, A., and Simard, J. (2002). In: *Selective estrogen receptor modulators: research and clinical applications*. Manni, A. and Verderame, M. F. (eds.). Humana: Totowa, NJ.
113. Morello, K. C., Wurz, G. T., and DeGregorio, M. W. (2002). *Crit. Rev. Oncol./Hematol.* **43,** 63–76.
114. Wardley, A. M. (2002). *Int. J. Clin. Pract.* **56(4),** 305–309.