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THE EFFECTS OF ESTROGEN AND PHYTOESTROGENS
ON FOCAL INFARCT, PTEN, AND p-AKT
EXPRESSION IN AGED RATS

By

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August 2010

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June 11, 2010

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ABSTRACT

THE EFFECTS OF ESTROGEN AND PHYTOESTROGENS ON FOCAL INFARCT, PTEN, AND p-AKT EXPRESSION IN AGED RATS

Karen Powell

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Premenopausal women have fewer and less severe strokes when compared to postmenopausal females and aged matched males. The most obvious factor that could account for the observed difference is the physiological activity of Estrogen. Numerous studies over the past two decades have investigated estrogens role as a neuroprotective agent against stroke damage. Many physiological mechanisms have been identified supporting neuroprotection; however, data also exist to suggest that estrogen may be harmful in certain situations such as those that produce high blood levels of estrogen or use estrogen in older models.

The Women's Health Initiative (WHI), a large epidemiological study, found that stroke incidence increased in women that were at least one year post menopausal and given hormone replacement therapy (HRT) containing estrogen. This conflicting information surrounding HRT has many women turning to soy-based phytoestrogens as an alternative therapy. Phytoestrogens are compounds made by plants that induce some response traditionally associated with the steroid hormone estradiol. The long term

consequences of phytoestrogen consumption are presently unknown. As more postmenopausal women choose to consume high levels of phytoestrogens either via diet or supplements, the question arises as to how estrogen and phytoestrogens interact. The current study is the first to explore the neuroprotective potential of phytoestrogens and the combined effects of estrogen and phytoestrogens against permanent focal ischemia in a middle aged model. It also explores the ability of estrogen and phytoestrogen in regulating apoptotic vs. proliferative pathways in injured neuronal tissue.

While estrogen and phytoestrogen did produce similar effects, neither was associated with neuroprotection. This study is important, however, because it adds substance to the growing body of reports that E2 does not mitigate neuronal damage due to primary infarct and it does not positively influence apoptotic pathways in aged female rats. It also provides a more realistic model for putting into context the negative effects documented in epidemiological studies such as the WHI, in which hypoestrogenic women suffered more frequent and detrimental stroke events when given estrogen replacement.

TABLE OF CONTENTS

	PAGE
ABSTRACT.....	iii
LIST OF FIGURES	viii
LIST OF TABLES.....	xi
INTRODUCTION	1
A brief history of phytoestrogens and animals	1
What is a phytoestrogen?	2
Phytoestrogen responses that are associated with 17 β -estradiol activity	4
17 β Estradiol and stroke: a conflicting history	5
Phytoestrogens and stroke	10
What is PTEN and p-Akt and how are they linked?.....	12
The anatomy of the middle cerebral artery and the expected outcomes of occlusion. .	16
Goals of the present study.....	19
MATERIALS AND METHODS.....	21
Animals.....	21
Choice of model.....	21

Experimental groups	22
Diet.....	23
Food consumption measurements.....	24
In vivo permanent cerebral ischemia	25
Histological preparation.....	26
Infarct measurements	26
P-Akt and PTEN measurements	28
Statistical analysis.....	31
RESULTS	33
Amount of phytoestrogens consumed by PE+ groups.....	33
Circulating blood estrogens levels produced via silastic implants in E2+ groups.....	35
Animal body weight between ovx and MCAo.	35
Infarct measurements.....	36
Raw infarct data in mm ³	37
Infarct data as % IL hemisphere	39
P-Akt/PTEN measurements as field area percent of the IL hemisphere	45
Cell type associated with p-Akt/PTEN	48
Effects of MCAo procedure on P-Akt/ PTEN regulation.....	51
Eliminating extraneous variables that could have contributed to non-significant infarct and p-Akt/PTEN data.....	55

CONCLUSIONS.....	59
Are physiological levels of E2 neuroprotective in reproductively senescent rats?	60
Additional effects of 17- β Estradiol that may confound infarct measurements	66
Comparing this study to the widely-referenced Dubal and Wise 2001 paper	67
Are dietary phytoestrogens neuroprotective in reproductively senescent rats?.....	72
What are the combined anti-ischemic effects of E2 and PE?.....	73
Do physiological levels of E2, dietary PEs, or ischemia alter PTEN and/or Akt expression	73
Is PTEN/Akt expression altered in neurons or astrocytes following MCAo?.....	75
Conclusion	75
REFERENCES	77
CURRICULUM VITAE.....	105

LIST OF FIGURES

	PAGE
Figure 1. The chemical structures of 17β estradiol, genistein, daidzein, and equol	3
Figure 2. The relationship between elevated PTEN and p-Akt levels on neuron survivability	13
Figure 3. Definition of terms used to describe infarct data.....	28
Figure 4. Antibody target area	29
Figure 5. No cortex damage (A) vs. cortex damage (B) in antibody target area.....	29
Figure 6 A-D. Food consumption by group by day between ovx and MCAo (mean + SE).	33
Figure 7. Food consumption across groups by day for the 7 days between ovx and MCAo (mean + SE).	34
Figure 8. Isoflavone consumption (mg/day) by PE+ groups	34
Figure 9. Plasma E2 levels in E2+ groups (mean + SE).....	35
Figure 10. Striatal infarct volume (mm^3) by group (mean + SE).	37
Figure 11. Cortex infarct volume (mm^3) by group (mean + SE).	38
Figure 12. Total infarct volume (mm^3) by group (mean + SE).	38
Figure 13. Striatal infarct volume as a % of total ipsilateral hemisphere volume by group (mean + SE).	39

Figure 14. Cortex infarct volume as a % of total ipsilateral hemisphere volume by group (mean + SE).	40
Figure 15. Total infarct volume (as a % of total ipsilateral hemisphere volume) by group (mean + SE).	40
Figure 16. Striatal infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean + SE).	43
Figure 17. Cortex infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean + SE).	43
Figure 18. Total infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean + SE).....	44
Figure 19. Field area percent of PTEN with Gfap in the IL hemisphere (mean + SE). ..	46
Figure 20. Field area percent of PTEN with NeuN in the IL hemisphere (mean + SE)..	47
Figure 21. Field area percent of p-Akt with Gfap in the IL hemisphere (mean + SE). ...	47
Figure 22. Field area percent of p-Akt with NeuN in the IL hemisphere (mean + SE)...	48
Figure 23. Immunoflourescent antibody treatment for p-Akt (red) /Gfap (green) in cortex neurons at high magnification (100x oil).....	49
Figure 24. Immunoflourescent antibody treatment for p-Akt (red) /NeuN (green) in cortex neurons at high magnification (100x oil).....	49
Figure 25. Immunoflourescent antibody treatment for PTEN (red) / Gfap (green) in cortex neurons at low magnification (20x).....	50
Figure 26. Immunoflourescent antibody treatment for PTEN (red) /NeuN (green) in cortex neurons at low magnification (20x).....	50

Figure 27. Field area percent of PTEN with Gfap in the IL hemisphere minus the CL hemisphere (mean + SE).....	52
Figure 28. Field area percent of PTEN with NeuN in the IL hemisphere minus the CL hemisphere (mean + SE).....	53
Figure 29. Field area percent of p-Akt with Gfap in the IL hemisphere minus the CL hemisphere (mean + SE).....	53
Figure 30. Field area percent of p-Akt with NeuN in the IL hemisphere minus the CL hemisphere (mean + SE).....	54
Figure 31. Correlation between body weight at time of MCAo and resulting cortex infarct volume.....	55
Figure 32. Correlation between body weight at time of MCAo and resulting total infarct volume.....	56
Figure 33. Correlation between mg isoflavones and resulting cortex infarct volume.....	56
Figure 34. Correlation between mg isoflavones and resulting total infarct volume.....	57
Figure 35. Correlation between E2 levels and resulting cortex infarct volume.....	57
Figure 36. Correlation between E2 levels and resulting total infarct volume.....	58

LIST OF TABLES

	PAGE
Table 1. Experimental groups.....	23
Table 2. Composition of soy protein in the PE- and PE+ diets as made by the Solae company.....	24
Table 3. Serial antibody treatments.	30
Table 4. Changes in body weight between ovx and MCAo.	36
Table 5. Mean striatal, cortical and total infarct measurements in mm ³ (+ SE) by group.	39
Table 6. Resulting mean + SE of striatal, cortex, or total infarct volume (% IL hemisphere) for four treatment groups.	41
Table 7. The presence of infarcted cortex measured as % IL hemisphere by treatment group.....	41
Table 8. Resulting mean + SE of striatal, cortex, or total infarct volume (as a % of total IL hemisphere volume) by group when cortex infarct is greater than zero.	42
Table 9. Number of rats in each group by percent of cortex damage (%IL).	45
Table 10. Field area percents of p-Akt/PTEN with Gfap/ NeuN in the IL hemisphere (mean + SE).	48
Table 11. Field area percents of p-Akt/PTEN with Gfap/ NeuN in the IL hemisphere minus the CL hemisphere (mean + SE).	54

Table 12. Comparison of present study design to Dubal and Wise, 2001	70
Table 13. Comparison of results of present study to Dubal and Wise, 2001.....	71

INTRODUCTION

A brief history of phytoestrogens and animals

Every good sheep farmer knows that one should never graze animals on pasture dominated by dinninup, dwalganup, and yarloop (Blood and Radostits, 1989). High consumption of these subterranean clovers can lead to infertility and a marked decrease in lambing percentages from the normal average of 80% down to 30% (Blood and Radostits, 1989). This phenomenon, first published in a 1946 edition of the Australian Veterinarian Journal by Bennets et al., is today a standard entry in large animal veterinary medicine text books. It is classified as “*poisoning caused by miscellaneous legumes*”. The etiology of the infertility is the high content of biologically active estrogenic chemicals, termed phytoestrogens (PE), found in the leguminous plants. The presence of estrogen-like chemicals in leguminous plants holds wide-spread implications for animals that ingest them. Some implications are advantageous, such as the example of California quail (*Callipepla californica*) switching feeding preference to legumes high in PE to reduce fertility in times of food shortage (Leopold et al., 1976), while other examples of artificial introduction of legumes in the diet have proven to be a disadvantage. Cheetahs have decreased fertility and increased liver disease when fed soy-based diets in captivity (Setchell et al., 1987). A study by Thigpen et al. (1987) showed that laboratory rodents fed a widely-used commercially available chow

had uterine weight gains similar to animals fed diets containing 6 parts per billion diethylstilbestrol, a pharmaceutical estrogen, suggesting that numerous scientific studies might have been inadvertently influenced by the presence of PE.

What is a phytoestrogen?

Defining a PE requires one to first consider the parallel concept of “what exactly is an estrogen”? The traditional dictionary and entry level anatomy class definition of an estrogen is any of several steroid hormones produced chiefly by the ovaries and responsible for promoting estrus and the development of female secondary sex characteristics (Merriam Webster Dictionary, 2005; Marieb and Hoehn, 2009). As we learn more about the wide array of physiological functions affected by estrogens, the definition has become increasingly more complex. We now know that estrogens have been found to directly or indirectly affect almost every system in the human body. They function at the classic receptor level to upregulate gene transcription and in many non-genomic ways. It isn't just about the female in heat anymore...and it really never was. The difficulties of defining estrogens carry over to trying to accomplish the same task with PE. In a 2001 publication, McLachlan defined PE as compounds made by plants that induce some responses traditionally associated with the steroid hormone estradiol. But as new estrogen responses are identified, the concept of PE must also continue to expand. To date, numerous plant compounds are widely accepted as PE, including the isoflavones genestein, daidzein, and the daidzein metabolite equol, which are studied in this work.

Figure 1. The chemical structures of 17 β estradiol, genistein, daidzein, and equol

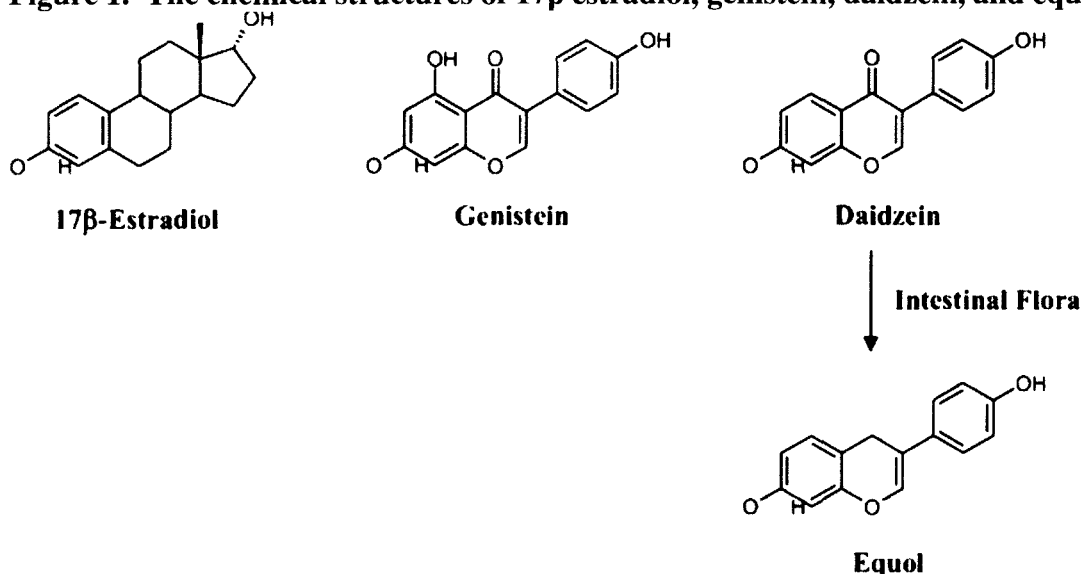


Figure from Zhao, L. et al. *Endocrinology* 2009;150:770-783. Reprinted with permission.

PE share a similar chemical structure (see Figure 1), specifically a phenolic group, with vertebrate estrogens, such as 17 β -estradiol (E2). These chemicals are found in leguminous plants in which one function is to initiate signaling pathways between the plant and nearby nitrogen-fixing bacteria (reviewed by McLachlan, 2001). These signals serve to recruit bacteria to the plant root hairs where they induce nodule formation for colonization. The bacteria produce NodD proteins, which interact with the phytochemicals to activate transcription of the genes necessary for the classic “symbiotic nitrogen-fixing relation” in which the host plant supplies CO₂ for the bacteria and in turn the bacteria transforms atmospheric nitrogen into a usable form for the plant (reviewed by McLachlan, 2001). This system has many similarities with the vertebrate endocrine system, in which chemicals are secreted, bind to receptors, and regulate transcription (McLachlan, 2001). The substrate on which "estrogenic" chemicals can act is highly

conserved across vertebrate taxa, with estrogen receptors having been found in mammals, birds, reptiles, and fish, and PE in particular have been acting on this substrate throughout much of vertebrate evolutionary history (McLachlan, 2001).

Phytoestrogen responses that are associated with 17 β -estradiol activity

There are two types of classic estrogen receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Most PEs bind to both but have higher binding affinity for ER β (Kuiper et al., 1997). Both ERs are widely expressed in different tissue types; however, there are some notable differences in their expression patterns. Systemically, ER α is more prevalent in tissues traditionally associated with female reproduction including the endometrium and breast, while ER β is more prevalent in other organs such as heart, lung, and kidney (Babiker et al., 2002; Morani et al., 2006). Both types of receptors have been identified in the brain, with ER α predominately located in the hypothalamus while ER β is located in extrahypothalamic brain regions such as the cerebral cortex and hippocampus (Shughrue and Merchenthaler, 2001). Dubal et al. (2006) demonstrated that ER α and ER β are expressed differently after an ischemic event, with ER α dramatically increased in the cerebral cortex. They also found that E2 was unable to protect the cortex in the absence of ER α receptors, suggesting that E2-mediated effects occur exclusively via ER α . Schreihofer (2005) found different results for PEs. At physiological concentrations, genistein and daidzein stimulated both ER α - and ER β -dependant transcription in cell culture, but these compounds were more active at ER β . Interestingly, the PEs did not antagonize estrogen activity at physiological concentrations. Using hippocampal neuron cultures to study the effects of E2 and PE on

Alzheimer's disease and memory function in postmenopausal women, Zhao et al. (2002) found that PEs exerted a neuroprotective effect at the plasma membrane but not at the level of neuronal mitochondrial viability when exposed to glutamate. In contrast, E2 afforded enhanced neuroprotection at both levels (Zhao et al., 2002). E2 also promoted neurite outgrowth and synaptogenesis while PE did not, leading the researchers to conclude that while PEs exert some neuroprotective effects, PE and E2 are not functional equivalents (Zhao et al., 2002). Since PEs bind both types of receptors (Kuiper et al., 1997), it is plausible that the presence of PE could be antagonistic to the effects of E2, as both would compete for the same ER α receptor. It is also plausible that through the action of ER β , PEs could produce a partial antagonist effect over that of E2 alone. A study of the combined effects of E2 and PEs will prove insightful.

17 β Estradiol and stroke: a conflicting history

Premenopausal women have fewer and less severe strokes; a decreased risk of mental and cardiovascular disease; experience increased cardiac output, and increased systemic blood flow in the face of decreased resting vascular tone and myogenic responses; but suffer higher incidences of uterine and breast cancer when compared to postmenopausal females and aged matched males as appropriate (Prelevic et al., 2002; Naftolin et al., 2004; Grodstein and Stampfer, 1995; Iafrafi et al., 1997; Karas and Hodgins, 1999; Ohkura, 1994; Wise et al., 2001; Dubal and Wise, 2001; Chen et al., 1999; Rau et al., 2003; Choi et al., 2004; Henderson 1993 and 1997; Nabulsi et al., 1993; Mendelsohn and Karas, 1999). The cardiovascular and neurologic advantages afforded to young females diminishes with the onset of menopause (Kolominsky-Rabas et al,

2001; Sacco et al., 1998; Stegmayr et al., 1997). The most obvious factor that could account for the observed difference is the physiological activity of E2. Numerous studies over the past two decades have investigated the role of E2 as a neuroprotective agent against stroke damage. The initial studies were promising. In 1988, Paganini-Hill et al. published some of the first data suggesting neuroprotection in women. Since 1997, a vast amount of experimental literature has been published on this subject, including the widely-referenced 2001 Dubal and Wise paper entitled, *Neuroprotective effects of estradiol in middle aged female rat*, which demonstrates profound neuroprotective effects of E2 at low and high doses in both young and middle aged female rats following an ischemic event. Many physiological mechanisms have been identified supporting neuroprotection; however, data also exist to suggest that E2 may be harmful in certain situations. Harmful side effects seem to be consistently associated with methods that produce high blood levels of E2 or use E2 in older models (see below). Several mechanisms, including increased excitotoxicity, hyper-immune responses, and miscellaneous age-specific changes have been reported that may help explain this phenomenon.

High levels of E2 and/or estrogen in older models have been demonstrated to promote neuron excitotoxicity. Brann et al. (1993) found an increase in mRNA for N-methyl D aspartate (NMDA) glutamate receptors in the cerebral cortex of rats following E2 treatment. While Weiland et al. (1992) showed that E2 upregulates NMDA binding sites, Foy et al. (1999) found that E2 enhances NMDA receptor-mediated EPSPs, indicative of increased seizure activity, when concentrations of E2 reach 10 nmol. Gu et

al. (1996) described an E2-mediated increase in kainite-induced currents via activation of cyclic AMP pathways, suggesting that decreased E2 may correlate with decreased brain activity. E2 has also been reported to decrease the uptake of L-glutamate by astrocytes, thus contributing to excitotoxicity (Sato et al., 2003).

High levels of E2 and/or other estrogens in older models may lead to hyper-immune responses. Nordell et al. (2003) reported that E2 given to older female rats exacerbated NMDA-induced brain inflammation as compared to younger counterparts. Sohrabji (2005) also found that E2 treatment in young animals decreased the inflammatory cytokine interleukin 1B (IL-1B) in response to brain injury while the same treatment in aged females increased IL-1B secretions dramatically. Looking at systemic immune responses in whole blood to lipopolysaccharide (LPS) injections, they also found that the aged had a heightened response to LPS and that E2 replacement further increased TNF- α expression (Sohrabji, 2005).

E2 produces miscellaneous age-specific changes in older models. One study found that E2 administration to aged non-overiectomized rats did not stimulate neurotrophin, a growth factor that promotes neuronal growth and repair, even though E2 has been shown to have this effect in surgically menopausal young females (Jeziarski and Sohrabji, 2001). Similar effects were also reported for neurotrophin receptor trkA in which E2 administered to young surgically menopausal animals upregulated trkA mRNA and subsequent number of trkA receptors while E2 administered to the aged, naturally menopausal animal had no effect (Gibbs, 2003; Sohrabji, 2005). Sohrabji (2005) found

that the blood brain barrier (BBB) is more permeable in reproductively senescent animals and that E2 replacement has no effect on this phenomenon in the aged except in the area of the hippocampus, where it decreases BBB effectiveness even more. Aged rats have a decrease in the number of hippocampal neurons that express ER α (Adams et al., 2002) and overall ER α distribution has been shown to change from a nuclear to a more cytoplasmic localization in older human females (Hestiantoro and Swaab, 2004).

Physicians are currently discouraging the use of hormone replacement therapy (HRT) for peri-menopausal and post-menopausal women. This recommendation is based largely upon the findings of the Women's Health Initiative (WHI) in which breast cancer, dementia, stroke, and cardiovascular morbidity and mortality increased in women that were chronically hypoestrogenic (at least one year post menopausal) and given a combination HRT of estrogen and progestin (Alving, 2004; Wassertheil-Smoller et al., 2003). A second phase of the WHI, utilizing estrogen only, unopposed by progesterone, in women that had previously had hysterectomies, found that cardiovascular risk was unaffected while stroke risk increased (Alving, 2004). Since unopposed estrogen has long been associated with uterine cancer, only women with no uterus were included in the study (Alving, 2004). The results of the WHI contradicts the large observational study known as the *Nurses' Health Study* in which women in the menopausal transitional phase were given HRT and experienced lower cardiovascular morbidity and mortality rates after the initial year of therapy (Grodstein, 2001). The WHI also contradicts the vast scientific literature available concerning the numerous benefits of E2 on an array of neurological functions in animal models (Wise et al., 2001, 2005).

What accounts for the disparity between the Nurses' Health Study, the *in vitro* and *in vivo* animal studies, and the WHI? The answer may lie, at least in part, in the model. The WHI studied women that were aged and had undergone natural menopause, in many cases years before the study; however, the Nurses' Health Study examined women that were perimenopausal. Many of the *in vivo* animal studies reporting positive benefits were done on young females, on males, with non-physiologic levels of E2, or with a very acute exposure. Detrimental effects are often reported with high supraphysiological levels of E2 or when models have experienced a period of hypoestrogenism. A more realistic animal model of the peri and menopausal human would be to use aged female retired breeder rats that had experienced the hormone fluctuations of pregnancy, lactations, and irregular cycling common to natural aging. Dubal and Wise (2001) used such a model. They demonstrated decreased infarct damage from middle cerebral artery occlusion in 9-12 month-old female Sprague-Dawley rats when given E2 replacement via slow release silastic capsule. Circulating estrogen levels were equivalent to those of a normal estrus cycle. These results conflict with those of the WHI. Because the Dubal and Wise model was profoundly accepted and widely cited in the literature at the time of experimental design, the current study mirrors it. The current study is based upon estrogens being neuroprotective with the novel aspect centering on whether or not PE alone or in combination with estrogen were neuroprotective in a like fashion.

Phytoestrogens and stroke

The conflicting information and the current FDA guidelines for limited and guarded estrogen and progestin treatment has resulted in many women receiving no HRT and instead turning to soy-based PE as an alternative therapy (Alving, 2004; Clarkson et al., 1998 and 2001; Clarkson, 2002; Davis and Simpson, 1999; Huntley, 2004). The long term consequences of PE consumption are presently unknown. Soybeans contain mg/g amounts of isoflavones, but these levels are highly variable depending upon strain and growth conditions (Burow et al., 2001; Davis and Simpson, 1999). Reports of correlations between high soy intake and decreased neoplastic incidence and cardiovascular risk have led to increased interest in the potential health benefits of soy (Clarkson, 2002). For example, an epidemiological study of Asian women showed a direct correlation between soy consumption and a reduction in breast cancer incidence (Lee et al, 1991). These positive effects were negated when Asian women adopted a western diet (Dixon, 2004). Other studies have focused on PE as an alternative for postmenopausal HRT (Alving, 2004; Clarkson, 2002; Clarkson et al., 1998 and 2001; Davis et al., 1995; Huntley, 200; Newton et al., 2002). Most PE studies focus on cardiovascular benefits (Anthony et al., 1997; Clarkson, 2002; Clarkson et al., 2001). An extensive literature search revealed very few published papers concerning the effects of PE on ischemic stroke. Most of the available literature utilizing female rats and thus, most pertinent to this study, is from the lab of D.A. Schreihofer. In 2005, this group compared the effects of a high PE diet (600 mg soy isoflavones/gram of food) with estrogen replacement pellet (0.25 mg 17 β -estradiol) in ovariectomized female rats (225-250 grams). Rat age was not specified, but extrapolating from standard growth curves for

Sprague-Dawley rats supplied by Harlan Laboratories (Indianapolis, Indiana) age can be estimated at twelve to fifteen weeks. The animals were exposed to the diet for a total of two weeks. The high PE diet was designed to produce blood levels equivalent to a prototypical Asian diet, while the estrogen pellet was chosen to deliver estrogen levels equivalent to those of proestrus (80-140 pg/ml) (Scheihofer et al., 2005). One week post ovariectomy the animals underwent permanent middle cerebral artery occlusion (pMCAo) via intraluminal occlusion and were sacrificed 24 hours later. Brains were sectioned into 2mm slices using a brain matrix and stained with triphenyltetrazolium chloride (TTC). Measured E2 blood levels at the time of sacrifice were 159 pg/ml +/- 21. Their data showed that the animals that had been ovariectomized, not provided estrogen replacement, and placed on an isoflavone-free diet (PE-/E2 -) suffered the largest strokes. The animals with estrogen replacement and on an isoflavone-free diet (PE -/E2+) had the least amount of stroke damage. The animals on the high PE diet (PE +/E2-) with no estrogen replacement had stroke damage intermediate to the other two groups. The PE+/E2- and PE-/E2+ groups were significantly different from the PE-/E2 - group. The study did not include a combination PE+/E2+ group. Through this experiment, Schreihofner et al. (2005) were the first to illustrate a beneficial role for PE against focal ischemia; however, the authors also point out that, due to the lessened protection offered by PE alone, PEs may not be as effective as estrogen in reducing infarct size. In 2006, Burguete et al. found that 8 week-old male rats that had been fed a high PE diet since weaning had a decreased infarct following transient MCAo. The remaining literature concerning the effects of PE on neuronal damage has been done *in vitro* using rat embryonic day 18 cortices. Results suggest that soy isoflavones can reduce cell death in

cultured cells exposed to glutamate toxicity (Zhao et al., 2002) and calcium misregulation by thapsigargin (Linford and Dorsa, 2002). PE results were similar to those seen with E2.

The claims of soy as a “natural” antineoplastic and cardioprotective agent, as well as an E2 replacement, has led to the availability of many over-the-counter, non-FDA regulated, soy based products. Soy-rich diets, especially those containing large amounts of tofu as are consumed in a traditional Asian diet, contain approximately 150 µg of daidzein and 250 µg of genistein per gram of soy protein (Dixon, 2004). Once ingested, the soy isoflavones genestein and daidzein are absorbed or daidzein is converted to equol by intestinal microbes. Equol is significantly more estrogenic than daidzein (Dixon, 2004). Soy consumption results in measurable blood and urine concentrations of these PE (Dixon, 2004). As more postmenopausal women on hormone replacement therapy adopt a soy based diet, the question arises as to how E2 and PE interact. The current study is the first to explore the neuroprotective potential of PE and the combined effects of estrogen and PE against permanent focal ischemia in a middle aged model.

What is PTEN and p-Akt and how are they linked?

PTEN (the phosphatase and tensin homolog deleted from human chromosome 10) is a protein tyrosine phosphatase that is known as a “tumor suppressor” gene because it suppresses cell proliferation and promotes programmed cell death via apoptosis (Omori et al., 2002). This action is desirable when cell proliferation may be unregulated, such as in epithelial neoplasias; however, it is unprotective and undesirable for amitotic cells such as neurons. In it’s active form, which is the non-phosphorylated state (Choi et al.,

2005), PTEN acts as a phosphoinositide 3 phosphatase which negatively regulates the phosphoinositide 3-kinase (PI3K) pathway by catalyzing the degradation of phosphatidylinositol (3,4,5) triphosphate, yielding phosphatidylinositol (4,5) diphosphate (Waite et al., 2005). Phosphatidylinositol (3,4,5) triphosphate is required for the activation of the pro-proliferative p-Akt dependent pathway (Waite et al., 2005). Akt is a serine threonine protein kinase which when activated by phosphorylation (p-Akt) promotes cell survivability in neurons via several mechanisms, including the inhibition of death-inducing proteins such as glycogen synthase kinase 3 (GSK – 3) (Kim et al., 2001 and Alloatti et al., 2004) and the mitogen activated protein kinase/ c-jun N terminal kinase (MLK3/JNK3) signaling pathways (Zhang et al. 2006). An increase in p-Akt has also been shown to upregulate Bcl-2 protein in neurons (Pugazhenthii et al., 2000). Bcl-2 is antiapoptotic and prevents neurons from undergoing programmed cell death from ischemic injury (Wise et al., 2001; Choi et al., 2004; Martinou et al., 1994). In the case of neuronal damage, because p-Akt prolongs cell survivability, higher levels are protective.

Figure 2. The relationship between elevated PTEN and p-Akt levels on neuron survivability

↑ PTEN = ↑ neuron apoptosis = ↓ neuron survivability

↓ PTEN = ↑ p-Akt = ↑ neuron survivability

The active form of Akt is well accepted to be the traditional phosphorylated form (p-Akt). Burgering and Coffey (1995) and Franke et al.(1995) both report that phosphorylation of residues Thr-308 and Ser -473 are required for Akt activity. Surprisingly, the active form of PTEN is actually the unphosphorylated form. Phosphorylation of PTEN regulates its conformational changes, thereby suppressing the activity of PTEN by controlling the recruitment of PTEN into the PTEN-associated complex (Adey et al, 2000; Vazquez et al., 2001). Choi et al. (2005) added credibility to the hypothesis that p-PTEN is the inactive form, as they found that the activation of Akt in the post-ischemic hippocampus of adult male rats following global ischemia mirrored that of p-PTEN expression. Since activated PTEN inhibits activated Akt, the two forms should not increase together.

Choi et al. (2004) showed that pharmaceutical doses of E2 greatly reduced ischemic damage after transient MCAo in adult male Sprague-Dawley rats. Animals administered E2 at 4 mg/kg and 10 mg/kg intraperitoneally (ip) 24 hours prior to and 5 minutes after reperfusion had significantly less infarct damage than did the control animals. Interestingly, 1 mg/kg E2 administered ip did not significantly decrease infarct damage over that seen in the control animals. Infarct damage paralleled measured p-Akt levels in the ipsilateral cortex with active Akt levels significantly increased in the groups receiving 4 and 10 mg/kg E2 over those receiving 1mg/kg E2 and control. This suggests that the protective effects of E2 seen in this study may be ascribed to the ability of E2 to upregulate Akt signaling pathways after ischemic injury. The investigators also looked at PTEN and p-PTEN levels, but reported the results as p-PTEN being the biologically

active form. Their results actually showed a disconnected relationship between active PTEN and p-Akt, as both increased together in a dose proportionate manner. Although the PTEN data are somewhat confusing, this body of work does provide evidence for E2, albeit at very high doses, regulating pathways that ultimately determine cell fate. This study raises the question of whether physiological, as opposed to pharmacological, levels of estrogen in female animals could also mitigate stroke damage by regulating Akt/PTEN pathways. Studies by Omori et al. (2002) and Choi et al. (2005) added an additional factor to be unraveled. Omori et al. (2002) found that the transient focal MCAo procedure itself upregulated PTEN activity over controls in the ischemic cortex. Choi et al. (2005) found that the phosphorylated forms of PTEN and p-Akt were upregulated in the hippocampus following a transient global ischemia procedure, which leads to another question addressed within the present study concerning what changes are actually due to treatment vs. due to the MCAo procedure? In an attempt to answer this question, protein measurements were taken from both ipsilateral and contralateral hemispheres, the contralateral hemisphere providing information about the systemic treatment while the ipsilateral, subjected to unilateral MCAo, provides information concerning the insult in conjunction with the interventions. A previous study has reported that both p-PTEN and PTEN are upregulated in the post ischemic brain with expression occurring predominately in neuronal cells of the cortex, but not in astrocytes (Omori et al., 2002). A different study reported that upregulation of PTEN activity occurs in astrocytes, not neurons, of the hippocampus after being subjected to transient ischemia (Choi et al., 2005) and kainic acid injection (Cho et al., 2002). This brings into question the cell type responsible for PTEN upregulation in response to ischemia.

Several studies performed in cell cultures are relevant to the work presented in this dissertation. Honda et al. (2000, 2001) and Wilson et al. (2002) have demonstrated that neuroprotection exerted by estrogen in primary culture cells is attributed to the ability of E2 to activate the PI3 kinase pathway. Supportive data from Waite et al. (2005) demonstrated a three-fold increase in PTEN levels when breast cancer cells were stimulated with 0.1nm genestein and, as would be expected, p-Akt levels decreased. Although these studies utilized neoplastic breast cells in culture, they are relevant to the current study because they illustrate that E2 and genestein affect the PTEN/p-Akt pathway, albeit in an opposite manner, suggesting that E2 increases p-Akt while PE has the opposite effect of increasing PTEN and thus decreasing p-Akt. To add to the possible antagonist effects of E2 and PE, Stoica et al., 2003 found that E2 increased p-Akt levels in breast cancer cell lines by 9-fold within 10 minutes: an action that was blocked by genestein.

Very few studies have directly looked at the roles of PE in regulating apoptotic vs. proliferative pathways. *In vivo* studies of aging female systems utilizing a chronic exposure to realistic levels of both E2 and dietary PE and their effects on the regulation of apoptotic/proliferative pathways is merited. Such a study of the combined effects of physiological levels of E2 and dietary soy PE may also prove insightful.

The anatomy of the middle cerebral artery and the expected outcomes of occlusion.

To fully comprehend the MCAo procedure, a good understanding of the MCA itself is merited, as infarct studies may be adversely affected by naturally occurring

vascular anomalies in the anatomy of the MCA and the point at which it is actually occluded. The main trunk of the middle cerebral artery originates in the circle of Willis and proceeds dorsally (Rubino and Young, 1988). From this point the anatomy is quite variable. Rubino and Young (1988) attempted to define the branching patterns of the MCA in Sprague-Dawley rats. They found that the MCA bifurcated into two major branches, the frontal and parietal, in 95% of animals studied. In addition to these predictable branches, two minor branches were identified in a smaller percentage of animals. The anteroventral pyriform branch was identified in 43% of animals while the posteroventral temporal branch was identified in 32% of animals. Seventeen percent had all four branches, 31% had the two major branches and one minor branch, 48% had only the two major branches and the other 5% only had a parietal branch. They found that when stained with Evans blue, selective occlusion of the frontal, parietal, or pyriform branches produced a cortical lesion that was conical in shape with deep apexes. Interestingly, selective occlusion of either the frontal or parietal branches resulted in tertiary lesions located outside the region supplied by the branch in 56% and 86%, respectively, of the animals studied. This study raises two critical questions concerning the MCAo procedure. Do the anatomical differences in arterial branching lead to variability in resulting infarct? Is the high percentage of tertiary lesions noted due to other processes, outside of primary striatal ischemia and cortex apoptosis, that are concurrently induced by the MCAo procedure?

The present study attempts to standardize primary infarct damage by studying infarct size limited to the brain areas served by the parietal and frontal branches, thus

eliminating the anatomical variance of the minor branches. To standardize occlusion, the intraluminal suture used had a consistent preformed bulb on the end, insertion length was measured according to body weight, and the internal carotid artery was ligated to anchor the intraluminal suture and decrease collateral perfusion.

The frontal and parietal branches of the middle cerebral artery collectively serve 60-70% of the cortical surface of each hemisphere (Rubino and Young, 1988). The territory served has undefined rostral limits of “much of the frontal cortex including the piriform cortex”, caudal limits of “part of the occipital cortex”, and most everything in between (Rubino and Young, 1988). Due to the published descriptions of areas served by the two major branches of the MCA and the well-documented fact that the intraluminal MCAo procedure does not predictably induce damage to the hippocampus, which requires a four-vessel occlusion including the vertebral arteries, the area of infarct damage measured in this study was limited to Bregma +2.2 through Bregma -2.8. Bregma +2.2 lies within the frontal cortex and has a well defined piriform cortex. Bregma -2.8 lies within the occipital lobe with minimal hippocampus but includes caudoputamen, internal capsule, and anterior thalamus, which were described by Longa et al. (1989) as areas rendered ischemic by intraluminal MCAo. To add credence to the choice of using these coordinates, Rau et al. (2003) stated that this region contained the area that undergoes injury with MCAo. Thus, the data should reflect focal cerebral necrosis to the striatum directly served by the two main branches of the MCA and the secondary results of ischemia to the overlying cortex. The cortex, while hypoperfused, has the benefit of collateral circulation from the anterior and posterior cerebral arteries.

Because of the differences in perfusion, the cells of the cortex undergo programmed cell death rather than necrosis (Rau et al., 2003). Given the area of the brain selected for study, one should expect to see uniform, near complete, striatal infarct across all experimental groups, with any variation due to treatment manifested as spared cortex. The current study aimed to clarify the neuroprotective potential of E2 and PE alone and in combination to selective neuronal injury associated with primary occlusion of the striatum served by the middle cerebral artery and subsequent apoptosis of overlying cortical neurons in middle aged females.

Goals of the present study

The present study was designed to test a more realistic model of the peri and menopausal female than many previous studies cited above by using aged female retired breeder rats that had experienced the hormone fluctuations of pregnancy, lactations, and irregular cycling common to aging. The hypothesis of this study was that *physiological levels of 17 β - estradiol (E2) and dietary soy phytoestrogens (PEs) produce similar but not additive effects on primary ischemic damage and apoptotic pathway regulation by PTEN/Akt, producing an outcome of neuroprotection in aging female systems*. To test this hypothesis, six specific questions were addressed:

1. Are physiological levels of E2 neuroprotective, as measured by infarct size after MCAo, in reproductively senescent rats?
2. Are dietary PE neuroprotective in reproductively senescent rats?
3. What are the combined anti-ischemic effects of E2 and PE?

4. Do physiological levels of E2 and/or dietary PE treatment alter PTEN and/or p-Akt expression compared to controls in the MCAo model?
5. Is PTEN/p-Akt activity altered in neurons or in astrocytes following MCAo?
6. Does the MCAo procedure upregulate PTEN/p-Akt activity as measured by comparing IL hemisphere protein levels to those of the corresponding CL hemisphere?

This study is important because it is the first to contribute to the understanding of realistic levels of PE alone and in combination with E2 to mitigate occlusive stroke damage in aged reproductively senescent rats. It also attempts to isolate primary ischemic damage from other secondary factors by limiting the area of the brain used for data to only the area known to be served by the middle cerebral artery in 98% of rats. In addition, it explores the ability of PE and E2 to regulate the PTEN/Akt pathway in a middle aged rodent model and attempts to identify cell type associated with PTEN/Akt proteins. Lastly, it will provide insight into whether or not the pMCAo procedure itself upregulates apoptotic proteins.

MATERIALS AND METHODS

Animals

Middle aged (10-12 months, 244-339 g body weight) female non-pregnant retired breeder Sprague-Dawley rats ordered from Harlan (location) were maintained in a 12 hours light/12 hours dark photoperiod (lights on 6AM). Animals were given a two week acclimation period after arrival prior to any experimentation, during which time they were fed a PE-reduced diet provided by the laboratory of Dr. Thomas Clarkson (Wake Forest University) using soy protein provided by the Solae Company (St. Louis, MO), made by subjecting soy protein to alcohol extraction to remove isoflavones, and provided free access to water. All procedures were approved by the University of Louisville IACUC.

Choice of model

Since rats do not have a menstrual cycle they technically cannot be said to enter menopause; however, they do experience age-related changes in their four-day estrous cycle (Hsueh et al., 1979; Lu et al., 1979). At 10-12 months of age, rats enter a period of irregular estrus cycling followed by a period of anovulatory constant estrous that transitions to a persistent diestrus state at approximately 25 months of age (Lu et al., 1979). Ovariectomizing 10-12 month old rats allow for the control of endogenous circulating estrogen (E2) while offering a naturally aged model. Additionally, retired

breeder rats may offer a more realistic model for women that have experienced the extreme hormonal changes associated with pregnancy and lactation as a part of their reproductive history over male rats or very young female rats with acute surgically induced hypoestrogenism. Initially, Fisher rats were proposed for use in this study due to a readily available source of retired breeders; however, they are unsuitable for the procedure due to their cerebrovascular anatomy (Dittmar et al., 2006). Sprague-Dawley rats were chosen instead to more closely parallel the Dubal and Wise (2001) and Scheihofer et al. (2005) studies.

Experimental groups

Each experimental group consisted of thirteen animals. All animals were bilaterally ovariectomized (ovx) under isoflurane anesthesia to eliminate endogenous E2 production and then implanted with a silastic capsule made by filling 1.47 mm x 1.96 mm silastic tubing (Dow Corning) with either microfiltered sesame oil (E2- group)(Sigma) or 189 ug/ml of 17 β -estradiol (Sigma) dissolved in microfiltered sesame oil (E2+ group). Each capsule contained 0.6ml of oil or E2 and was sealed at each end with medical grade silicone sealant. Each animal received two capsules implanted subcutaneously in the scapular region. This dose is consistent with previous studies where it is reported to produce physiological circulating E2 blood levels of approximately 20 pg/ml in Sprague-Dawley rats (Dubal and Wise, 2001; Rau et al., 2003). Immediately post ovx, buprenorphine (0.5 mg/kg) was administered via a subcutaneous route for analgesia. Rats were provided with a diet of intact soy protein (PE+ group) or a diet of soy protein that had been subjected to alcohol extraction of phytoestrogens (PE- group). All

experimental animals were on the respective diet for 1 week after ovx. Fresh food was provided daily along with free access to water.

Table 1. Experimental groups.

Denotation	Silastic implant	Diet	Role
E2-/PE-	Oil (-)	Soy -	Negative control
E2+/PE-	E2 (+)	Soy -	E2 effects only
E2-/PE+	Oil (-)	Soy +	Phytoestrogens effects only
E2+/PE+	E2 (+)	Soy +	Combined effects of E2 and Phytoestrogens

Diet

Rats were provided with a diet of 25 grams/day containing either intact soy protein (PE+ group) or a diet of soy protein that had been subjected to alcohol extraction of phytoestrogens (PE- group). Both diets were prepared by the laboratory of Dr. Tom Clarkson (Wake Forest University) using soy protein provided by the Solae Company (St. Louis, MO), which also provided analysis of isoflavone content (Table 2). The final diet made by the Clarkson lab consisted of 14.83% of the PE+ or PE- soy protein. The PE+ diet was estimated to provide an intake of 144 mg of PE/1800 calories per day. This dosage is equivalent to human intake when consuming a diet high in soy protein and has been used successfully in several studies of rats (Clarkson et al., 1998; Clarkson et al., 2001; Clarkson, 2002). Animals were provided fresh food daily along with free access to water.

Table 2. Composition of soy protein in the PE- and PE+ diets as made by the Solae company.

Composition	PE -	PE+
Protein (%)	86.8	87.6
Calcium (mg/100g)	110	183
Ash (%)	4.23	4.17
Fat (%)	2.25	1.91
Moisture (%)	4.4	3.7
Phosphorus (mg/100g)	740	807
Potassium (mg/100g)	1240	644
Sodium (mg/100g)	1090	824
Genistein-containing compounds ($\mu\text{g/g}$ protein)	30	2090
Daidzein-containing compounds ($\mu\text{g/g}$ protein)	30	900
Glycitein-containing compounds ($\mu\text{g/g}$ protein)	10	150
TOTAL AGLYCONES ISOFLAVONES ($\mu\text{g/g}$ protein)	40	1910
TOTAL ISOFLAVONES ($\mu\text{g/g}$ protein)	70	3140

Food consumption measurements

The food utilized in this project had a consistency similar to play dough. When formed into a ball and placed in a wire bar rack, the animals were able to bite off pieces with very little waste. Food consumption was measured in a subset of experimental animals by recording initial weight of food provided and the weight of the food remaining 24 hours later to get an estimate of daily food intake. These measurements

were taken on an average of 8 animals per treatment group on 5 different days during the week post ovx and prior to ischemic event. Isoflavone intake was calculated based on grams of food consumed x 14.83% percent PE+ or PE- protein x total isoflavone content per gram of protein.

In vivo permanent cerebral ischemia

One week post-ovariectomy and oil or E2 treatment, all animals underwent permanent middle cerebral artery occlusion (MCAo). MCAo is a widely used procedure in rats to mimic human occlusive stroke (Longa et al., 1989; Wise et al., 2001; Rau et al., 2003; Choi et al., 2004). Briefly, under isoflurane anesthesia, a 4/0 monofilament suture, coated with poly L lysine, with a standardized plastic coated tip (Doccal Company, Redlands, CA) was inserted into the left internal carotid artery and advanced to the middle cerebral artery. Insertion length was determined according to body weight and measured from the bifurcation of the internal and external carotid arteries. The suture was secured in position by placing a ligature around the internal carotid artery. This procedure results in complete blockage of blood flow to the striatum of the left hemisphere, which is served by MCA, with resulting necrotic cell death. In addition, it drastically reduces blood flow to the overlying cortex but because the cortex has collateral perfusion from branches of other cerebral arteries, it provides a model to study programmed cell death in cortical neurons. During the MCAo procedure body temperature was monitored with a rectal probe and maintained at normothermic (+/- 1°C) with a heating pad. Buprenorphine (0.5 mg/kg) was administered immediately post operatively via a subcutaneous route for analgesia.

Histological preparation

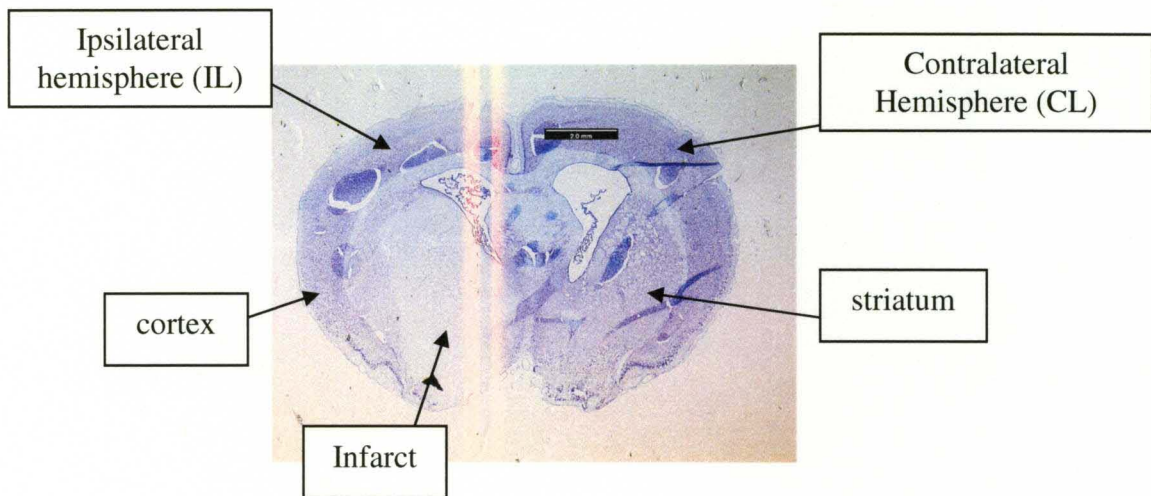
Twenty-four hours after undergoing MCAo, rats were injected with 0.35 ml (total dose 26.25 mg ketamine/1.75 mg xylazine per animal) of a 3/1 ketamine (100 mg/ml)/xylazine (20 mg/ml) mixture IM to induce deep anesthesia, euthanised via thoracotomy, and then intracardially perfused with 10 ml room temperature 0.1M PBS followed by ice cold 4% paraformaldehyde at a flow rate of 15 ml/min for 15 minutes per rat. Prior to perfusion, intracardiac blood samples were drawn for analysis of E2 plasma levels and sent to the UVa Center for Research in Reproduction Ligand Assay and Analysis Core Laboratory (Charlottesville, VA) for fee for service ELISA testing. After perfusion the brain was collected and cryoprotected in stepwise 12.5% to 25% sucrose in 0.1M PBS at 4°C before being frozen at -80°C until subsequent Nissl staining and immunofluorescent antibody processing occurred. Brains were sliced on a cryostat into four series of 20 µm sections at -18°C. All sections were accounted for in order to calculate total thickness between sections for infarct volume measurements.

Infarct measurements

One series of 20µm sections was subjected to a standard Nissl stain and analyzed for striatal, cortical, and total infarct volumes. Infarcted areas did not take up the blue Nissl stain, leaving clear demarcations between infarcted and non-infarcted tissue (see Figure 3). Brain sections that spanned Bregma +2.2 to -2.8 were photographed on a Leica synchroscopy microscope and saved in tagged image file format (TIFF). This

region contains the area of the brain served by the middle cerebral artery, which includes a portion of the frontal lobe and the lateral surface of the temporal and parietal lobes that undergo primary injury (Rau et al., 2003). Pictures were resized to 900 pixels and adjusted for contrast using Adobe Photoshop version 6.0 (Adobe Systems, San Jose, Calif., USA). Infarcted areas were measured using an image analysis system (Scion Image v4.02, Scion Corp., Frederick, MD, USA) calibrated to 76 pixels/mm for all pictures taken at 1.25 magnification or 60 pixels/mm for all pictures taken at 1.0 magnification. Measurements were collected on infarcted striatum, infarcted cortex, total ipsilateral (IL) hemisphere, and total contralateral hemisphere (CL). Infarct volume data were collected in two ways, as raw measurements in mm^3 and as a percent of the total IL hemisphere volume (%). Raw measurements allow for comparison with the results of Dubal and Wise (2001), which is the model study for this research. The later measurements were to account for infarct associated edema as described in Scheihofer et al (2005) and allowed for comparison of results with the most closely matched PE study in the literature to date. Infarct volumes were calculated by multiplying the cross-sectional infarct area of striatum or cortex by thickness from one measured section to another to arrive at infarct volume (mm^3), then dividing by total cross-sectional area of IL hemisphere to arrive at infarct volume as a % of IL hemisphere. All section measures for each brain were then added for total mm^3 or % IL hemisphere.

Figure 3. Definition of terms used to describe infarct data



P-Akt and PTEN measurements

Two other series of 20 μm sections from six animals per experimental group were processed for double label immunofluorescence histochemistry. Sections were from brains that had non-infarcted cortex. Some of the original thirteen per group were not eligible for antibody studies due to presence of infarcted cortex in the study area. The antibody study area was defined as superficial lateral cortex, in line with the lateral ventricles, and within Bregma +0.8 to -0.92 (Figure 4). This area of the somatosensory cortex was chosen for antibody study because it tended to be the variable area, either being totally damaged or totally spared (Figure 5).

Figure 4. Antibody target area

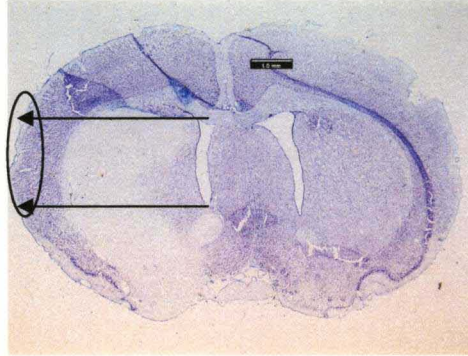
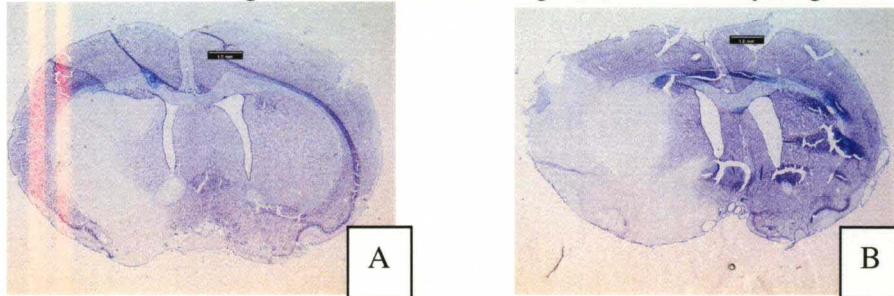


Figure 5. No cortex damage (A) vs. cortex damage (B) in antibody target area



Sections were blocked with normal goat serum (60 mg/ml, Jackson ImmunoResearch Labs, West Grove, PA., USA) for one hour at room temperature. The sections were then incubated with primary antibodies overnight at 4°C in a combination of a rabbit monoclonal antibody against p-Akt (Ser473, Cell Signaling Technology, Beverly, Mass., USA, diluted 1:50), or a rabbit monoclonal antibody against PTEN (138G6, Cell Signaling Technology, diluted 1:200) and either a mouse anti-gial fibrillary acidic protein (Gfap) monoclonal antibody (Chemicon International, Temecula, Calif., USA, diluted 1:400) or mouse anti-neuronal nuclei (NeuN) monoclonal antibody

(Chemicon, diluted 1:400). All antibody dilutions were made using a sterile antibody dilution buffer consisting of 40mls 1X PBS, 0.4 mls bovine serum albumen, and 120 ul triton X. Sections were then incubated for 2 hours at room temperature with a mixture of secondary antibodies consisting of Rhodamine Red conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, diluted 1:500) and Fluorescein (FITC) conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, diluted 1:50). All incubations occurred within a zero light humidity chamber. Prolong antifade reagent (Molecular Probes, Eugene, OR) was applied to all sections prior to cover slipping.

Table 3. Serial antibody treatments.

Protein Marker	Cell Type Marker	Effects with Secondary Antibody
p-Akt	Gfap	red proteins with green astrocytes
PTEN	Gfap	red proteins with green astrocytes
p-Akt	NeuN	red proteins with green neurons
PTEN	NeuN	red proteins with green neurons

Immunofluorescent staining was visualized and recorded using a Zeiss laser deconvolution microscope using a 20x objective and quantified with the accompanying Axiovision 4.4 image analysis software. Each of the four original treatment groups (E2-/PE-, E2-/PE+, E2+/PE-, E2+/PE+) were subjected to all four antibody combinations (p-Akt/Gfap, p-Akt/NeuN, PTEN/Gfap, PTEN/NeuN) so that expression of each protein in glia vs. neurons could be assessed in each treatment group. Five images per hemisphere (CL and IL) from three sections per slide for six different trials were analyzed resulting in

2,880 pieces of data collected for this study. Data are reported as *field area percent*, which refers to the sum of the areas of all regions (mm^2) that meet defined immunofluorescent criteria divided by the entire field area (mm^2) x 100. The field area was the same for all measurements and is determined by the aperture of the 20x lens used for all protein data collection. In other words, this parameter measures the percent of the field, visible with the 20x objective, occupied by fluorescing areas within a defined range of brightness and size. Although sections were double labeled, when measuring Akt or PTEN, only red fluorescence is measured, not both markers

Statistical analysis

Food consumption, blood estrogen levels, and changes in body weight between ovx and MCAo data are presented as means (+/- SE). Total isoflavones consumed is presented as average mg/day (+/- SE) intake over the six day period between ovx and MCAo. Differences in infarct volume, p-Akt, and PTEN expression was assessed separately by two way analyses of variance (ANOVA) with E2 and PE exposure as independent factors using Sigma Stat software. In some cases, data did not pass tests of equal variance or normality, and so were ranked before analysis. Separate analyses on infarcts included striatal, cortical, and total infarct volumes, as raw measurements in mm^3 , as % of ipsilateral hemisphere volume, and as specific subset data including only animals in each group that did sustain cortex damage. (+/- SE). Subset data were analyzed using a PROC MIXED in SAS. P- Akt and PTEN expression were analyzed with each co-marker (NeuN, Gfap) separately. Levels were compared from the ipsilateral hemisphere and from the ipsilateral minus the contralateral hemisphere, in which case

post-hoc multiple pairwise comparisons using the Holm-Sidak method was warranted. A difference of $p < 0.05$ was considered statistically significant. Marker effects were analyzed using a type 3 tests of fixed effects in SAS. Pearson product moment correlation coefficients were calculated for body weight at time of MCAo, E2 levels, and PE consumption in relationship to cortex and total infarct volume as a % of ipsilateral hemisphere volume (+/-SE).

RESULTS

Amount of phytoestrogens consumed by PE+ groups

Food consumption during the week immediately following ovx up to MCAo was found to be consistent among the groups (see Figures 6 A-D). As expected, all groups demonstrated a trend of increased consumption with consecutive days post ovx. Average food intake for day one was 6 grams (± 0.39 g) with a steady increase of 2 to 3 grams per day. By day six, the average intake had increased to 19g (± 0.57 g).

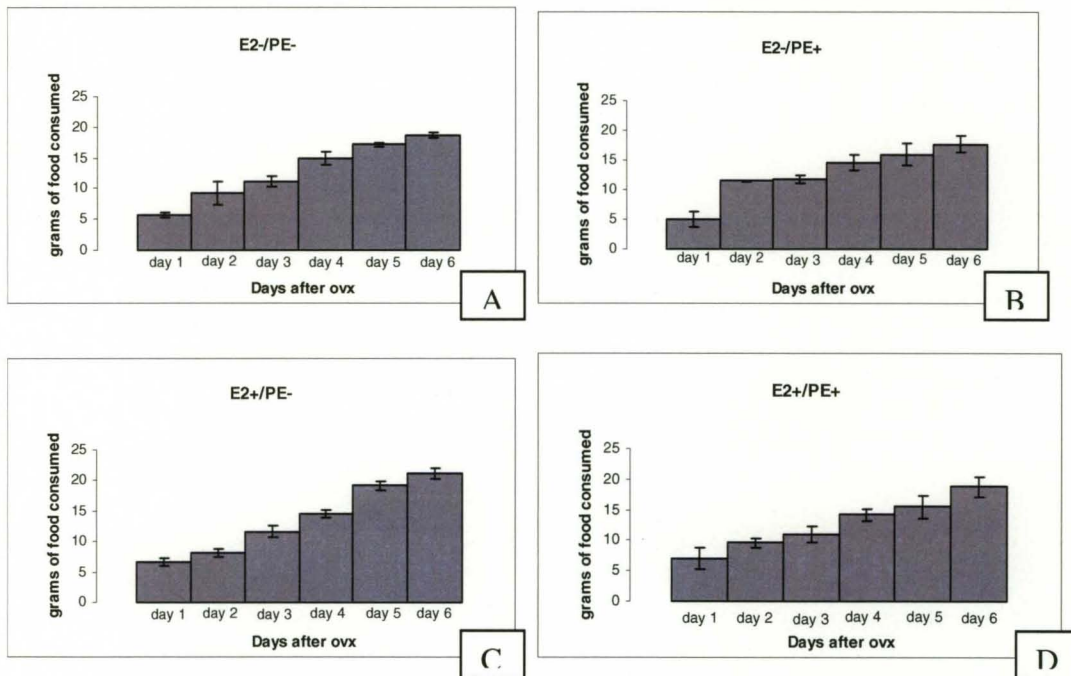


Figure 6 A-D. Food consumption by group by day between ovx and MCAo (mean \pm SE).

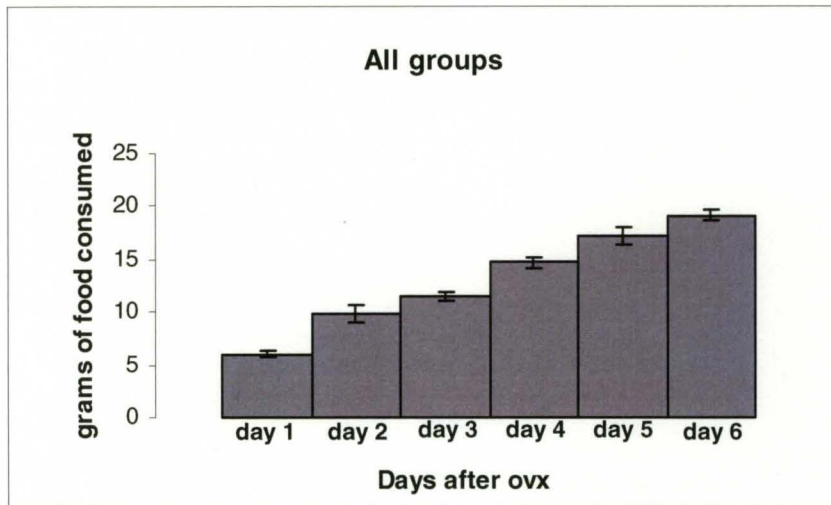


Figure 7. Food consumption across groups by day for the 7 days between ovx and MCAo (mean \pm SE).

Total isoflavones consumed by the PE+ groups reached 48.6 mg/day (\pm 2.59 mg) in the E2-/PE+ group and 51.71 mg/day (\pm 4.21 mg) in the E2+/PE+ group (see Figure 8).

There was no significant difference between groups.

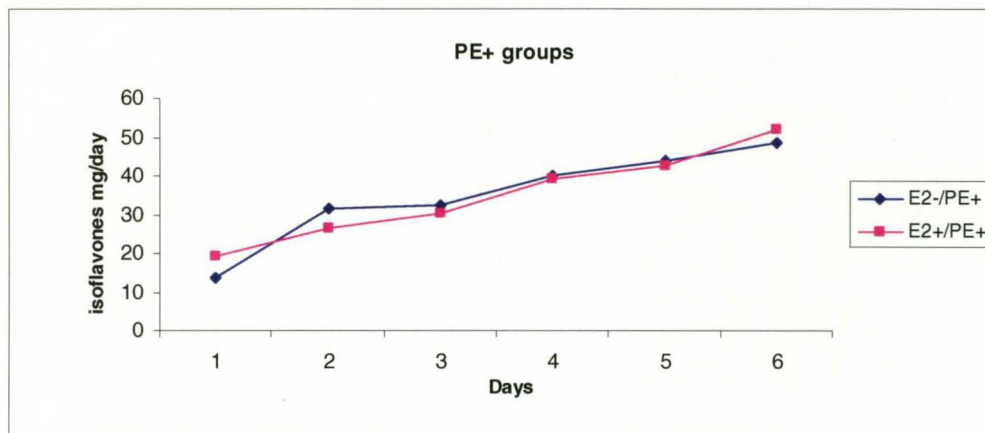


Figure 8. Isoflavone consumption (mg/day) by PE+ groups

Circulating blood estrogens levels produced via silastic implants in E2+ groups

Circulating plasma E2 levels were measured from blood harvested at time of animal sacrifice. The silastic implants produced average physiological blood levels of 40 pg/ml (24-67 pg/ml), which is consistent with levels of E2 during diestrus. The E2+/PE- and E2+/PE+ groups had very similar plasma levels of E2, 38.65 +/- 1.88 pg/ml and 40.48 +/- 3.66 pg/ml, respectively (see figure 9).

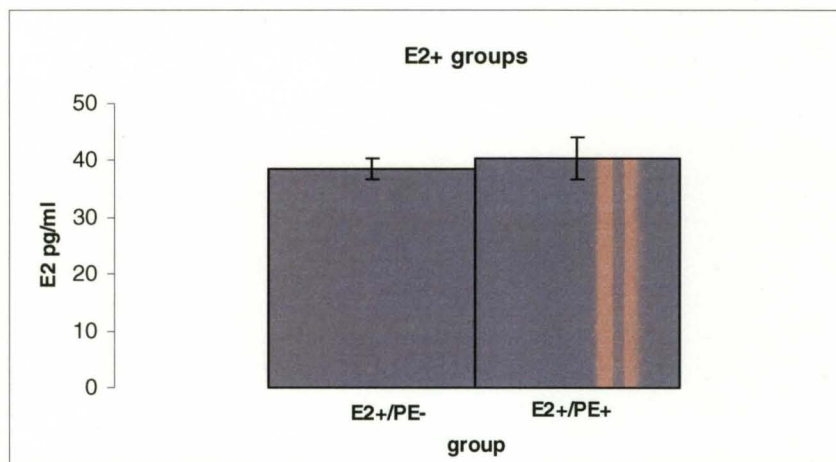


Figure 9. Plasma E2 levels in E2+ groups (mean \pm SE)

Animal body weight between ovx and MCAo.

The average weight per group was similar at the time of ovx; however, much variation existed within each group (see Table 4). As expected, the E2+ groups lost the most weight with the E2+/PE- group losing an average of 6.6% body weight between ovx and MCAo and the E2+/PE+ group losing an average of 6.8% body weight during the

same time frame. The E2-/PE+ group lost the least amount with only an average 2.6% reduction in body weight following ovx.

Table 4. Changes in body weight between ovx and MCAo.

Group	Body weight at ovx (g)	Body weight at MCAo (g)
E2-/PE-	305 +/-90	292 +/-82
E2-/PE+	305 +/-107	297 +/- 81
E2+/PE-	305 +/- 91	285 +/- 73
E2+/PE+	307 +/- 60	286 +/- 51

Infarct measurements

Infarct volume data is presented in two forms, as raw measurements in mm³ and as a percent of the total IL hemisphere volume. Raw measurements allow for comparison with the results of Dubal and Wise (2001), which is the model study for this research. The later measurements were to account for infarct-associated edema as described in Scheihofer et al (2005), and allowed for comparison of results with the most closely matched PE study in the literature to date. No significant effects were detected in striatal, cortex, or total infarct volume by two-way ANOVA using raw infarct volume (mm³) or % IL hemisphere as measures. Since there was no ANOVA effect, post hoc multiple comparisons were not performed. Power for all analyses was low (0.05), but with all p values above 0.7 the risk of a Type II error is very low. While all measurements passed equal variance testing, only the striatal infarct data passed

normality testing; thus, cortex and total infarct measurements were ranked prior to analysis. The striatal infarct volume was essentially the same in all groups, suggesting that the infarct procedure was equalized between groups (see Figures 10, 12 and Table 5). Statistical analysis is addressed within the section specific to measurement type.

Raw infarct data in mm³

There was no significant difference among the ANOVA factors (E2, PE, and E2 x PE interaction) in striatal (E2 p= 0.78, PE p= 0.80, E2 x PE p =0.73), cortex (E2 p= 0.84, PE p= 0.96, E2 x PE p =0.72), or total infarct volume (E2 p= 0.99, PE p= 0.89, E2 x PE p =0.71), when measuring infarct in mm³ (see Figures 10, 12 and Table 5).

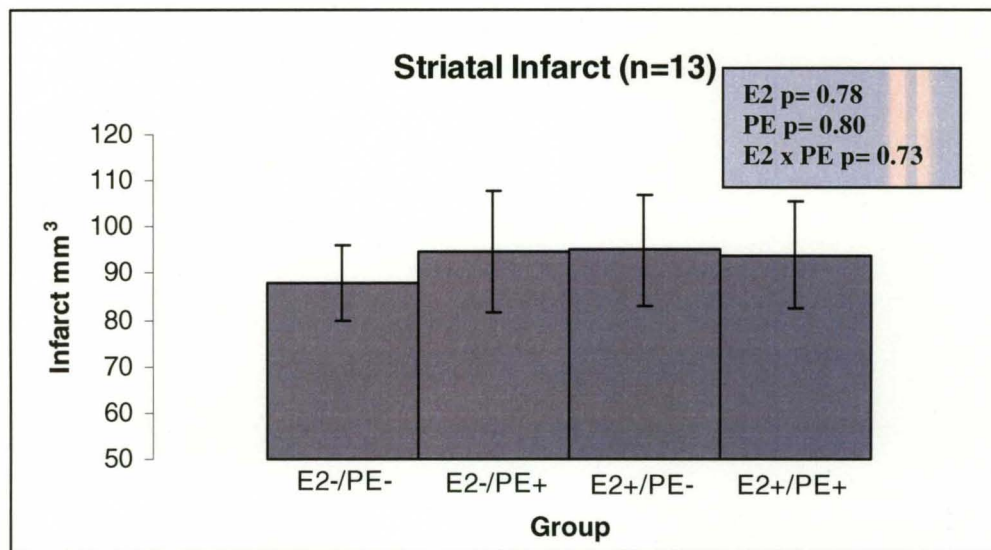


Figure 10. Striatal infarct volume (mm³) by group (mean ± SE).

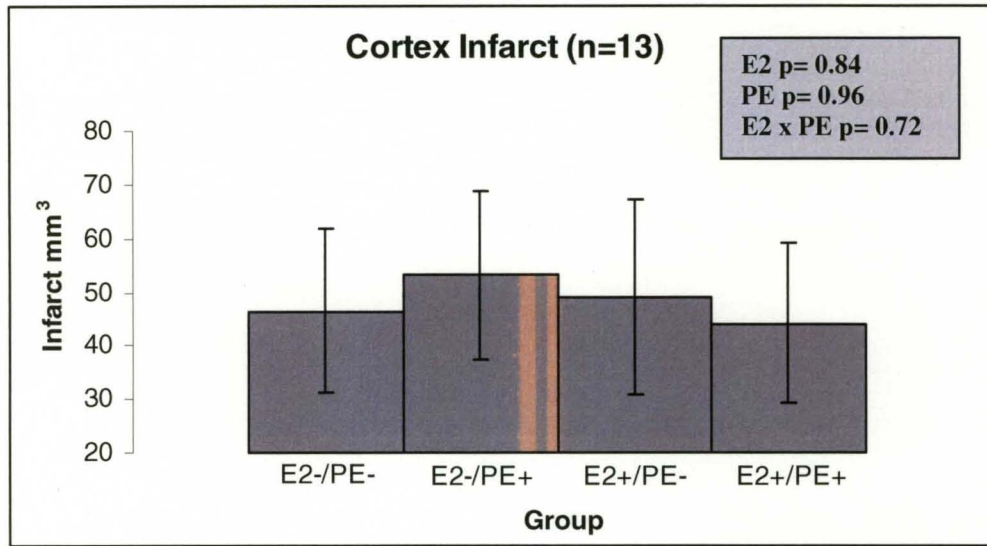


Figure 11. Cortex infarct volume (mm³) by group (mean \pm SE).
Note: These data failed normality testing and so were ranked before analysis.

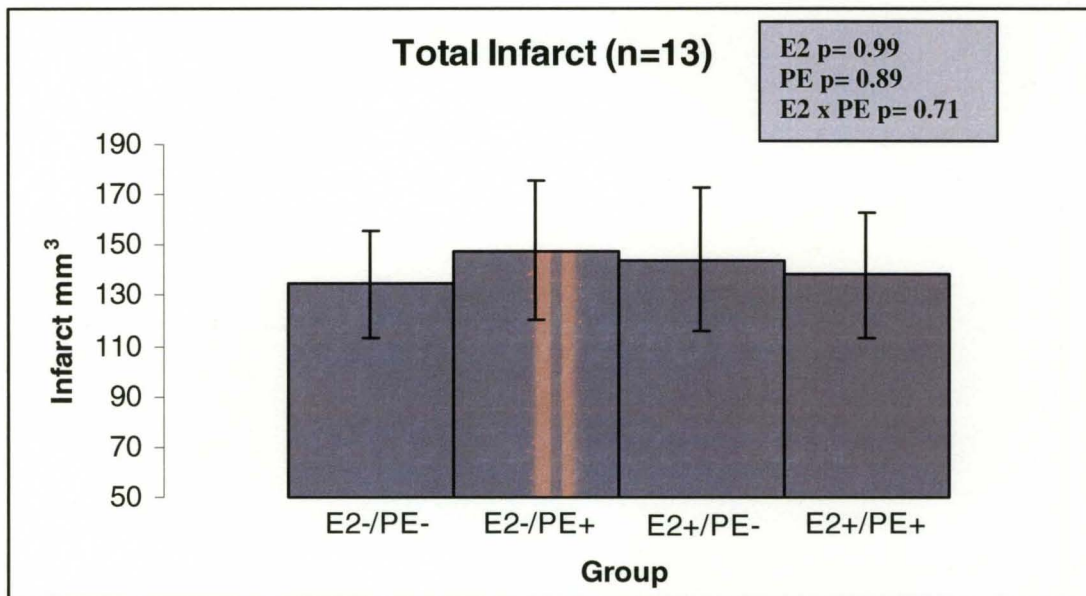


Figure 12. Total infarct volume (mm³) by group (mean \pm SE).
Note: These data failed normality testing and so were ranked before analysis.

Table 5. Mean striatal, cortical and total infarct measurements in mm³ (\pm SE) by group.

Group	Striatal infarct volume mm ³	Cortical infarct volume mm ³	Total infarct volume mm ³
E2-/PE-	88.00 (+/-7.99)	46.46 (+/-15.19)	134.46 (+/-21.43)
E2-/PE+	94.77 (+/-13.16)	53.15 (+/-15.67)	147.92 (+/-27.72)
E2+/PE-	95.08 (+/-11.99)	49.15 (+/-18.23)	144.23 (+/- 28.48)
E2+/PE+	94.00 (+/-11.54)	44.08 (+/-14.87)	138.08 (+/- 24.71)

Infarct data as % IL hemisphere

There was no significant difference among the ANOVA factors (E2, PE, and E2 x PE interaction) in striatal (E2 p= 0.92, PE p= 0.92, E2 x PE p =0.84), cortex (E2 p= 0.63, PE p= 0.93, E2 x PE p =0.89), or total infarct volume (E2 p= 0.69, PE p= 0.92, E2 x PE p =0.98), when measuring infarct as percent IL hemisphere (see Figures 13, 14, 15 and Table 6).

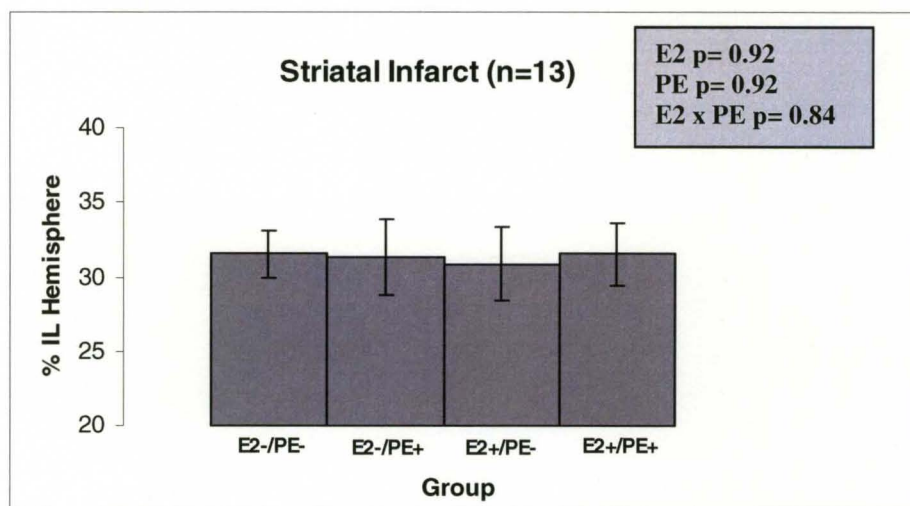


Figure 13. Striatal infarct volume as a % of total ipsilateral hemisphere volume by group (mean \pm SE).

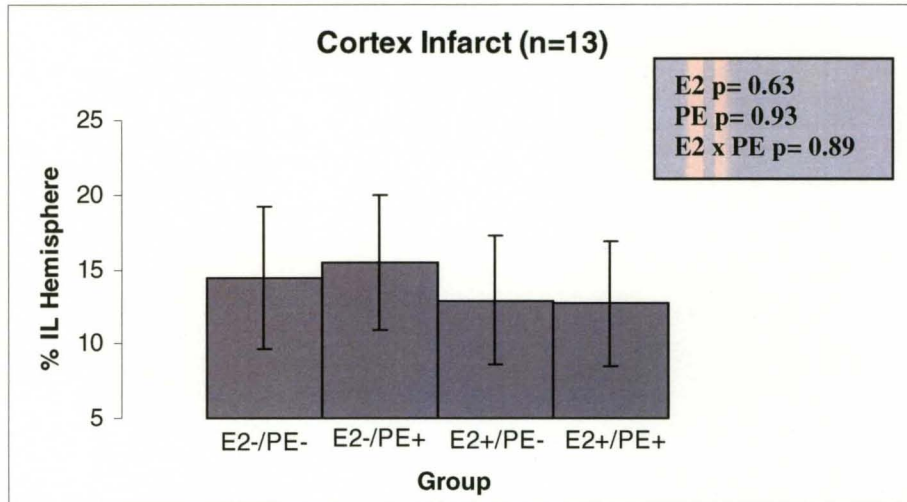


Figure 14. Cortex infarct volume as a % of total ipsilateral hemisphere volume by group (mean \pm SE).

Note: These data failed normality testing and so were ranked before analysis.

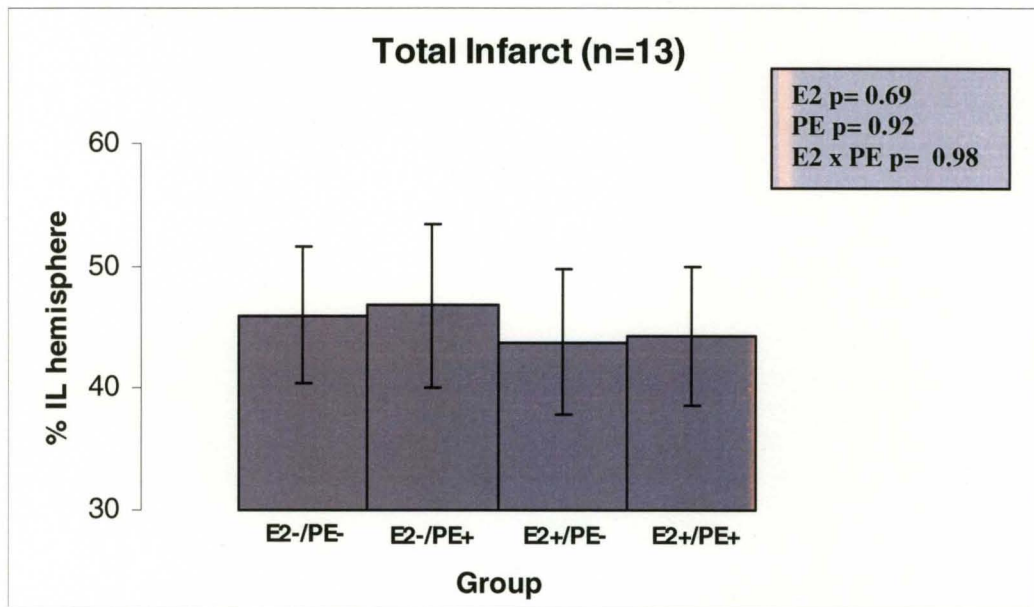


Figure 15. Total infarct volume (as a % of total ipsilateral hemisphere volume) by group (mean \pm SE).

Note: These data failed normality testing and so were ranked before analysis.

Table 6. Resulting mean \pm SE of striatal, cortex, or total infarct volume (% IL hemisphere) for four treatment groups.

Group	Striatal Infarct Volume % IL hemisphere	Cortical infarct volume % IL hemisphere	Total infarct volume % IL hemisphere
E2-/PE-	31.54 (+/-1.55)	14.46 (+/-4.79)	46.00 (+/-5.64)
E2-/PE+	31.31 (+/-2.56)	15.46 (+/-4.51)	46.77 (+/-6.68)
E2+/PE-	30.85 (+/-2.47)	12.92 (+/-4.37)	43.77 (+/-5.96)
E2+/PE+	31.54 (+/-2.15)	12.69 (+/-4.17)	44.23 (+/-5.68)

Due to the non-significant results across the entire treatment groups (n=13) for the three factors tested, two additional questions were addressed using a subset from each original group. The first question was whether or not the presence of cortex damage depended upon treatment group. The second question was, if cortex damage is present, does the amount of damage depend upon treatment group? The subset to be included in this analysis is defined in Table 7 as those animals in each group that did sustain cortex damage. All animals with no cortex damage were excluded from the analysis testing the second question.

Table 7 . The presence of infarcted cortex measured as % IL hemisphere by treatment group

Cortex Damaged?	E2-/PE-	E2-/PE+	E2+/PE-	E2+/PE+
NO (zero cortex damage)	5	6	5	5
YES	8	7	8	8
TOTAL	13	13	13	13

Table 8. Resulting mean \pm SE of striatal, cortex, or total infarct volume (as a % of total IL hemisphere volume) by group when cortex infarct is greater than zero.

Group	Striatal Infarct Volume (%IL)	Cortical infarct volume (%IL)	Total infarct volume (%IL)
E2-/PE-	32.56 (+/-1.84)	20.89 (+/-5.75)	53.44 (+/-6.71)
E2-/PE+	38.00 (+/-2.08)	28.71 (+/-3.48)	66.71 (+/-4.26)
E2+/PE-	34.88 (+/-1.94)	21.00 (+/-5.38)	55.88 (+/-6.11)
E2+/PE+	35.67 (+/-1.75)	18.33 (+/-4.98)	54.00 (+/-5.57)

A logistic regression model using PROC MIXED in SAS revealed that the presence of cortex damage did not depend upon treatment group ($p=0.84$). There was also no significant factor effect (E2 $p=0.78$, PE $p=0.78$, E2/PE interaction $p=0.41$). Thus, to answer the first question, the presence of cortex damage does not depend upon treatment group.

The second question, if cortex damage is present, does the amount of damage depend upon treatment group, was analyzed in the same manner. Resulting graphs are presented in Figures 16, 17, and 18.

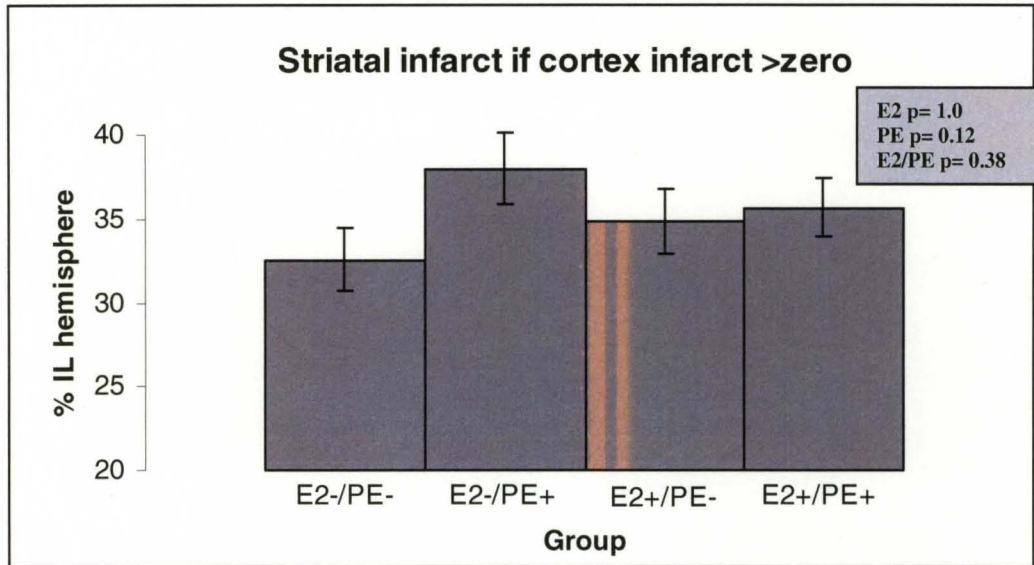


Figure 16. Striatal infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean \pm SE).

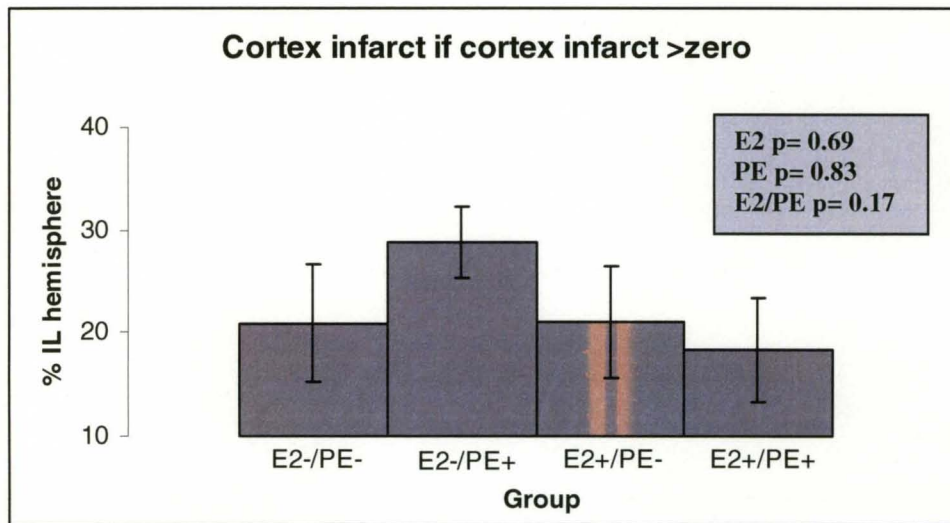


Figure 17. Cortex infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean \pm SE).

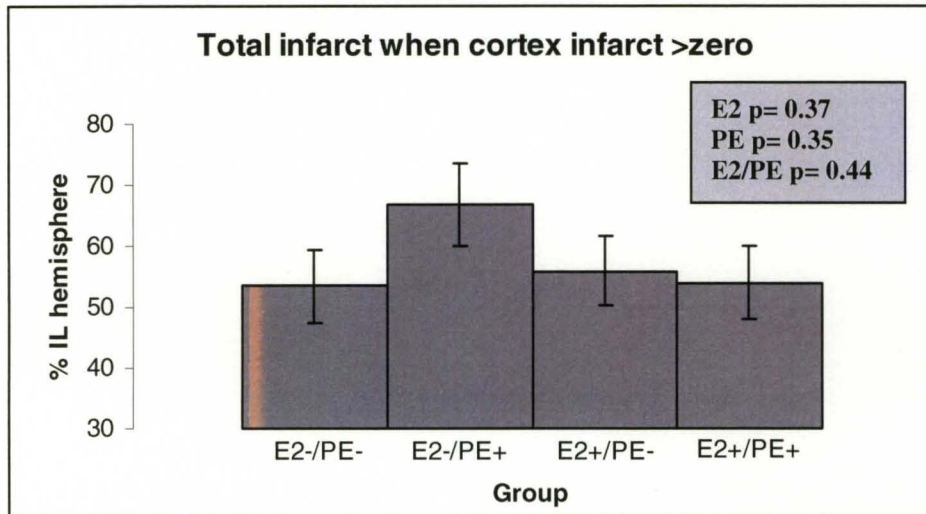


Figure 18. Total infarct volume (as a % of total ipsilateral hemisphere volume) by group when cortex infarct is greater than zero (mean \pm SE).

The resulting p values for E2, PE, and E2 x PE interaction for striatal (E2 p= 1.00, PE p=0.12, E2/PE interaction p=0.38), cortex (E2 p=0.69, PE p=0.83, E2/PE interaction p=0.17), and total damage (E2 p=0.37, PE p=0.35, E2/PE interaction p=0.44) were not significant; however, the interaction effects in the cortex did approach significance (p=0.17) when measuring cortex damage only. Thus, to answer the second question, if cortex damage is present, the amount of damage does not depend upon treatment group.

The fact that both questions resulted in non-significant findings is not surprising when one considers that the number of animals per group sustaining cortical injury was nearly identical and when damage was present, it occurred very consistently among groups (see Table 4 and 6).

Table 9. Number of rats in each group by percent of cortex damage (%IL).

Percent cortex damage	E2-/PE-	E2-/PE+	E2+/PE-	E2+/PE+
0%	5	6	5	5
1-25%	3	2	5	4
26-50%	5	5	3	4
50 -100%	0	0	0	0
Total	13	13	13	13

P-Akt/PTEN measurements as field area percent of the IL hemisphere

There were no significant differences in PTEN {(PTEN/Gfap E2 p=0.68, PE p=0.55, E2/PE interaction p=0.096) and (PTEN/NeuN E2 p=0.54, PE p=0.78, E2/PE interaction p=0.91)} or p-Akt {(p-Akt/Gfap E2 p=0.47, PE p=0.94, E2/PE interaction p=0.68) and (p-Akt/NeuN E2 p=0.94, PE p=0.88, E2/PE interaction p=0.71)} levels when comparing ANOVA factors for the field area percent in the IL hemisphere (see Figures 19, 20, 21, 22 and Table 7). Interestingly, when double labeled with Gfap, the interaction between E2 x PE for PTEN approaches significance (0.09). This phenomenon is not seen when PTEN is double labeled with NeuN. This suggests that there may have been a marker effect. A type 3 test of fixed effects performed in SAS failed to demonstrate a marker effect for p-Akt (p=0.60) but did demonstrate a significant marker effect for PTEN (p=0.0045). This helps explain why the level of expression of PTEN varies with secondary marker. This may be due to the fact that both PTEN and p-Akt appear to reside within the neurons (see Figures 23, 24, 25, 26) and some immunofluorescent signal

could have been lost due to co-localization or physical overlap despite the red and green immunofluorescent pigments being measured separately. Because of the marker effect, it may be more realistic to evaluate protein expression when double labeled with Gfap and use the NeuN data for protein localization.

It should also be noted that PTEN and p-Akt levels could only be measured on brains with non-damaged cortex in the target area as unviable tissue would most likely contain only denatured proteins. This eliminated the inclusion of the most damaged brains and thus biased data towards not finding a difference.

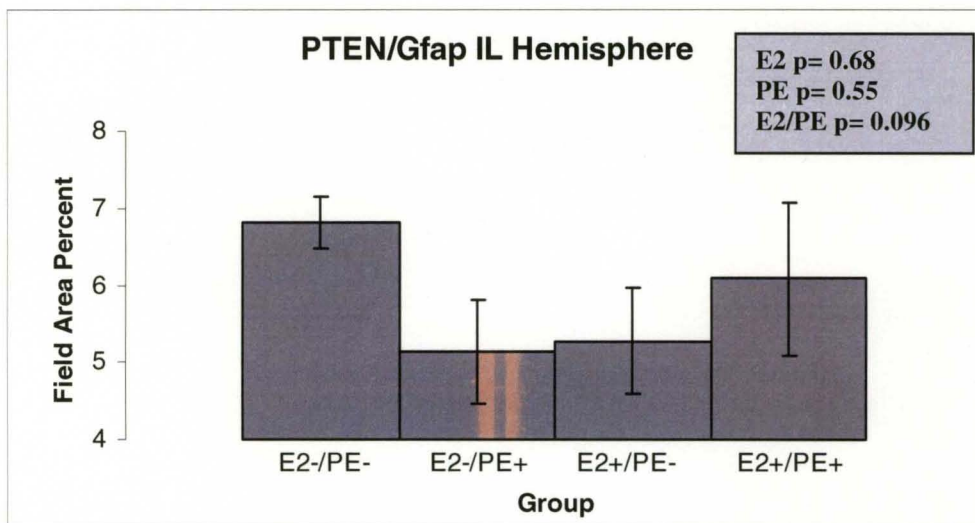


Figure 19. Field area percent of PTEN with Gfap in the IL hemisphere (mean \pm SE).

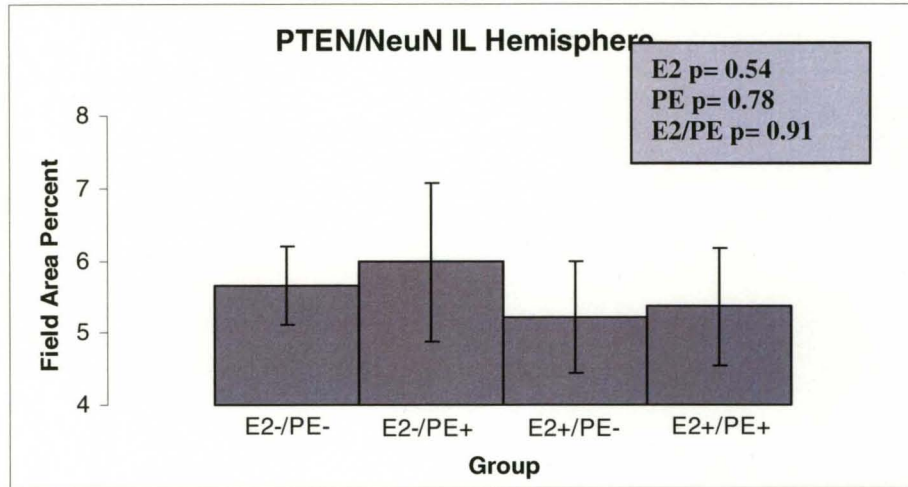


Figure 20. Field area percent of PTEN with NeuN in the IL hemisphere (mean \pm SE).

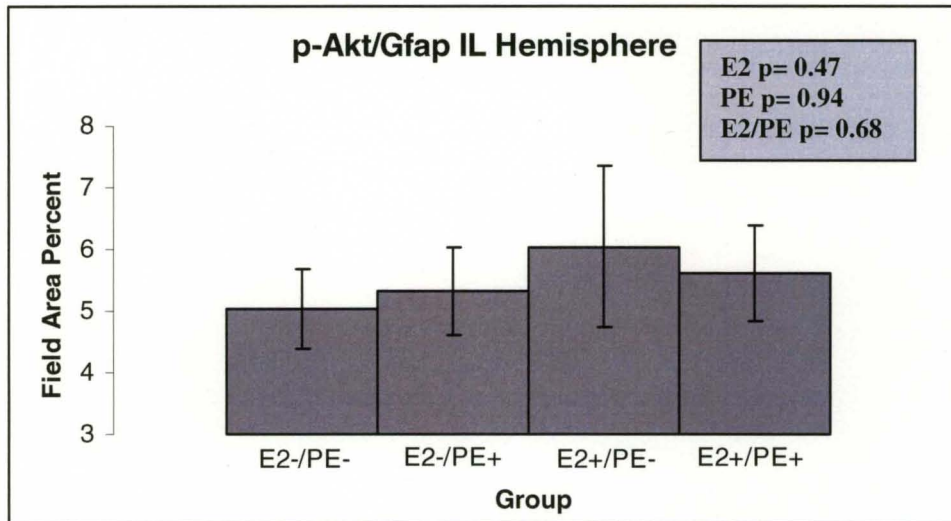


Figure 21. Field area percent of p-Akt with Gfap in the IL hemisphere (mean \pm SE).

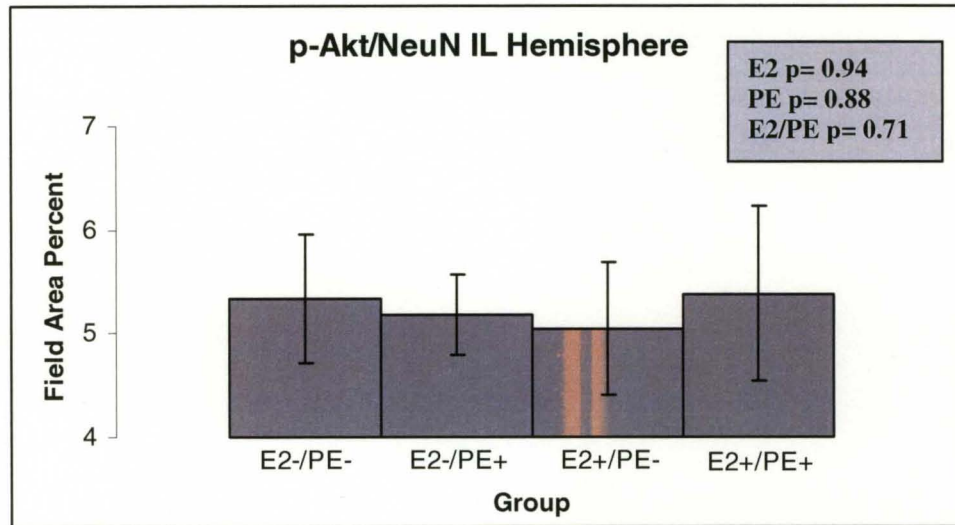


Figure 22. Field area percent of p-Akt with NeuN in the IL hemisphere (mean \pm SE).

Table 10. Field area percents of p-Akt/PTEN with Gfap/ NeuN in the IL hemisphere (mean \pm SE).

Protein/marker	E2-/PE- (%)	E2-/PE+ (%)	E2+/PE- (%)	E2+/PE+ (%)
PTEN/Gfap	6.83 (+/-0.34)	5.14 (+/-0.68)	5.28 (+/-0.69)	6.09 (+/-0.99)
PTEN/NeuN	5.65 (+/-0.54)	5.98 (+/-1.10)	5.22 (+/-0.77)	5.37 (+/-0.81)
p-Akt/Gfap	5.03 (+/-0.65)	5.33 (+/-0.70)	6.05 (+/- 1.31)	5.61 (+/- 0.79)
p-Akt/NeuN	5.34 (+/-0.62)	5.18 (+/-0.39)	5.05 (+/-0.64)	5.38 (+/-0.84)

Cell type associated with p-Akt/PTEN

PTEN and p--Akt expression occurred predominantly in the neurons, instead of the astrocytes, of the ischemic cortex following focal pMCAo. These proteins are clearly expressed within the somas of neurons (Figures 24, 26) and not co-expressed with the glial marker (Figures 23, 25).

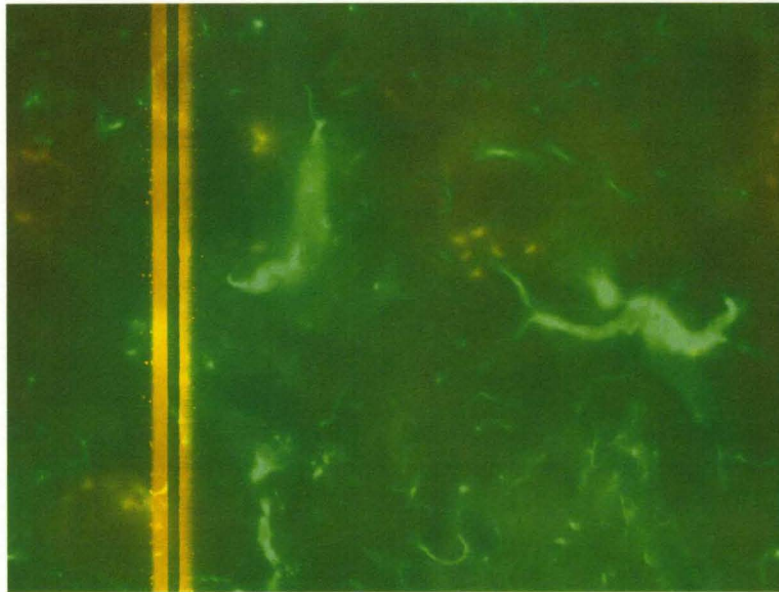


Figure 23. Immunofluorescent antibody treatment for p-Akt (red) /Gfap (green) in cortex neurons at high magnification (100x oil)

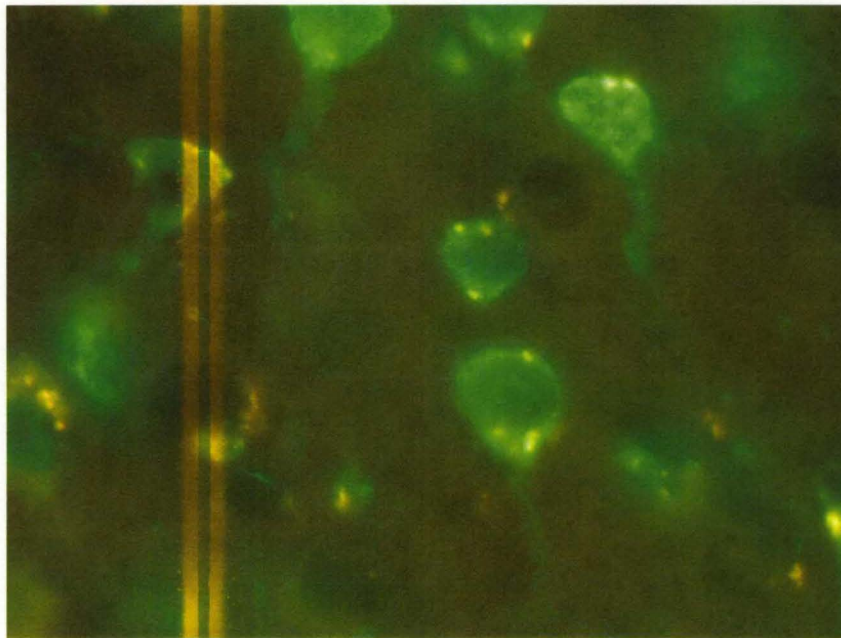


Figure 24. Immunofluorescent antibody treatment for p-Akt (red) /NeuN (green) in cortex neurons at high magnification (100x oil)

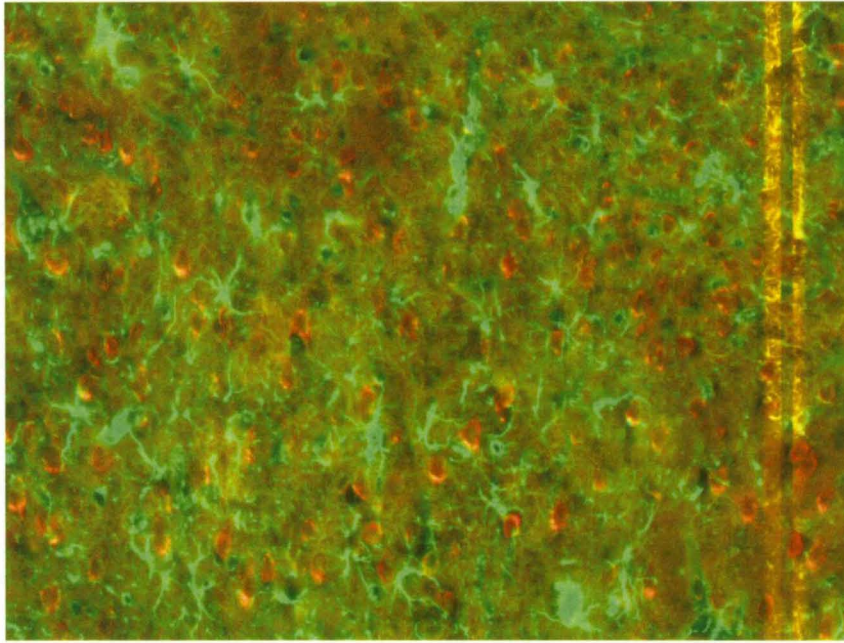


Figure 25. Immunofluorescent antibody treatment for PTEN (red) / Gfap (green) in cortex neurons at low magnification (20x)

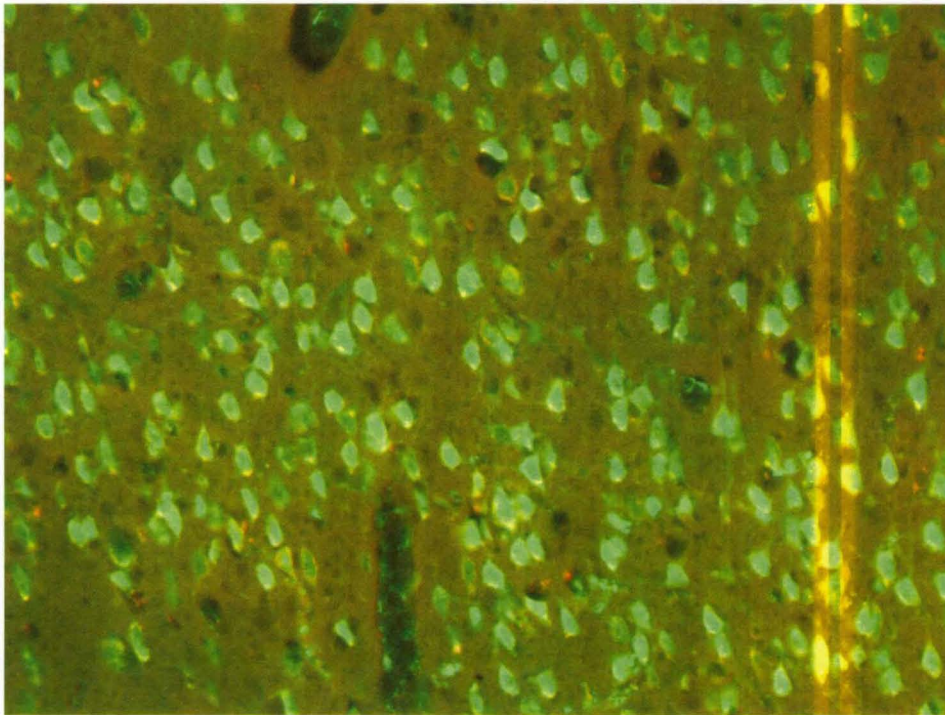


Figure 26. Immunofluorescent antibody treatment for PTEN (red) / NeuN (green) in cortex neurons at low magnification (20x)

Effects of MCAo procedure on P-Akt/ PTEN regulation

P-Akt and PTEN levels were compared between hemispheres (IL-CL) to determine if ischemia upregulated PTEN and/or p-Akt. If the procedure itself was upregulating proteins, all data included in Figures 27, 28, 29, 30 and Table 8 would be positive, indicating higher IL measurements than CL measurements. The field area percent for the CL hemisphere was subtracted from the IL hemisphere (IL-CL) in order to determine if the injury itself upregulated p-Akt and/or PTEN in aged models. This phenomenon has been reported in adult female rats following global ischemia (Cai et al., 2009) and in adult male rats following transient MCAo (Omori et al., 2002 and Choi et al., 2004) but, until this work, had not yet been explored in aged female rats following pMCAo. Most resulting values were close to zero, indicating no procedure-specific effects on expression.

When analyzing PTEN/Gfap the ANOVA factors of E2 and PE did not demonstrate a significant difference ($p=0.61$ and $p= 0.26$ respectfully) but had low power ($E2 = 0.05$ and $PE = 0.08$). A statistically significant difference was found for the E2 x PE interaction ($p=0.019$) with a resulting power of 0.61. Post -hoc multiple pairwise comparisons of the interaction factors showed that the E2-/PE- group differed from the E2+/PE- group and the E2-/PE+ group, which did not differ from each other. The E2+/PE+ group was intermediate to the others and did not differ significantly from either. This suggests that having a single type of estrogen present prevents a difference in PTEN levels between hemispheres. With standard error all but the E2-/PE- group are very close to zero. The positive value of the E2-/PE- group suggests an upregulation of PTEN in the

IL hemisphere in this model. These data failed normality testing and so were ranked before analysis.

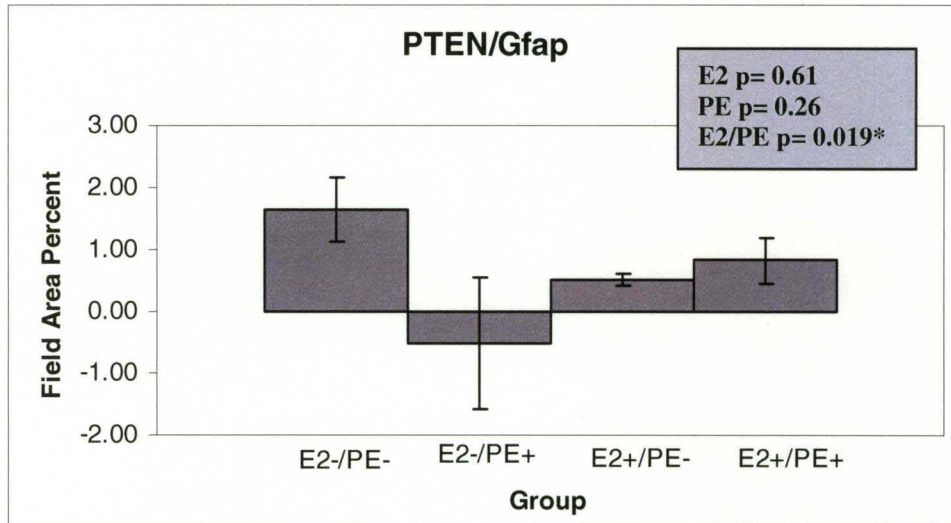


Figure 27. Field area percent of PTEN with Gfap in the IL hemisphere minus the CL hemisphere (mean \pm SE).

Note: These data failed normality testing and so were ranked before analysis.

The ANOVA factors of E2 (p=0.68), PE (p=0.07), and the E2 x PE interaction (p=0.82) for PTEN/NeuN did not demonstrate significant difference. The PE did approach significance and had a 0.3 power of performance.

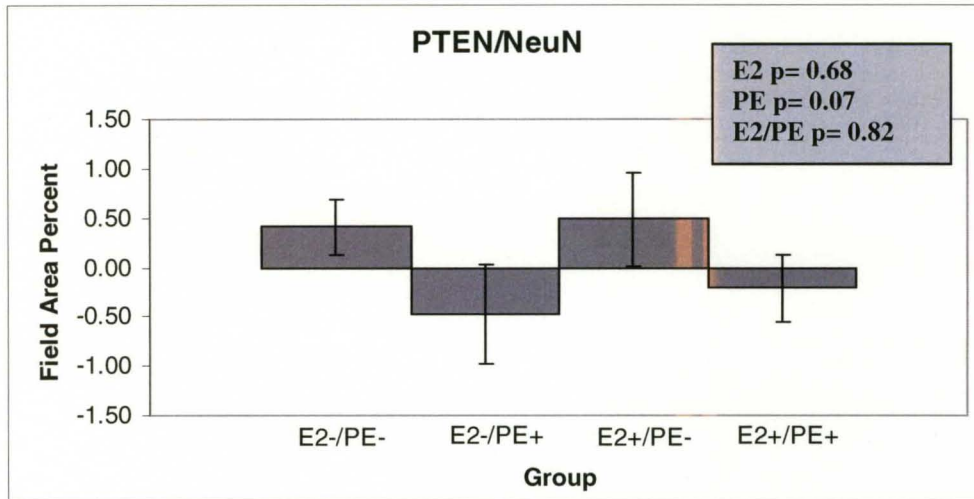


Figure 28. Field area percent of PTEN with NeuN in the IL hemisphere minus the CL hemisphere (mean ± SE).

The ANOVA factors of E2 (p=0.29), PE (p=0.46), and the E2 x PE interaction (p=0.07) for p-Akt/Gfap did not demonstrate significant difference with respective power of analyses of 0.06, 0.05, and 0.31.

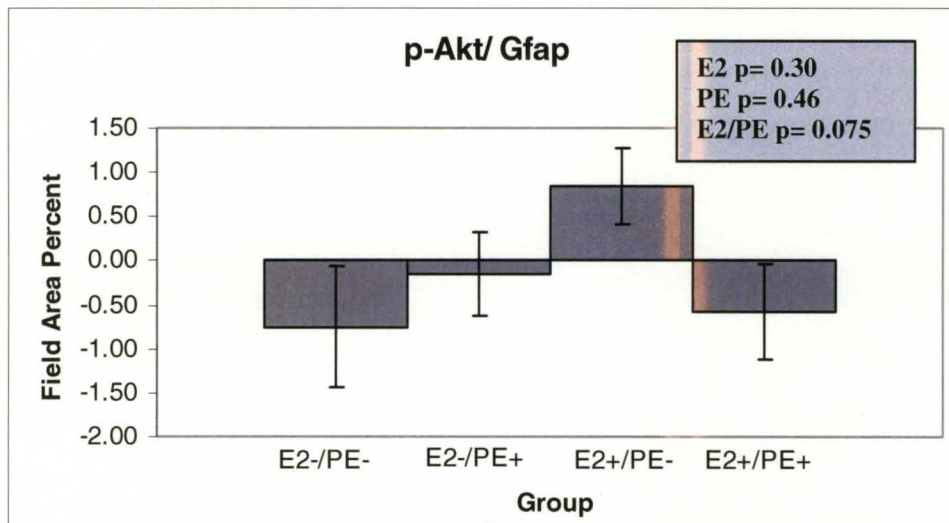


Figure 29. Field area percent of p-Akt with Gfap in the IL hemisphere minus the CL hemisphere (mean ± SE).

The ANOVA factors of E2 ($p=0.49$), PE ($p=0.17$), and the E2 x PE interaction ($p=0.71$) for p-Akt/NeuN did not demonstrate significant difference. Power for all analyses was low (0.05).

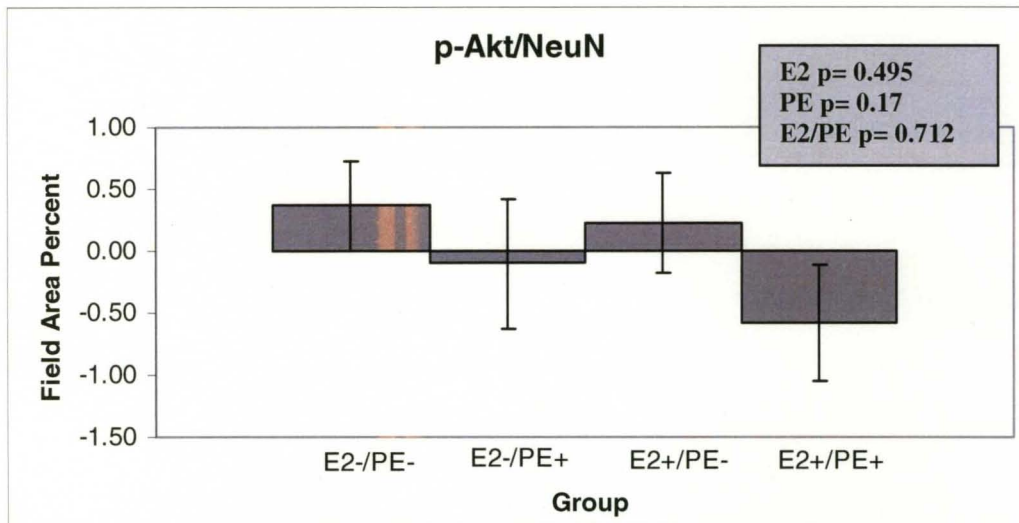


Figure 30. Field area percent of p-Akt with NeuN in the IL hemisphere minus the CL hemisphere (mean \pm SE).

Table 11. Field area percents of p-Akt/PTEN with Gfap/ NeuN in the IL hemisphere minus the CL hemisphere (mean \pm SE).

Protein/marker	E2-/PE- IL-CL (%)	E2-/PE+ IL-CL (%)	E2+/PE- IL-CL (%)	E2+/PE+ IL-CL (%)
PTEN/Gfap	1.65 (+/-0.52)*	-0.52 (+/-1.05)	0.52 (+/-0.10)	0.83 (+/- 0.37)
PTEN/NeuN	0.41 (+/-0.28)	-0.47 (+/-0.51)	0.49 (+/-0.48)	-0.21 (+/-0.34)
p-Akt/Gfap	-0.75 (+/-0.69)	-0.14 (+/- 0.48)	0.85 (+/-0.42)	-0.57 (+/-0.54)
p-Akt/NeuN	0.37 (+/-0.37)	0.10 (+/- 0.52)	0.22 (+/-0.40)	-0.58 (+/-0.47)

*- significant difference from other groups for same measure

Eliminating extraneous variables that could have contributed to non-significant infarct and p-Akt/PTEN data.

Since a wide range of body weights existed within groups at the time of MCAo, a correlation analysis was merited to determine if this could have been a contributing factor to the results. Figures 31 and 32 reveals no correlation between body weight at the time of MCAo and resulting infarct volume. Resulting Pearson correlation value was -0.19 for cortex infarct and -0.26 for total infarct, indicating no correlation between the two parameters.

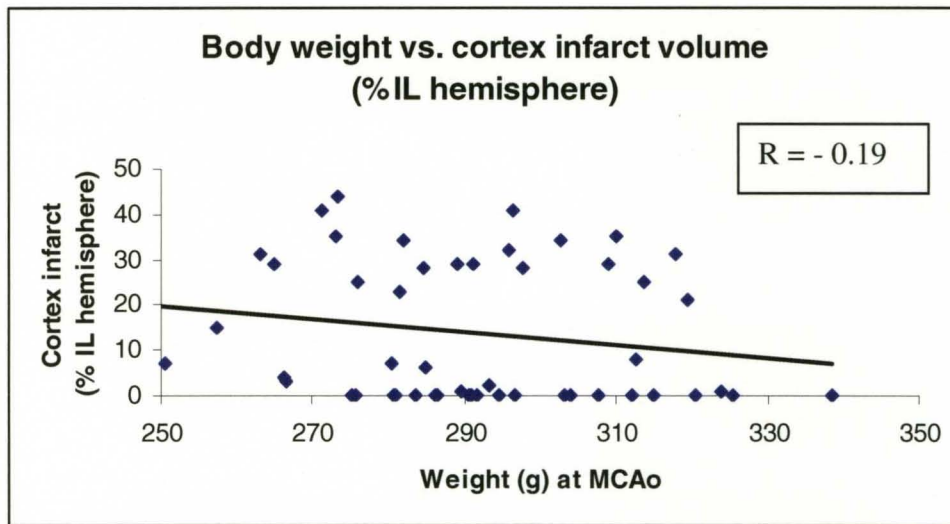


Figure 31. Correlation between body weight at time of MCAo and resulting cortex infarct volume.

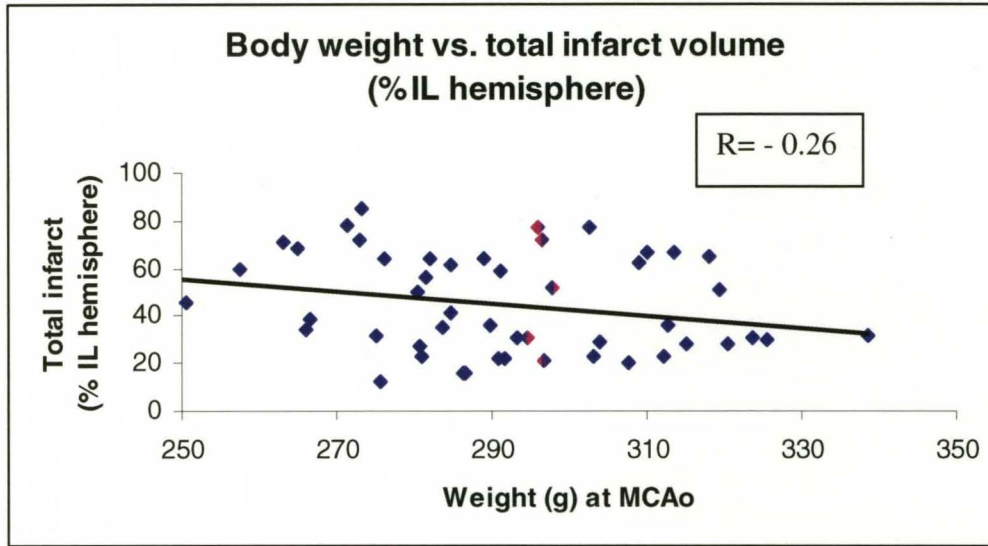


Figure 32. Correlation between body weight at time of MCAo and resulting total infarct volume.

To determine if varying levels of PE consumption introduced variability into the infarct volume data set, Pearson correlations were performed (see Figures 33 and 34). Values were 0.04 and -0.02, respectively, indicating no correlation of the two parameters with infarct volume.

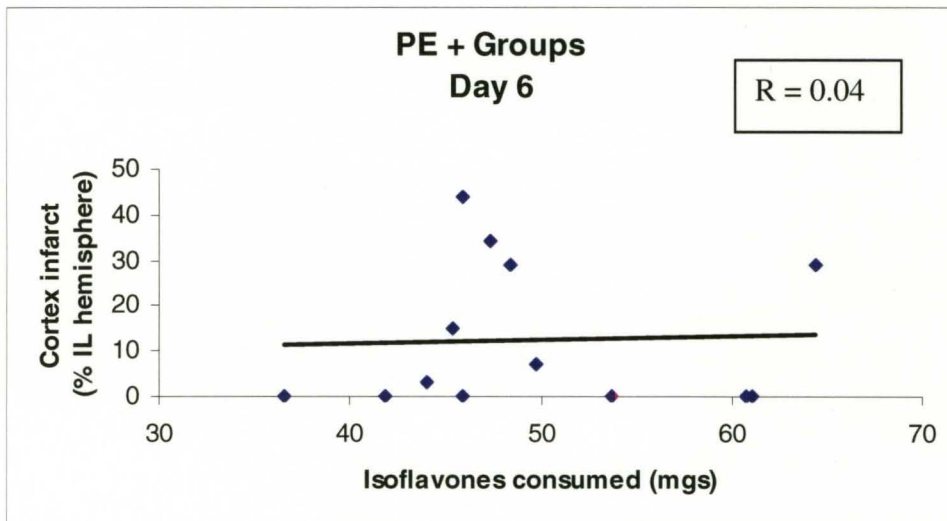


Figure 33. Correlation between mg isoflavones and resulting cortex infarct volume.

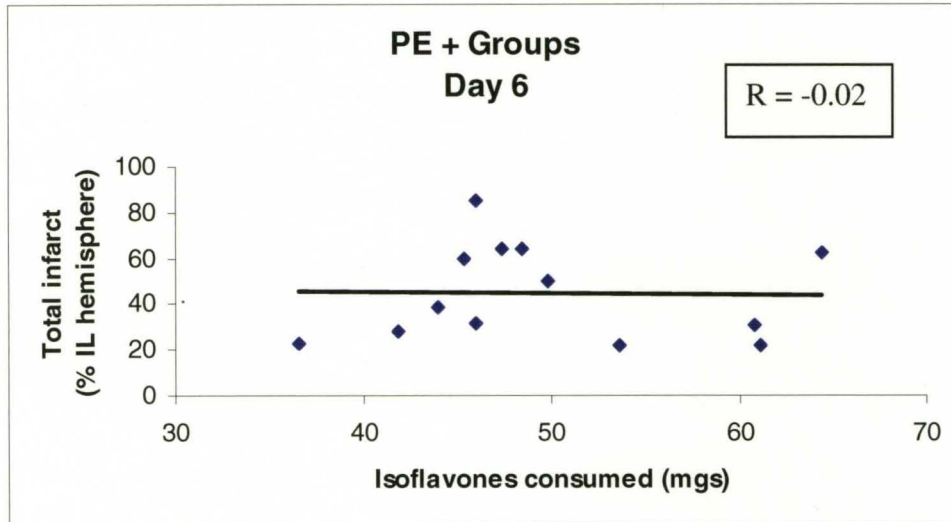


Figure 34. Correlation between mg isoflavones and resulting total infarct volume.

To determine if varying E2 levels introduced variability into the infarct volume data set, Pearson correlations were performed (see Figures 35 and 36). R values were 0.01 for cortex infarct volumes and 0.08 for total infarct volumes indicating no correlation between circulating estrogen levels at time of sacrifice and resulting cortex or total infarct volume.

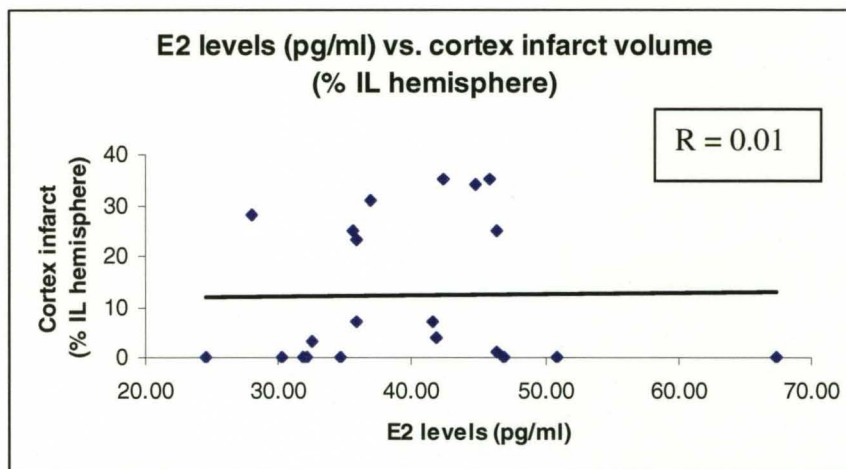


Figure 35. Correlation between E2 levels and resulting cortex infarct volume.

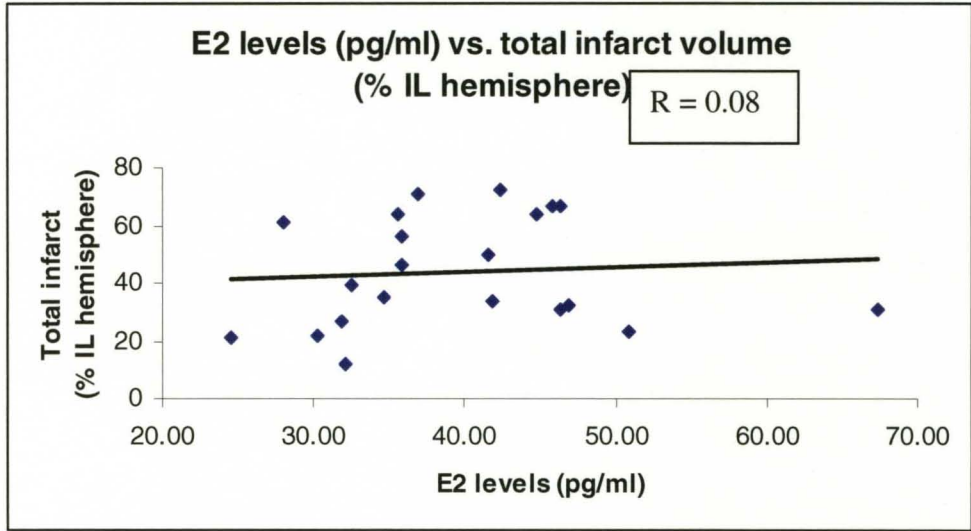


Figure 36. Correlation between E2 levels and resulting total infarct volume.

CONCLUSIONS

This body of work was performed to provide insight into six specific questions as follows.

1. Are physiological levels of E2 neuroprotective, as measured by infarct size after MCAo, in reproductively senescent rats?
2. Are dietary PE neuroprotective in reproductively senescent rats?
3. What are the combined anti-ischemic effects of E2 and PE?
4. Do physiological levels of E2 and/or dietary PE treatment alter PTEN and/or p-Akt expression compared to controls in the MCAo model?
5. Is PTEN/p-Akt activity altered in neurons or in astrocytes following MCAo?
6. Does the MCAo procedure upregulate PTEN/p-Akt activity as measured by comparing IL hemisphere protein levels to those of the corresponding CL hemisphere?

Throughout this section, findings for each question will be discussed and placed in context with the overall hypothesis of this study.

Are physiological levels of E2 neuroprotective in reproductively senescent rats?

The present study demonstrated that physiological levels of E2 delivered via silastic capsule to aged female Sprague-Dawley rats does not result in neuroprotection against pMCAo. No statistically significant differences in infarct volumes of striatum, cortex, and total infarct measured as mm³ and % of total IL hemisphere were detected due to treatment. These results were very surprising, as the current study was designed based upon the established evidence (Dubal and Wise, 2001; Rau et al., 2003; and Wise et al., 2001) that E2 reduces ischemic infarct size, with the intentions of comparing the ability of PE to mimic E2. A vast amount of literature exists that attests to the neuroprotective effects of E2 against cerebral ischemia in rats, much of which is mentioned in the introduction of this thesis. The apparent disparity of results may be due in part to the numerous variables between infarct studies. When one examines the stroke research as reported in the literature, it doesn't take long to determine that there are many variations in actual experimental design. A focal infarct, mimicking stroke, can be induced by electrocoagulation (Bingham et al., 2005; Carswell et al., 2004; Farr et al., 2006), irradiation (Strom et al., 2009), chemically (Selvamani and Sohrabji, 2008), or by using an intraluminal monofilament suture (Choi et al., 2004; Dubal and Wise, 2001; Rau et al., 2003). The suture can be inserted into the right or left middle cerebral artery (MCA) with the left option reported as producing larger injury (Gao and Zhang, 2008). The intraluminal suture can be left in place for a permanent MCAo (Dubal and Wise, 2001; Bingham et al., 2005; Rau et al., 2003) or removed after a specified time to produce a transient MCAo (Burguete et al., 2006; Choi et al., 2004; Omori et al., 2002; Santizo et al., 2002). The MCA can also be exposed transcranially by removing a piece

of the zygomatic bone and the artery can be occluded with a microclip producing a transient MCAo (Theodorsson and Theodorsson, 2005). Permanent and transient MCAOs produce two very different pathological scenarios as the transient option incorporates reperfusion injury. An additional method for inducing brain injury is the four vessel occlusion method (Harukuni et al, 2001). This induces not focal ischemia, but global ischemia and models cardiac arrest instead of a stroke event. It involves complete hypoperfusion to the entire brain for a short duration followed by reperfusion. Animal models vary, including several species but mainly using mice and rats. Male and female rodents of all ages ranging from pups (Strom et al., 2009) to 18 months of age (Strom et al., 2009) are used, with the majority being young adults, defined as 2-4 month old females (Dubal and Wise, 2001; Farr et al., 2006; Zhang et al., 2009). A study by Wappler et al., 2010 found that the age of the model is a factor that worsens the neuronal cell loss and behavioral functions of aged gerbils following transient brain ischemia. Common characteristics of most studies are that females are ovariectomized and supplemented with some form of E2 replacement; however, the time from ovariectomy to MCAo procedure varies greatly as does the administration route and resulting dose of E2 produced. Common ways to deliver E2 include commercially available pellets (Toung et al. 2004; Rusa et al, 1999; Schreihofner et al., 2005; Theodorsson and Theodorsson, 2005; Vergouwen et al., 2000), injections (Pelligrino et al, 1998; Choi et al. 2005; Park et al., 2005; Raval et al., 2007), silastic capsules (Dubal et al. 1998; Dubal and Wise, 2001), and recently minipumps (Zhang et al., 2009). The timing of E2 replacement ranges from immediately (Dubal et al. 1998; Dubal and Wise, 2001) to 10 weeks post ovariectomy (Zhang et al., 2009) and doses vary by over a thousand fold as some are administered in

microgram/kg body weight concentrations (Dubal and Wise, 2001; Rau et al., 2003) while other are given in milligram/kg body weight concentrations (Carswell et al., 2004; Choi et al. 2005).

Strom et al. (2009) recently published a review of E2 effects on rat cerebral ischemia. They attempted to only include studies sharing a set criterion including rat models that have undergone ischemic injury with E2 administration as the only variable between groups. A placebo group and direct measurement of brain damage also had to be included in the design. Their search revealed sixty-six studies meeting these criteria. Of the sixty-six, fifty-six concluded that E2 was neuroprotective but surprisingly four articles reported no difference between placebo and E2 treatments (Farr et al., 2006; Goodrow et al., 2005; Santizo et al., 2002; Vergouwen et al., 2000) and six articles reported detrimental effects with E2 (Bingham et al., 2005; Carswell et al., 2004; Gordon et al., 2005; Harukuni et al., 2001; Theodorsson and Theodorsson, 2005; Yong et al., 2005). Neuroprotection from E2 was mainly seen in studies involving young female rats that had undergone surgically induced, thus instant, menopause with immediate low level E2 replacement (Dubal and Wise, 2001 and Strom et al., 2010). In contrast, detrimental effects were most often seen following the use of high pharmaceutical doses of E2 or commercial pellets that have been found to produce a “supraphysiological burst” of E2 when first administered (Singh et al., 2008 and Strom et al., 2010), or following prolonged E2 deprivation. Interestingly, a recent article by Lebesgue et al. (2010) showed that E2, administered intracerebroventricularly, immediately post tMCAo, was neuroprotective in middle aged rats that were chronically E2 deprived. This suggests that

the ability to respond to E2 is maintained both in aged and hypoestrogenic animals and that the problem may arise from the logistics of a systemic delivery system.

Since this study found no significant neuroprotection by E2, a closer examination of similarities between it and those studies reported in the literature as finding no effect or a negative effect is merited. Farr et al. (2006), found no significant differences in infarct volume, functional recovery in behavioral tests, or gross synaptogenesis between placebo and E2 treatment groups when ovariectomized 12 week old female rats were subjected to focal ischemia via intracranial electrocoagulation of MCA. A key point of this study was that rats experienced a two week E2 deprivation prior to MCA. They received E2 through a pellet. Harukuni et al. (2001) found that five minutes of ischemia produced by four-vessel occlusion resulted in equivalent neuronal damage in 12 week old ovariectomized, ovariectomized plus E2, or intact females. Ten minutes of ischemia produced significantly worse injuries in the ovariectomized +E2 group and intact group over the animals that were ovariectomized and not supplemented with E2. They also demonstrated a highly significant correlation between increased cell loss and E2 level, with higher levels of E2 (within physiological range ≤ 30 pg/ml) being associated with increased neuronal cell death. Gordon et al. (2005) ovariectomized two month old female rats and immediately implanted them with either an E2 or placebo pellet. Two weeks later all underwent pMCAO via intraluminal suture and were sacrificed twenty-four hours later. Brains were cut into 6 μ m sections, stained, and resulting infarct measured from 12.5 mm anterior to 0.05 mm posterior of the interaural line. The E2 group experienced infarcts that were 118 % greater than placebo (area of brain measured not

listed). They also measured a byproduct of lipid peroxidation, 4 hydroxynonenol (4 HNE), which damages proteins in neurons and glial cells. They found almost none of this compound in the contralateral hemisphere of study animals but found it significantly increased in E2-treated infarcted cortex. The volume of cortex tissue with increased 4 HNE extended beyond the primary infarct suggesting that mechanisms of damage extend beyond primary infarct area and more than one mechanism of damage is initiated by MCAo. Also, the ratio of 4 HNE positive cells to infarcted cells was greater in the E2 group than placebo, suggesting that E2 could be causing an overexaggerated response to the injury. Carswell et al. (2004) found that ovariectomized four month old female rats provided immediate E2 replacement via pellets in two concentrations, 0.025 and 0.25 mg per animal, suffered increased infarcts that were dose dependant following pMCAo by electrocoagulation. Rats with the lower dose pellet averaged 65% larger infarcts than placebo and those with the higher dose had a 96% larger infarct than placebo. Similarly, Bingham et al. (2005) followed this up by using the same experimental design, except in twelve week old female rats, and found that both low and high dose treatment groups experienced significantly more damage than placebo. A study by Theodorrson and Theodorrson (2005), found that infarct damage was doubled in the E2 treatment group vs. placebo when the brain was measured from bregma +4 through -4 in 12-14 week old female rats that were ovariectomized with immediate E2 replacement by pellet. Selvamani and Sohrabji (2008) compared damage from a chemical-induced MCA occlusion model in middle aged (9-11 month) retired breeders and 6 to 7 month old active breeder Sprague-Dawley rats. This procedure caused a gradual constriction of the MCA lasting around 16 hours followed by reperfusion. All animals had previously been

overiectomized with immediate E2 replacement via pellet. They found that both cortical and striatal lesions were significantly greater in older females compared to younger females. These were also significantly worse in the middle aged group with E2 versus the middle aged control groups. These results suggest that E2 exacerbated the lesions over placebo in middle aged animals. Zhang et al. (2009) found that three month old female Sprague-Dawley rats overiectomized with immediate E2 supplement via a subcutaneous minipump had significantly lower infarct volumes, decreased NADPH oxidase activity, and superoxide levels, in specific regions of the hippocampus than placebo when subjected to global ischemia. However, when deprived of E2 for 10 weeks and implanted with pump one week before being subjected to global ischemia, all protection was lost. While cell survivability reached 50% (extrapolated from graph) in the animals receiving immediate E2 treatment, it dropped to 10% (extrapolated from graph) in animals deprived of E2 ten out of eleven weeks prior to ischemic event. In this instance there was no difference between placebo and the long term E2-deprived group treated with delayed E2 replacement.

These studies suggest that E2 loses its ability to protect neurons against ischemic damage after long term deprivation (2 weeks-10 weeks of hypoestrogenism) and in older animals, which are likely to have experienced periods of E2 deprivation as a normal aging process. Delivery of E2 via a slow release pump doesn't negate neuroprotection associated with E2 after a 10 week deprivation (Zhang et al., 2009). Such findings are in agreement with studies such as the WHI in which many participating women were several years beyond menopause (Alving, 2004). Negative effects are also commonly

seen with very high replacement doses of E2 after prolonged deprivation; however, this study did not use such doses. Relevant findings to this study are that older animals provided physiological levels of E2 via slow release replacement have been found to be at no advantage over controls and in some instances suffer worse damage.

Additional effects of 17- β Estradiol that may confound infarct measurements

Several articles published after this study was designed and implemented cite both protective and damaging effects of E2 that are not directly associated with a primary response to infarct but yet may very well influence measured infarct volumes in ancillary ways. These findings are relevant to explaining discrepancies between the results of the current study to those of the model study by Dubal and Wise (2001). For example, Brown et al. (2008) found that low dose E2 administered via silastic capsule downregulated nitric oxide production resulting from induced NOS2 gene expression in mice following permanent MCAo in both sham and injured cortex, suggesting that one mechanism by which E2 can exert neuroprotection is by downregulating critical components of the inflammatory response. Along the same lines, Park et al. (2005) found that, compared to age matched ovx females, young 2-3 month old intact females and ovx females with E2 replacement expressed lower levels of inducible nitric oxide synthase (iNOS), ICAM 1 (a leukocyte adhesion molecule) and NADPH subunit gp91^{phox} (presumed free radical producer), suggesting less inflammation. Crosby et al. (2007) found that activated caspase 12 was significantly increased in infarcted areas in E2 treated animals compared to controls. Caspase 12 is deregulated by endoplasmic reticulum stress and leads to increased apoptotic cell death. A corresponding increase in

terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (tunnel) staining in infarcted areas was also found, indicating increased DNA fragmentation. These studies all support the ability of E2 to regulate various components of inflammation following ischemic insult. In some instances E2 appears to be anti-inflammatory while in others it actually increases inflammatory responses and apoptosis. All above mentioned studies were performed in young animals. A very recent study by Traub et al., 2009 demonstrated neuroprotection from E2 delivered via a commercial pellet in aged rats that were subjected to transient global ischemia eight weeks post ovx. This discrepancy may be due in part to the mechanisms underlying neuronal death following focal and global ischemia models as well as, the differences in E2 action in the areas of the brain primarily damaged by the two procedures (Traub et al., 2009). Acute inflammation may play more of a role in damaged from focal ischemia than global ischemia (Traub et al., 2009 and Suzuki et al., 2007). The secondary effects of E2-mediated inflammatory changes on aged, E2-deprived brains following focal permanent MCAo is still to be determined but are most likely real and present.

Comparing this study to the widely-referenced Dubal and Wise 2001 paper

When comparing the model of ischemia studied in this project to all studies currently in the literature, not many exist with enough similarities for outright comparison. In fact, a thorough review of the literature revealed only one study that utilized silastic capsules for E2 delivery and performed permanent MCAo via intraluminal occlusion on middle aged rats. That study is the widely-referenced 2001 Dubal and Wise paper entitled *Neuroprotective effects of estradiol in middle aged female*

rats, the very one upon which this study was based, which demonstrates neuroprotective effects of E2 at low and high doses in both young and middle aged female rats. Both studies used 9-12 month old females that were ovariectomized and immediately implanted with silastic capsules containing either a total concentration of 180 ug/ml 17 β -estradiol (Sigma St. Louis, MO) or vehicle (sesame oil, Sigma). One week post ovariectomy and implant treatment, all rats underwent permanent MCAo via the intraluminal suture method (Longa et al. 1989). Unlike the Dubal and Wise study, this study used a commercially available suture with preformed bulb for MCAo in an attempt to decrease the variability introduced by forming the bulb via melting the tip of the suture (Gordon et al., 2005). Dubal and Wise (2001) do not reference creating such a bulb on the end of the intraluminal suture, but they do describe using a fire tipped suture in mice in a later paper (Dubal et al., 2006). In the present study, suture insertion length was measured from the bifurcation of the carotid artery and determined by animal weight. Dubal and Wise (2001) do not mention exact length of suture inserted. They do reference inserting the suture until resistance is felt.

A major deviation from the Dubal and Wise study is the amount of brain tissue that was measured for infarct. Dubal and Wise sectioned the entire brain (bregma +4.2 through -3.8) into *1mm* sections using a brain matrix while this study sectioned the brains into *20 μ m* sections and calculated total infarct from bregma +2.2 through -2.8 only. The rationale for using this area is two-fold: it reduces the variability introduced by the anatomical differences in the branching pattern of the MCA, as 95% of animals have the frontal and parietal branches that serve this area, thus providing a better measurement of

primary infarct damage with subsequent apoptosis of the cortex. In turn, it should decrease the contributions of inflammation and other E2-mediated confounds by eliminating brain tissue adjacent to but not part of the primary infarct territory. Following permanent MCAo, cells of the striatal core undergo necrotic cell death primarily as a result of hypoxia-induced glutamate excitotoxicity (Storm-Mathisen et al., 1992; Zerangue and Kavanaugh, 1996; Danbolt, 2001). The overlying cortex is less severely damaged due to collateral circulation and thus is subjected to cell death via apoptotic rather than necrotic pathways. Because apoptotic cell death is the outcome of intracellular events occurring inside a single cell and does not involve the release of chemicals into the interstitial area, primary apoptosis doesn't significantly contribute to the expansion of the infarct beyond the affected area (Crosby et al, 2007). Thus any cortex damage resulting from primary infarct-induced apoptosis should not be outside of the primary area of damage, although it is realized that other factors may be at work within this area of cortex. While it is impossible to tease out all the processes that are occurring within the primary affected cortex, one thing seems certain: any damage that is measured outside the area of the MCA territory cannot be attributed to primary infarct or infarct-induced apoptosis and must be mediated by other pathways and processes. Thus, a whole brain measurement could be misleading, as it accounts for numerous effects. The current study attempted, as much as possible, to tease out primary damage from other secondary contributors by limiting the measured area to that part of the brain served by the MCA in 95% of animals and the corresponding overlying cortex. This difference in amount of brain measured may contribute to the stark difference in reported outcomes between the current study and that of Dubal and Wise (2001). Interestingly, bregma +2.2

through -2.8 is the area later used and described by the same lab group (Rau et al., 2003) as the region that contains the area that undergoes injury with MCAo.

Table 12. Comparison of present study design to Dubal and Wise, 2001

Parameter	Present Study	Dubal and Wise, 2001
Animal model	9-12 month old female Sprague-Dawley rats (retired breeders)	9-12 month old female Sprague-Dawley rats
Overiectomized	Yes	Yes
Implants	Silastic capsule loaded with 180 $\mu\text{g/ml}$ E2 or sesame oil vehicle	Silastic capsule loaded with 180 $\mu\text{g/ml}$ E2 or sesame oil vehicle
Permanent MCAo and scarified 24 hours post	Yes	Yes
Left or Right MCAo	Left	Right
Suture size	4/0 Suture with preformed bulb on end	3/0 monofilament suture with heat tipped bulb
Suture insertion length	Measured from bifurcation of common carotid according to animal weight	Inserted until resistance met
E2 blood levels	40 pg/ml	20 pg/ml

Histological preparation	Brains were sectioned on a cryostat into 20 μm slices and stained with Nissl stain.	Brains were sectioned into 1mm coronal sections using a brain matrix and stained with TTC
Length measured	Bregma +2.2 to -2.8 = 5 mm	Bregma +4.2 to -3.8 = 8 mm

Table 13. Comparison of results of present study to Dubal and Wise, 2001

Infarct Measurement	Dubal and Wise oil results*	Present Study Oil results	Dubal and Wise low E2 results*	Present study low E2 results
Striatum infarct	75 mm ³	88 mm ³	70 mm ³	95 mm ³
Cortex infarct	100 mm ³	46 mm ³	40 mm ³	49 mm ³
Total infarct	250 mm ³	134 mm ³	140 mm ³	144 mm ³

*- extrapolated from graph

The low E2 results are essentially the same between the two studies. The largest difference is in the oil treatment cortex and thus total infarct volume. The average volume of the total ipsilateral hemisphere within the measured area of bregma +2.2 to -2.8 in this study was 279 mm³. To reach a total infarct volume of 250 mm³ would require an average 90% infarct throughout the entire measured area. Thus the differences seen in the oil treatment infarct volume may be attributed to secondary neuronal death occurring

in brain areas rostral and caudal to the primary area served by the MCA and captured via the extended measurement area utilized by Dubal and Wise (2001).

Are dietary phytoestrogens neuroprotective in reproductively senescent rats?

The present study demonstrated that dietary levels of PE equivalent to a prototypical Asian diet fed to aged female Sprague-Dawley rats did not result in neuroprotection against pMCAo. Infarct volumes of striatum, cortex, and total infarct did not differ from control groups. This contrasts the findings of Schreihofner et al. (2005) in which similar PE diets were found to be neuroprotective following pMCAo in young (age extrapolated from weight) female Sprague-Dawley rats. In the same study, isoflavones were found to be less effective than estrogen in reducing infarct size after MCAo in young rats. In light of those findings, it is not surprising that, given that no significant E2 effect was found in the present study, PE treatment was not found to be protective. No other references were found that examined the effects of dietary PEs against ischemic injury in an aged animal model. A few studies exist that have studied the possible mechanisms for the PE-based neuroprotection reported in young animals. Lovekamp-Swam et al. (2007) tested several parameters found to be affected by estrogens in stroke recovery (Rau et al., 2003). They found that in female rats (age not specified) subjected to transient MCAo, PE decreased DNA fragmentation, reduced caspase 3 activity, and increased the expression of antiapoptotic genes in the ischemic area. The remaining literature concerning the effects of PE on neuronal damage has been done *in vitro* using rat embryonic day 18 cortices. Results suggest that soy isoflavones can reduce cell death in cultured cells exposed to glutamate toxicity and oxygen glucose deprivation (Scheihofner and Redmond,

2009). PE results were similar to those seen with E2. The present study hypothesized similar results as those obtained by Schreihof et al. (2005). While the methods were essentially the same, accountable differences include animal age, reproductive status, and estrogen levels. Recent literature attests to these variables being major factors in the ability of estrogen or estrogen-like compounds to negate ischemic injury.

What are the combined anti-ischemic effects of E2 and PE?

There were no apparent agonistic or antagonistic effects of PE on E2 or vice versa on infarct size, as the combination was statistically no different from either treatment alone or from control. This was the first study to examine the combined effects in an aged model following pMCAo.

Do physiological levels of E2, dietary PEs, or ischemia alter PTEN and/or Akt expression?

No change was seen in PTEN or p-Akt levels following E2, PE, or a combination with one exception: PTEN when double labeled with Gfap did demonstrate a significant difference in levels between the IL and CL hemispheres in the E2-/PE- group when compared to groups that received either E2 or PE but not both. It has yet to be determined, however, if this differs significantly from zero. This parallels the non-protective infarct results of this study but is in contrast to studies cited in the introduction of this paper, as well as several recent publications. Cai et al. (2009) found p-Akt to be significantly upregulated in the cortex 24 hours after ischemic event; however, PTEN was not significantly upregulated over sham until 3 days post procedure (Cai et al.,

2009). When Scheihofer and Redmond (2009) used a PI3 kinase inhibitor, all neuroprotection imparted to embryonic 18 day old neuron cultures against oxygen glucose deprivation by genestein, daidzein, and equol was lost, suggesting that PEs mediate neuroprotection, at least in part, via the Akt pathway. A recent study by Anastasius et al. (2009) showed that human breast cancer cells, which were positive for estrogen receptors, had a diminished response to estrogen after a long-term genestein exposure (10 weeks). Genestein levels within the range of those measured in humans on high soy diets significantly inhibited E2-stimulated growth and downregulated the Akt signaling pathway, a dominant growth stimulatory pathway in human breast cancers (Anastasius et al., 2009). While E2 has been shown to activate the Akt signaling pathway, at least in part via ER α (Lee et al., 2005), physiological doses of genestein decreased Akt pathways in the absence of any change in ER α expression (Anastasius et al., 2009), suggesting that genestein could be mediating the Akt signaling pathway via a different mechanism than does E2. Alternatives include binding to ER β , or extranuclear receptors, or inhibiting tyrosine kinase, the last of which is a known action of genestein at high doses (Akiyama and Ogawara, 1991). These papers indicate that the interplay between E2 and PEs in regulating Akt pathways may be, at least in part, non-genomic. However interesting the interactions may be, the results of this paper allude to the fact that any control over the PTEN/Akt pathway seen in young animals or in cell cultures by low level E2 and/or dietary PEs is lost in aging brains subjected to infarct. We also found no differences in PTEN or Akt measurements between IL and CL hemispheres, indicating that the procedure itself did not upregulate these proteins as was reported by Omori et al. (2002).

Is PTEN/Akt expression altered in neurons or astrocytes following MCAo?

PTEN/Akt activity occurred predominantly in the neurons, instead of the astrocytes, of the ischemic cortex following focal pMCAo. In fact, when double labeled with Gfap, the proteins were visible in a pattern resembling the shape of the somas of neurons and clearly outside of astrocytes. This is in agreement with Omori et al. (2002) and Cai et al., 2009, in which PTEN levels were found to be elevated in cortical neurons following tMCAo. It contrast the findings of Choi. et al (2005), in which PTEN and Akt proteins were found in astrocytes of the hippocampus following transient four vessel occlusion. Since PTEN immunoreactivity has been found to be high in both cortex and hippocampus of normal rat brains (Cai et al., 2009), the difference in tissues studied is not likely to account for this discrepancy.

Conclusion

The hypothesis of this study was that *Physiological levels of 17 β -estradiol (E2) and dietary soy phytoestrogens (PE) produce similar but not additive effects on primary ischemic damage and apoptotic pathway regulation by PTEN/Akt, producing an outcome of neuroprotection in aging female systems.* While E2 and PE did produce similar effects, neither was associated with neuroprotection, thus, the hypothesis is rejected. This study is important, however, because it adds substance to the growing body of reports that E2 does not mitigate neuronal damage due to primary infarct and it does not positively influence PTEN/Akt apoptotic pathways in aged female rats. It also provides a more realistic model for putting into context the negative effects documented in

epidemiological studies such as the WHI, in which hypoestrogenic women suffered more frequent and detrimental stroke events when given E2 replacement. The complex effects of E2 on the brain is yet to be determined. However enough multiple effects and numerous neurological processes have been identified to declare it a complex set of interactions. Which specific mechanisms predominate in a given study may largely depend upon type and severity of injury, treatment paradigm, and age of model. Evidence is mounting in support of the case that E2 levels seem to determine which pathways are activated, while reproductive age and hypoestrogenic status seem to determine how the model can respond at the classic receptor level, in mediating inflammation, in regulating various apoptotic pathways, and/or via non-genomic pathways, many of which are still not identified at present. Despite literature support for the neuroprotective effects of E2 and PE, data from this study supports a lack or loss of neuroprotection in middle aged females when treated with physiological levels of E2 via slow release capsule and/or normal dietary levels of PE. This study is the first to demonstrate non-neuroprotection against ischemic insult when supplementing E2 by slow release capsule immediately post ovx. To date there have been no published findings in which low levels of slowly released E2 via silastic capsules have been associated with either a negative or no significant effect in middle aged females after permanent MCAo induced by the intraluminal filament method.

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CURRICULUM VITAE

Karen Powell

CURRENT POSITION : Staff Veterinarian and Assistant Professor,
University of Louisville School of Medicine

EDUCATION

- Graduate student, University of Louisville, seeking Ph.D. in biology with emphasis on neuroendocrinology and the molecular mechanisms of environmental endocrine disruptors, expected completion date summer 2009 (3.75 GPA)
- M.S. in biology with emphasis on immunology and parasitology, May 1998, Western Kentucky University (4.0 GPA)
- D.V.M., June 1994, Auburn University College of Veterinary Medicine (3.4 GPA)
- B.S. in chemistry and biology, May 1990, Western Kentucky University (3.2 GPA)

PROFESSIONAL EXPERIENCE

Clinical Experience

Practicing Veterinarian Graystone Pet Hospital – AHAA certified (July 1994 – January 1997)

Associated technical skills: various orthopedic and soft tissue surgeries; chemotherapy, total parental nutrition, and various fluid therapy administration; advanced ocular treatments; diagnostic testing including urinalysis, blood chemistries, plain and contrast radiography, ECGs, and cultures with antibiotic sensitivities; jugular, peripheral venous, and arterial catheter placements; histopathology/cytology including bacterial, fungal, and parasite identification; preventative medicine; internal medicine; preparing specimens for shipment to specialty diagnostic labs; patient referrals; administration of various intramuscular and gas anesthetics; utilization of scheduled drugs; safe handling of zoonotic pathogens; AHAA, OSHA, USDA, and FDA compliance regulation; client education; staff management; emergency services; and aseptic technique/quarantine.

Research Experience

Ph.D. research, University of Louisville (started March 2006) – *The effects of estrogens and/or plant based estrogens on occlusive stroke recovery in a post menopausal Sprague Dawley rodent model*

Associated technical skills: performed ovariectomies, middle cerebral artery occlusions, thoracotomies, and intracardiac blood collection; made and surgically placed extended release hormone delivery implants; administered isoflurane and ketamine/xylazine anesthetics; completed food consumption studies; administered buprenorphine and buprenorphine analgesics; paraformaldehyde perfusions; tissue cryopreservation; cryostat sectioning; immunofluorescent antibody studies; nissl staining; brain infarct analysis; cell cultures; and utilized the biological toxin iberiotoxin.

M.S. research, Western Kentucky University (January 1997 – May 1998) -

Evaluation of the success of adoptive versus passive transfer of immunity against Trypanosoma cruzi in C3HeB/FeJ mouse model.

Associated technical skills: worked with a biohazard level two pathogen; administered intraperitoneal injections; monitored parasitemias; harvested and processed whole spleen extracts for adoptive transfer; harvested and processed serum for passive transfer; daily health and environment monitoring; cervical dislocations; collection of peripheral blood samples; maintaining breeding pairs; sexing offspring; and all aspects of rodent husbandry.

Student research assistant, Auburn University (Summer 1992) – student employee
in Dr. Eva Sartin’s lab

Associated technical skills: neurofibroma and other neoplastic cell collections,
setting up and maintaining neoplastic cell cultures, preparing media, and sterile
techniques.

Student research assistant, Auburn University (Summer 1991) – student employee
in Dr. Ralph Paxton’s lab

Associated technical skills: study of the activity of the liver enzyme branched
chain alphaketoacid dehydrogenase; harvesting tissues from slaughter house;
purifying enzymes via chromatography; and enzyme identification utilizing
radioactive phosphorus

Teaching Experience (other than current position)

- Teaching Assistantship, University of Louisville (Spring 2006)
- Adjunct Professor, Western Kentucky University, (1998-1999)
- Teaching Assistantship, Western Kentucky University, (1997-1998)

Courses Taught

- Biology 242 Principles of Biology lab (U of L Spring 2006)
- Biology 131 Anatomy and Physiology class (WKU 1998 - present)
- Biology 131 Anatomy and Physiology lab (WKU 1997 - present)

- Biology 275 Pathophysiology (WKU 2000 – 2005)
- Biology 275 Pathophysiology Web Course (WKU 2006 – present)
- Biology 113 General Biology class and lab (WKU 1998 – present)
- Human Anatomy for non-science majors (WKU 1998)
- Zoology lab (WKU 1998)

Other Appointments

- Human Subjects Review Board (2001–present)
- Director of the Regional Science Resource Center (2000–2007)
- Director of the Consortium for Evidence Based Research in Rural Educational Setting (CEBRRES) (2004–2006)
- University Promotion and Tenure Review Committee (2006-2008)
- Guidelines for Tenure and Promotion Revision Committee, BGCC (2005–2007)
- Southern Association for Colleges and Schools (SACS) On Site Focus Groups (2005)
- WKU Faculty Search Committees - BGCC Dean (2006), Biology (2004-2005), Health Care Information Systems (2004-2005 and 2007-2008), Geography (2003-2004), Liberal Arts and Science Division Chair (2003 -2004), Biology – chair of the committee (2002-2003), and History (2000-2001)
- Task Force for Diversity (2004-2007)
- University Assessment Committee (2003 –2005)
- University Diversity Committee (2002 –2004)

- Chair of the BGCC Faculty Awards Committee and member of the University Awards Committee (2003)
- NASA Steering Committee for Saturday Academy Reform (2003)
- Text book reviewer for Addison Wesley/Benjamin Cummings (2002 and 2007)
- President's Award for Diversity Selection Committee (2002)
- Academic Probationary Committee (Fall 2001)

Continuing Education

Conferences

- Gordon Research Conference: Environmental Endocrine Disruptors (2008)
- Society for Behavioral Neuroendocrinology Annual Meeting (2006, 2007)
- Politics of the Yellowstone Ecosystem (2006)
- NEH Landmarks of American History and Culture Workshop – Mammoth Cave: People, Place, and History (2006)
- The Kentucky Council on Post Secondary Education Annual Conference (2002, 2004, 2005)
- Human Anatomy and Physiology Society Annual Conference (2003, 2005)
- Kentucky Academy of Science (1999, 2004)
- The Funds for the Improvement of Post Secondary Education (FIPSE) Annual Project Directors Conference (2001, 2002, 2003)
- All One Nation Conference (2003)

- 14th International Conference on College Teaching and Learning (2002)
- National Association of Biology Teachers Annual Conference (2001)
- Action Agenda Teacher Educational Summit I and II (2001, 2002)

Lectures/Workshops

- IACUC Level II Training (2005 with online renewal in 2008)
- Preventing Sexual Harassment (2007)
- Being Efficient and Effective: Teaching Based on Research on Learning (2007)
- The One Minute Manager Meets the Monkey (2007)
- Family Education Rights and Privacy Act (2007)
- Why We Get Sick: The New Science of Darwinian Medicine (2007)
- Emerging Zoonotics (2006)
- Estrogen: The Mother Earth of all Hormones (2006)
- Scholarly Writing and Proposal Writing (2005)
- Blackboard Training (2003, 2005)
- Women in Science and Engineering Leadership Workshop (2004)
- Blood Borne Pathogen Training (2003)
- Teaching Portfolios (2003)
- NASA Program Review and Focus Group for Saturday and Summer Academies (2003)
- Legal Aspects of Instruction (2000)
- Staying Legal - Research Compliance Issues (2000)

- After the Award: Administering your Grant (2000)
- Faculty Evaluations: What I need to know for Tenure (2000)
- Maintain Academic Quality and Still Meet Students Needs (2000)
- Searching for Funding Opportunities Online and Preparing Grant Budgets (1999)

Invited seminars and posters

The effects of estrogens and/or plant based estrogens on occlusive stroke recovery in a post menopausal Sprague Dawley rodent model (Invited poster)

- Gordon Research Conference (2008)
- Society for Behavioral Neuroendocrinology (2007)

How I use the instructor's resource CD rom, Interactive Physiology Modules, and blackboard to teach an all inclusive one semester A and P course

- Human Anatomy and Physiology Society Regional Conference (2006)

Infusing your classroom with critical thinking

- WKU (2008)

Intersexuality

- Liberal Arts and Sciences Diversity Awareness Lecture (2005, 2006)

A successful collaboration between middle school and post secondary education communities the Regional Science Resource Center at the Bowling Green

Community College of Western Kentucky University

- Kentucky Academy of Science (2004)
- Funds for the Improvement of Post Secondary Education (FIPSE) Annual Project Directors Conference (2003)
- Bowling Green Community College Board of Advisors (2002)
- Council on Post Secondary Education Action Agenda Teacher Educational Summit II (2002)
- 14th International Conference on College Teaching and Learning (2002)

The Consortium for evidence based research in rural educational setting (CEBRRES)

- The Kentucky Council on Post Secondary Education Annual Conference (2005)

Using the Biology Place interactive website in general education biology classes

- Benjamin Cummings Strategies workshop *Hands on the Biology Place*, University of Kentucky (2001)

Professional and Community Service

- Volunteer for Brigadoon State Nature Preserve (2007 – present)
- Member of The Friends of the Clarks River (2007 - Present)

- The Regional Science Resource Center (2000 –2007)
- Member of the Southern Kentucky Regional Science Fair Board (2000 – 2006)
- Chair of the Science Review Committee and Institutional Review Board for the Southern Kentucky Regional Science Fair (2000-2006)
- Co-host of the Southern Kentucky Regional Science Fair (2003-2006)
- Science fair judge for various schools including Drakes Creek Middle School, North Metcalfe Elementary, and Metcalfe County High School (2002-2006)
- Co-director of the NAACP/NASA Science and Math Saturday Academy (2002 and 2003)
- Grand Awards Judge for Medicine and Health at the Intel International Science Fair, Cleveland, Ohio (2003)
- Hosted and taught science components for various WKU K-12 programs such as Science Olympiad, Girls in Science, Super Saturdays, Vampy, Whitney Young Scholars, and Project Aims (2000 –2006)
- Hosted programs with and taught science components for various community K-12 groups including the Bowling Green Housing Authority, Clark’s River National Wildlife Refuge, Kentucky State Nature Preserves, Warren Juvenile Detention Center, Barren River Home School Association, Gear Up, Girl Scouts, Boys and Girls Club of Butler County, Mammoth Cave National Park, Region Two Service Center, Green River Regional Educational Cooperative (GRREC), NASA, and the NAACP (2000 –2006)

- Hosted various K-12 teacher professional development opportunities including; Problem Based Learning in Middle School Science, Bowling Green Elementary Teacher PD seminar, Region Two Science Academy follow ups, and the Regional Science Resource Center / Region Two Service Center Midsummer Science Challenge (2000 –2006)
- National proctor for Emergency Medical Technicians (2000 –2005)
- Co-sponsored “Funding your Best Ideas” presented by Joan Straumanis of the Department of Education’s Funds for the Improvement of Post Secondary Education (2002)

Professional Societies

- Kentucky Veterinary Medical Association (1994 –1998 and 2006 - present)
- Society for Behavioral Neuroendocrinology (2006 - present)
- American Veterinary Medical Association (1990 –1998)
- WKU Biotechnology Center (2006-present)
- Human Anatomy and Physiology Society (2001 – present)
- Kentucky Academy for Science (2000 – 2002 and 2004 – 2005)
- National Association for Biology Teachers (2000-2002)

Honors

- WKU Exemplary Achievement Award (2005)

- Auburn University College of Veterinary Medicine “Young Achiever” Award (2004)
- Bowling Green Human Rights Commission Woman of the Year in Science (2003)
- SGA Excellence in Teaching (2002)
- Community College Award for Public Service (2002 and 2006)
- Outstanding M.S. graduate student (1998)
- Presidents scholar M.S. graduate of Western Kentucky University (1998)
- Cum Laude graduate of Auburn University College of Veterinary Medicine (1994)
- Whitley Ophthalmology Award Auburn University College of Veterinary Medicine (1994)

Publications

Powell, K (1998) Evaluation of the success of adoptive versus passive transfer of immunity against *Trypanosoma cruzi*. *M.S. thesis*.

Grants Funded

- National Wildlife Foundation Friends Group Grant (2008-2009: \$10,000)
- National Wildlife Foundation, “Nature of Learning” Grant (2004-2007: \$11,000)

- Environmental Protection Agency, “Expanding Environmental Discovery within Rural Communities” (2005-2006: \$30,000)
- Environmental Protection Agency, “Expanding Environmental Discovery within Rural Communities” (2006-2008: \$41,741)
- The Department of Education’s Funds for the Improvement of Post Secondary Education (FIPSE), “Regional Science Resource Center (RSRC)” (2000–2004: \$295,411)
- Provost’s Action Agenda, “Staff Position” (2001–2004: \$58,346)
- Provost’s Action Agenda, “Midsummer Science Challenge” (2002-2003: \$18,489)
- Provost’s Action Agenda, “RSRC Vehicle” (2002-2003: \$11,800)
- NAACP/NASA “Saturday Academy” (2002-2003: \$5200)
- Provost’s Action Agenda, “RSRC Continuation Operational Expenses” (2004-2006: \$25,000)
- Teacher Education Action Agenda, “CEBRRES Pilot” (2004-2006: \$86,661)
- Provost’s Action Agenda, “Outreach Assessment” (2004-2005: \$11,605)
- Enterprise Community/Bowling Green Housing Authority, ‘Centering on Science’ (2004–2005: \$13,000)