Oppong-Gyebi, Anthony, The role of aging and length of hypogonadism on the neuroprotective effects of dietary genistein following focal cerebral ischemia. Doctor of Philosophy (Biomedical Sciences), May 2021, 226 pp, 8 tables, 30 illustrations, bibliography, 537 titles

The risk of ischemic stroke increases with increasing age. Women beyond menopause have an exponential increase in stroke risk with worse post-stroke prognosis and mortalities compared to men of similar ages. One of the key reasons for this discrepancy is the sudden and drastic drop in the levels of the circulating principal female sex hormones estrogen and progesterone after menopause. Both sex hormones have been shown in several studies to provide neuroprotection against ischemic insults in stroke models and other disease models including Alzheimer's Disease and Parkinson's Disease. However, from clinical studies, neither estrogen nor progesterone alone or in combination has met clinical needs for the prevention of chronic cardiovascular diseases. These clinical failures were mainly evidenced by the absence of benefits in the human population or an increased predisposition to adverse side effects. Reports from studies including the Women's Health Initiative and Nurse's Health Study showed that the timing of initiation and age of recipients significantly influence the outcome of estrogen therapy.

In this dissertation project, we investigated the plant-based estrogenic compound genistein as a possible alternative to estrogen therapy. It was hypothesized that the neuroprotective benefits of genistein will be less sensitive to the length of hypogonadism and age under experimental ischemic conditions. We used a rodent model of transient middle cerebral artery occlusion under varied lengths of estrogen deprivation and age to test the neuroprotection of dietary genistein. Findings from this dissertation show that early initiation of dietary genistein after hypogonadism improves aspects of cognition, an effect that is diminished following the long absence of circulating

estrogen. Furthermore, pre-treatment with dietary genistein improves age-associated locomotor deficits after long-term hypogonadism after stroke.

This dissertation, therefore, provides new considerations on the time-dependent sensitivity of the brain to genistein's effect as a potential therapeutic option to improve aspects of cognition and reduce the severity of stroke in the target population with low circulating estrogens.

THE ROLE OF AGING AND LENGTH OF HYPOGONADISM ON THE NEUROPROTECTIVE EFFECTS OF DIETARY GENISTEIN FOLLOWING FOCAL CEREBRAL ISCHEMIA

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# THE ROLE OF AGING AND LENGTH OF HYPOGONADISM ON THE NEUROPROTECTIVE EFFECTS OF DIETARY GENISTEIN FOLLOWING FOCAL CEREBRAL ISCHEMIA

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

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Fort Worth, Texas

May 2021

# ACKNOWLEDGMENT

I thank God for the good life, wisdom and resources I have been gifted with thus far to complete this journey. It all belongs to Him.

A very special thank you to my professor and mentor Dr. Derek A. Schreihofer for your selfless contributions and guidance, financial support, and patience throughout this period. I am most grateful for prioritizing my Ph.D. training and ensuring I made the best of my time in school and the resources available to me to shape me into the scientist I am today.

To my committee members, Dr. Meharvan Singh, Dr. Nathalie Sumien, Dr. Shao-Hua Yang and Dr. Xiangrong Shi, thank you for your willingness amidst your busy schedules to serve on my academic committee. I appreciate your support, constructive feedbacks, guidance and letters of reference throughout this journey.

Thank you, Dr. Rebecca Cunningham, Dr. Stella Goulopoulou and Dr. August Woerner for your contributions in diverse ways to make my Ph.D. training successful. A posthumous acknowledgment for my late undergraduate supervisor and mentor Dr. George A. Koffuor for your training that boosted my interest in science for which I made the efforts to pursue my Ph.D.

To past and present members of the Schreihofer Lab and lab managers who helped with my training, thank you for your contributions.

To my family and friends whose support saw me through this journey, with notable friends Deborah, Ella and Johnny, thank you very much for your prayers, sacrifices and encouragement to realize this moment.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's Disease |
| AngII | Angiotensin II |
| ATR1 | Angiotensin II Receptor Type 1 |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived-neurotrophic-factor |
| CBF | Cerebral blood flow |
| CCA | Common carotid artery |
| CCL2 | C-C Motif Chemokine Ligand 2 |
| CD16 | Cluster of Differentiation-16 |
| CPP | Cerebral perfusion pressure |
| E2 | Estrogen |
| ECA | External carotid artery |
| GAP43 | Growth-Associated Protein 43 |
| HSP70 | Heat Shock Protein 70 |
| IACUC | Institutional animal care and use committee |
| ICA | Internal carotid artery |
| IL-1β | Interleukin-1β |
| LDL | Low-density lipoproteins |
| NHANES | National Health and Nutrition Examination |

| | |
|---|---|
| OVX | Ovariectomy |
| OEF | Oxygen extraction fraction |
| PARP | Poly-ADP ribose polymerase |
| PD | Parkinson's disease |
| PPAR-γ | Peroxisome proliferator-activated receptor subtype gamma |
| Pgrmc1 | Progesterone-receptor-membrane-component-1 |
| ROS | Reactive oxygen species |
| TGFβ1 | Transforming growth factor- β1 |
| TNF-α | Tumor necrosis factor alpha |
| tPA | Tissue-type plasminogen activator |

# CHAPTER 1

## 1 Stroke

The brain accounts for about 2% of the body's total mass, yet it consumes about 20% of the blood's oxygen, accounting for almost 15% of cardiac output [1]. Stroke, less commonly referred to as a cerebrovascular accident, occurs when there is a sudden loss of function (ictus) due to an inadequate supply of oxygen and nutrients to the brain. There are several types of stroke including ischemic, hemorrhagic and subarachnoid hemorrhage [2]. Ischemic stroke accounts for approximately 87% of all stroke cases in the US and results from a complete blockade by a clot or atheroma of a major artery that supplies a target brain region [2, 3]. Hemorrhagic stroke accounts for 10% of all stroke cases and is caused by a rupture of a vessel due to acute blood pressure elevations or conditions including brain tumors and coagulation disorders that make blood vessels less pliable [2, 4, 5]. Subarachnoid hemorrhage makes up for the remaining 3%, and it is caused by vascular bed leakage from aneurysms unto the brain surface. The loss of function due to stroke invariably results in permanent neurological and motor deficits, while increasing the risk of death depending on the stroke type and brain region affected [6].

### 1.1 Stroke Demographics

According to the National Health and Nutrition Examination Survey 2013-2016, the national stroke prevalence in the United States hovers around 2.5%. That translates into an estimated 7 million Americans who are 20 years old or more [7]. It was projected in 2012 that by 2030, there would be an approximate 20.5% increment in stroke prevalence in the adult population accounting for nearly 3.4 million additional cases [8]. It is estimated that 795,00 new and recurrent strokes

occur annually with a stroke happening every 40 seconds. Stroke incidence and prevalence increase with increasing age in both males and females. On average, 10% of all stroke cases happen between the ages of 18 and 50 years with about 75% occurring in persons over 65 years [9, 10].

Following hypertension control efforts since the 1970s and increased awareness about cardiovascular health, the U.S. population has seen a significant drop in stroke incidence and its accompanied mortalities [11-13]. Even though recent data further show a declining overall stroke incidence [14, 15], aging and cumulative cardiovascular risk factors temporally increase lifetime cerebrovascular disease risk. According to the Global Burden of Disease 2016 Lifetime Risk of Stroke Collaborators, the overall lifetime stroke risk increased from 22.8% in 1990 to 24.9% in 2016 [16], Ethnically, stroke prevalence is highest among the female black population (3.8%) and lowest in non-white Asian males (1.1%). This suggests an ethnicity-related discrepancy in stroke considering that the Framingham Heart study between 1990 and 2012 had previously reported a decline of stroke, one that was mainly seen in the white population [14].

## 1.2 Pathophysiology of Ischemic stroke

There is a limited supply of oxygen and nutrients to the brain, hence, to maintain normal brain function, there is a need for substantial perfusion which allows adequate delivery of both oxygen and required nutrients as well as optimum clearance of waste materials. For these reasons, it is important to ensure a tightly controlled vascular system to sustain the basic functions of the brain. Atherosclerosis and high blood pressure are the two most common underlying pathologies for cerebrovascular disease [17].

### 1.2.1 Atherosclerosis

Atherosclerosis serves as the main underlying pathology for both coronary heart disease and cerebral artery disease [17]. Atherosclerosis is initiated by the attachment and trapping of low-density lipoproteins (LDL) in the endothelial wall [18]. The attached LDLs undergo oxidation by reactive oxygen species (ROS) leading to recruitment and flux of leukocytes to the affected arterial wall [19, 20]. This subsequently results in endothelial dysfunction, a key and early step in atherosclerosis formation. Previous evidence in apolipoprotein E-deficient ($ApoE^{-/-}$) mice suggests that atherosclerosis is characterized by chronic inflammation due to elevated oxidative stress, a finding which is attributed to the activity of NADPH oxidase 2 [21, 22]. Further experiments to establish a strong linkage between oxidative stress and vascular dysfunction showed that the ROS scavenger tempol and NADPH oxidase inhibitor apocynin can both reverse impaired nitric oxide responses in $ApoE^{-/-}$ mice endothelial walls [23, 24]. These suggest the involvement of Nox2-derived superoxide in the development of atherosclerosis (Figure 1.1). Large vessel strokes as evident mostly in ischemic strokes have involvement of atherosclerotic component [25]. Atherosclerosis causes stroke through two main processes: cardiac-related events (coronary/aortic arch atherosclerosis) and carotid-related events (carotid atherosclerosis), both of which produce emboli that result in end vessel occlusion and stroke [26, 27]. A strong association between aortic atherosclerotic plaques and ischemic stroke has been established through several case-control and pathology studies. Amarenco et al. and Khatibzadeh et al. have shown through pathology studies that ulcerated aortic plaques were significantly associated with cyptogenic strokes and arterial embolism [28, 29]. This observation is further strongly associated with the thickness of plaques, mostly $\geq$ 4mm independent of carotid stenosis and atrial fibrillation [28-30], hence establishing the integral involvement of atherosclerosis and its extent in stroke. Carotid atherosclerosis, a blockade within the carotid artery, especially the internal carotid artery results

in a sudden endothelial wall breach from atheromatous plaque accumulation leading to hemorrhage and ulcerations. The ensuing endothelial damage promotes platelet aggregation and the activation of clotting factors to cause thromboemboli that further restrict blood flow through end vessels, hence the occurrence of stroke [31, 32].

### 1.2.2 Hypertension

Hypertension negatively affects cerebral flow and poses a key risk factor for cerebrovascular disease, cognitive decline and dementia [33]. There is ample evidence to show the formation of atheromatous plaques within cerebral vessels and the involvement of NADPH oxidase-mediated oxidative stress in hypertension-induced cerebral circular dysfunction [34-38]. Recently, Angiotensin II (Ang II) has been shown to play integral roles in cellular changes and endothelial remodeling during chronic hypertension, hence a major contributor to stroke [33, 39]. Blood pressure and ROS levels are elevated by Ang II through the production of peroxynitrite within the vascular walls [38, 40-42]. Ang II further has been reported to influence inflammation induced by hypertension. Elevated Ang II causes leukocyte and platelet adhesion in the cerebral vasculature depicting that immune cells are involved in the etiology and progression of hypertension (Figure 1.1) [43]. In spontaneously hypertensive rats, inhibition of Angiotensin II Receptor Type 1 (ATR1) has achieved a substantial reduction in cerebral inflammation associated with stroke [44, 45]. These pieces of evidence suggest that oxidative stress and Ang II-induced inflammation underlie chronic hypertension which increases predisposition to cerebrovascular disease. Furthermore, chronic hypertension results in compromised integrity of the arterial smooth muscle cells. This is due to progressive damage and replacement of the tunica media by fibrous collagenous tissues that could lead to the occlusion of the endothelial wall and subsequently lacunar strokes [46]. An increase in stroke risk with increasing blood pressure has been identified to be continuous and

progressive for which an optimal reduction can be beneficial to the hypertensive individual [47]. It is established that instituting blood-lowering medications including angiotensin-converting enzyme blockers, calcium channel blockers, beta-blockers and diuretics significantly reduce stroke risk (35-44% reduction) across all ages [48]. However, just a small fraction of the global population (25%) responds adequately to antihypertensive medications [49]. The differences in response to antihypertensives and a potential reduction in stroke thereof stem from health disparities among age groups, ethnicity, and the presence of comorbidities, mostly within the minority populations [10]. For these reasons, special attention needs to be paid to such minority groups to make available more patient-specific precision medications. This will improve the

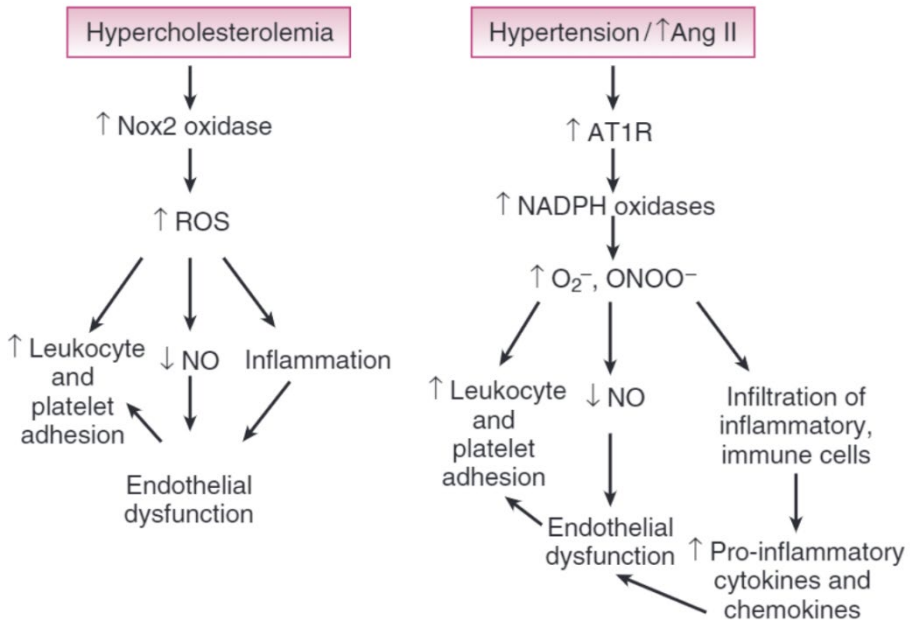


**Figure 1.1. Schematic diagram of Hypercholesterolemia, Hypertension and Angiotensin II on vascular effects** (Adapted from Stroke Pathophysiology, Diagnosis and Treatment 6th edition)

efficacy of antihypertensives with the goal of reducing cardiovascular risk including stroke.

### 1.2.3 Changes in Cerebral Blood Flow during Ischemic Stroke

The brain has intrinsic compensatory mechanisms that ensure enough cerebral blood flow over a wide range of cerebral perfusion pressure (CPP) between 70 and 150 mmHg, a phenomenon referred to as autoregulation [50]. However, when CPP falls below the lower limit of autoregulation, a drastic drop in cerebral blood flow (CBF) ensues [51]. Under conditions of low CBF (<20ml/100g/min), increased vasodilation occurs to maintain optimum cerebral oxygen extraction fraction (OEF) and normal brain function over long periods [52-54]. When OEF can no longer sustain low CBF and CPP to maintain the structural and functional integrity of the brain, a series of deleterious biochemical processes are kickstarted. First, there is a progressive decline in protein synthesis followed by a disruption of normal cellular function. Neuronal electrical excitability is impaired and diminished over time, ATP stores are depleted, pH drops due to lactate accumulation and ultimately cell death ensues, all of which will be described in detail in the next section [6, 55, 56].

### 1.2.4 Ischemic cell death sequelae (Figure 1.2)

Neuronal and glial cells depend almost exclusively on aerobic respiration for energy production and maintenance of normal physiological function [57]. Under low CBF (<20ml/100g/min), well below that which changes in OEF can compensate for, an impaired ionic gradient across the cell membrane occurs due to depleted ATP stores at the core of the stroke [56]. This leads to anoxic depolarization that triggers the release of large amounts of glutamate into the extracellular space [6, 55]. Failure of the ATPase pumps leads to $Na^+$ and $Cl^-$ influx accompanied by the passive flow of water resulting in edema [6]. Within the penumbra of stroke, the energy stores are also depleted but to a lesser extent hence, anoxic depolarization and $K^+$ efflux may be absent [56]. The glutamate released further causes an elevation of intracellular $Ca^{2+}$ by interacting with glutamate receptors mainly *N*-methyl-D-aspartate receptor (NMDARs) and **α-amino** -3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPARs) [55]. The high intracellular $Ca^{2+}$ promotes activation of calcineurin, a serine-threonine phosphatase that dephosphorylates Bad a pro-apoptotic protein [55, 58, 59]. Bad in turn binds Bcl-2 and Bcl-xL to release bound Bak and Bax in the mitochondria whose units coalesce to form channels in the mitochondrial membrane. The channels formed allow the intrusion of cytochrome c which initiates the apoptotic process through the activation of downstream caspases [60]. Necrosis as another fate of unsalvageable ischemic cells, with necroptosis as a more specific neuronal necrotic process slightly differs from apoptosis and ensues

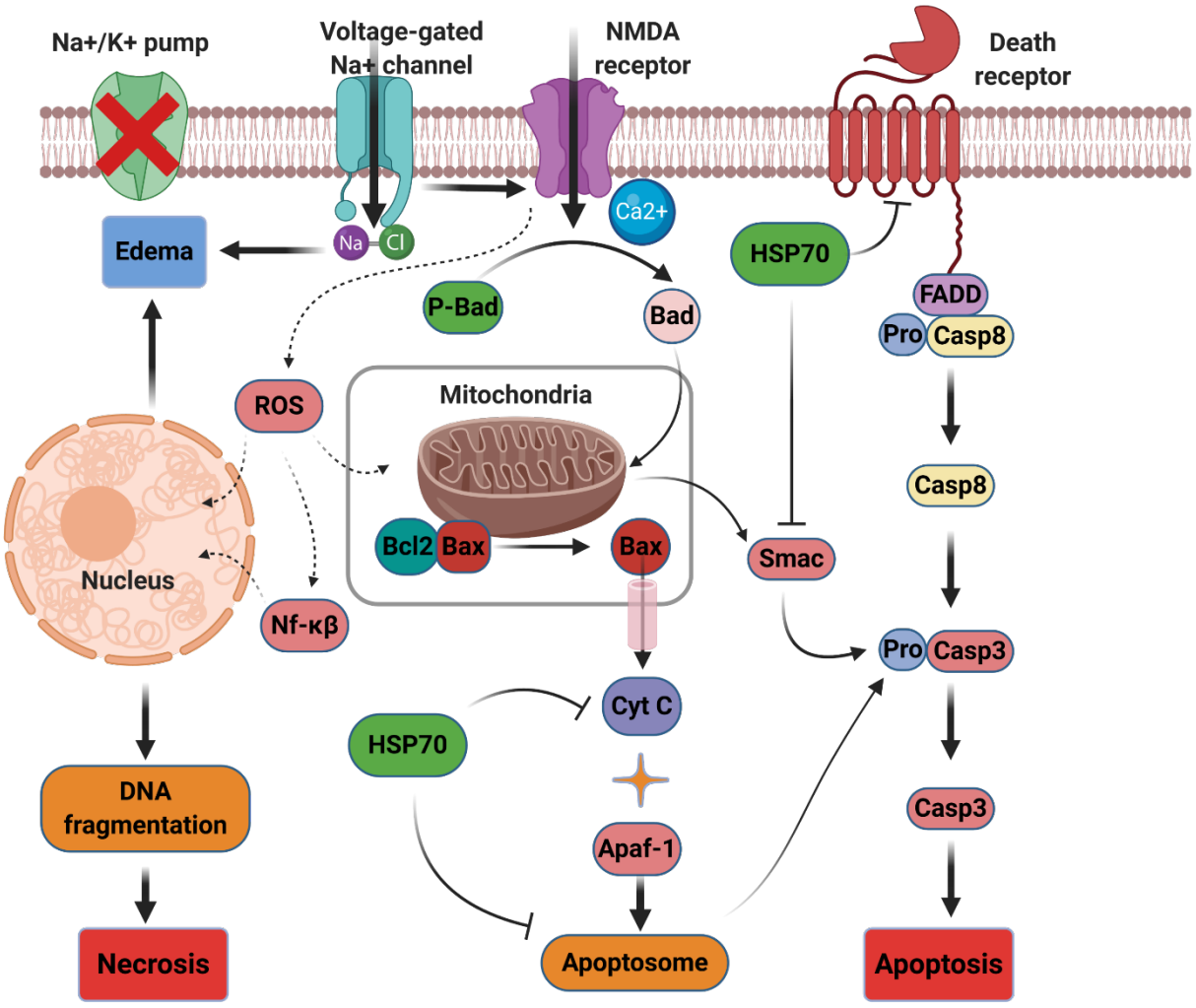


**Figure 1.2 Diagrammatic representation of ischemic stroke cell death sequelae** (Created with Biorender)

in a caspase-independent manner [61, 62]. This involves swelling of the cell and disruption of its organelles coupled with clumping of its genetic materials, hence an ultimate loss of the cell's integrity [62-64]. The Tumor Necrosis Factor-Tumor Necrosis Factor Receptor-1 (TNF-TNFR1)-mediated pathway with receptor-interacting protein (RIP) has been proposed as the signaling cascade that governs the process of necroptosis. Under ischemic conditions, both apoptosis and necrosis may be antagonized by growth factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) through the PI3K/Akt pathway to preserve neuronal function [65]. Autophagy also provides another mammalian cell death endpoint. This is a catabolic process characterized by the generation of energy from the digestion of organelles within cells with the involvement of lysosomes [66, 67]. Autophagy is a homeostatic mechanism that allows energy-deprived cells to reallocate and redistribute resources from less energy-demanding processes to more pro-survival activities [68, 69]. Unlike necrosis, autophagy is not characterized by chromatin condensation nor DNA fragmentation [70]. Rather, it involves the fusion of autophagosomes and lysosomes to confer cytotoxic effects or possible cytoprotective effects as seen in the case of ischemic injury [71, 72].

### 1.2.5 Post-stroke inflammation

Ischemic stroke induces an inflammatory response that may persist for days or even weeks after the initial injury [73-75]. This inflammation can be likened to that which is presented by the activation of microglia and astrocytes within the brain [76]. Large amounts of proinflammatory cytokines including TNF-α (tissue necrosis factor-alpha), interleukin-6 (IL-6) and interleukin 1β (IL-1β) are highly expressed at the site of injury in both animal models and humans [74, 76, 77]. The proinflammatory cytokines promote the overexpression of cell adhesion molecules and selectins which in turn cause increased transmigration of leukocytes to the brain parenchyma to

worsen the initial injury [76, 78]. Several studies have shown the detrimental effects of post-stroke inflammation. This experimental evidence has been demonstrated in several ways that include leukocyte depletion, inhibition of adhesion molecules with antibodies and prevention of the interaction between leukocytes and selectins [79-83]. The mechanisms by which post-stroke inflammation and immune response exacerbate ischemic injury are thought to involve the generation of ROS and reactive nitrogen species (RNS) as well as macro and microvascular occlusions due to the infiltrating leukocytes and activated platelets [78, 84, 85]. Based on the extent of ischemic injury, brain-resident microglia undergo intra-transformation between the classical

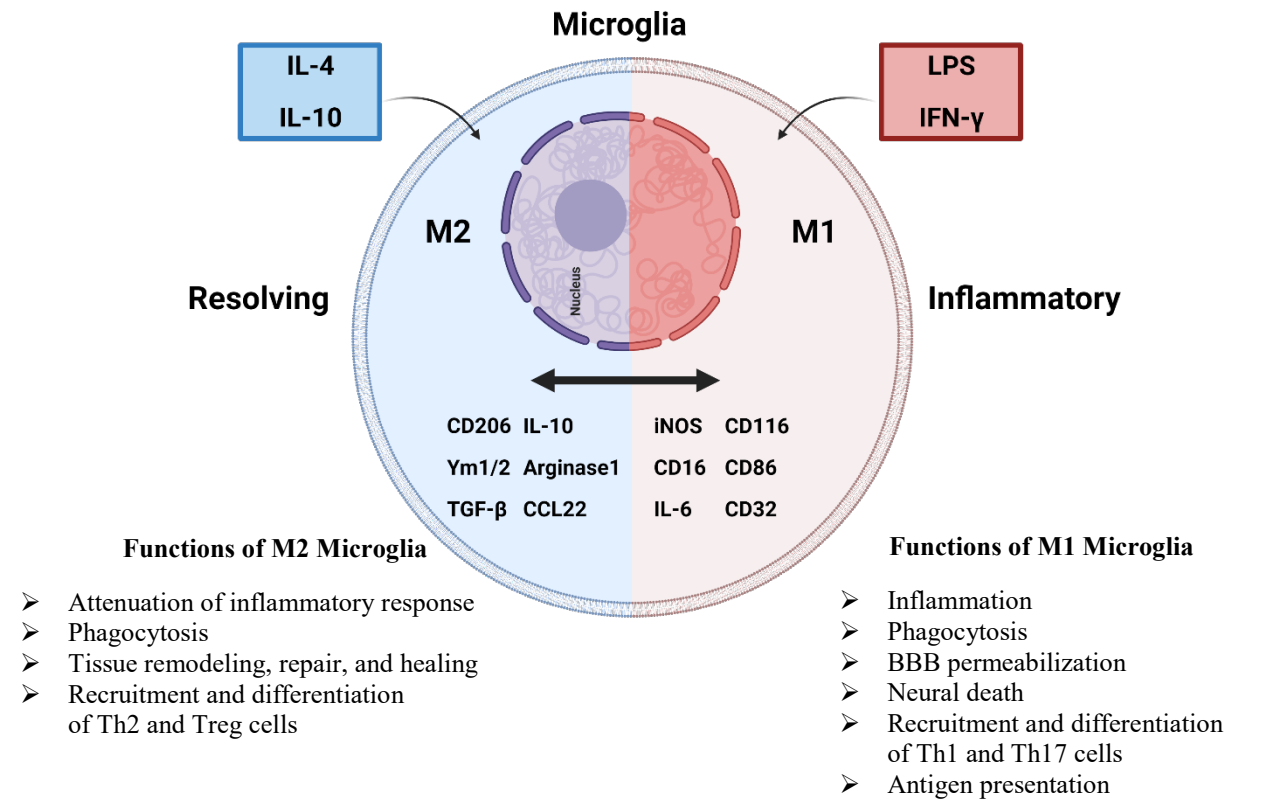


**Figure 1.3 Intra-transformation of Microglial phenotypes following activation.** Proinflammatory cytokines and inducers including lipopolysaccharides (LPS) and interferon-gamma (IFN-γ) promotes M1 phenotype manifestation to cause inflammation, blood-brain barrier (BBB) permeabilization. Anti-inflammatory cytokines including interleukin-4 (IL-4 and 10) on the other hand promote M2 phenotype to attenuate inflammation while promoting tissue repair). CD-cluster of differentiation, TGF-transforming growth factor, CCL22-C-C motif chemokine 22, iNOS-inducible nitric oxide synthase (Created with Biorender)

pro-inflammatory M1 phenotype or alternative pro-restorative M2 phenotype (Figure 1.3) [86]. The extent of brain damage has been linked to the microglia phenotype and associated inflammatory response at play during and after the ischemia [86-91]. Considering the bimodal action of microglia, M2 phenotype can prove vital in the prognosis of stroke and functional outcome [92].

#### 1.2.5.1 Interleukin-6 (IL-6) as a poststroke pro-inflammatory marker

Interleukin-6 (IL-6) is globally induced in the brain following ischemia [93]. Its induction mainly occurs in neurons and microglia within the ischemic hemisphere, the ischemic hippocampus and to an extent, the contralateral cortex [94]. As an integral component of ischemic injury, the heightened IL-6 production is not fully understood, but it is thought that they are synthesized from endothelial cells, microglia and astrocytes [94, 95]. Differential effects of IL-6 have been observed under ischemic conditions where it presents as both a pro- and anti-inflammatory cytokine. In IL-6 deficient mice, the inflammatory response of astrocytes and microglia to ischemic injury was substantially reduced, suggesting IL-6 mediates a pro-inflammatory response in the pathophysiology of stroke [96]. On the other hand, Loddick et al demonstrated that administering IL-6 confers neuroprotection against ischemic stroke that depicts the anti-inflammatory properties of exogenous IL-6 [93]. The contrasting findings on IL-6 suggest that more exploration in the field is required to ascertain the clear-cut roles of IL-6 during stroke.

#### 1.2.5.2 Involvement of Transforming growth factor-β1 (TGF-β1) in poststroke inflammation

Transforming growth factor-β1 is mostly confined to the epithelium and meninges of the choroid plexus under physiological conditions [97]. However, its expression pervades the brain following an insult. TGF-β1 mRNA levels are elevated after at least 15 mins focal cerebral ischemia with

the circulating levels dependent on the duration of the ischemia [98]. Previous findings have demonstrated that delivery of TGF-β1 before or after ischemic injury reduces infarct size thereby offering neuroprotection [99-103]. TGF-β1 is mainly derived from microglia and macrophages after stroke [104] with optimum expression at 1-week post-stroke and persists in the ischemic penumbra even up to 3 weeks after the initial injury [104]. Even though the exact mechanisms of TGF-β1's actions in the neurovascular unit are not fully elucidated, some studies have identified the involvement of plasminogen activator inhibitor- 1 (PAI-1) through an intermediate action of astrocytes [105]. More importantly, TGF-β1 reduces the differentiation and proliferation of microglia both *in vitro* and *in vivo* to rescue neurons from ischemic injury [106-108]. Furthermore, TGF-β1 induces overexpression of the anti-inflammatory proteins bcl-2 and bcl-x1 to confer neuronal survival, making it a therapeutic potential against detrimental inflammatory responses after stroke [109, 110].

#### 1.2.5.3 Growth-Associated Protein (GAP43) as a measure of functional recovery after stroke

Growth-associated protein 43 is produced during neurite growth and development within the hippocampus and cortical regions where it mainly modulates axonal growth and synaptic plasticity [111-114]. GAP43 concentrations within the brain are elevated especially at the ischemic region and its gene and protein expression can persist for several weeks post-stroke, making GAP43 a biomarker for stroke prognosis and recovery [115-118]. Expression of GAP43 during stroke has been linked with the expression and activation of microglia and macrophages during stroke. Madinier et al showed that decreasing microglia activation with 3-aminobenzamide as was detected with OX-42 staining was associated with decreased GAP43 expression following focal cerebral ischemia [119]. Shin et al also demonstrated that treatment of mice with noggin, a bone morphogenetic protein, increases microglial activation with an accompanied GAP43 elevation

within the ipsilateral injured brain [120]. However, the authors observed that the activation was mainly due to M2 microglia, a pro-restorative immune cell activation rather than the more pro-destructive M1 phenotype. This was evidenced by reduced markers for M1 including IL-1β, TNF-α, IL-12, C-C motif chemokine ligand 2 (CCL2) and CD86 (cluster of differentiation 86) and increased markers for M2 including interleukin-10 (IL-10), arginase 1, mannose receptor 1 (CD206) and Ym1 [120]. Conversely, a peripheral nerve injury study by Cobianchi et al showed that treadmill exercise, both short- and long-term, reduces microglia and astrocyte activation as were detected with CD11b and GFAP stains respectively while increasing the levels of GAP43 [121]. This observation was associated with the improvement in the regenerative and functional recovery processes [121]. Similarly, Noguchi et al demonstrated that treatment of nerve root avulsion injured rats with erythropoietin reduces microglia activation while significantly increasing GAP43 in the surviving motor neurons [122].

#### 1.2.5.4 Heat shock protein-70 (HSP70) and poststroke inflammation

Heat shock proteins play significant roles in how brain cells respond to ischemic injury [123, 124]. HSP70 has been identified as a key heat shock protein that is induced under stress conditions [125, 126]. The increased expression of HSP70 has been associated with events that cause protein denaturation including ischemia, heavy metal poisoning and acidemia [125, 127-129]. Due to reduced energy output within the ischemic core, HSP70 production at this site may not be apparent during protracted hypoxic/anoxic conditions [130, 131]. However, HSP70 mRNA is substantially upregulated with minimal occlusions in and around the ischemic core, mainly confined to the blood vessels, astrocytes, and microglia [132-134]. HSP70 protein is mostly expressed within the technically defined ischemic penumbra that surrounds the infarct core, an area where progressive protein denaturation is observed [132-134]. Induction of HSP70 has been identified as a

cytoprotective mechanism against ischemic injury even though the extent to which it protects largely depends on the magnitude of the initial injury [135-138]. HSP70's neuroprotection has been demonstrated in the hippocampal ischemic study by Plumier et al. where the authors observed protection by HSP70 overexpression within the hippocampus of HSP70-transgenic mice [139]. Raj et al recapitulated this observation where overexpression of HSP70 conferred neuroprotection against permanent middle cerebral artery occlusion in transgenic mice [140]. Contrasting effects of HSP70 have been observed depending on the region of its expression. Within the cytosol, HSP70 decreases pro-inflammatory response including Nuclear Factor κB, matrix metalloproteinases and ROS. However, in the extracellular matrix, HSP70 binds to toll-like receptors on macrophages, dendritic cells and microglia in the extracellular matrix to increase pro-inflammatory responses [141, 142]. Tyrosine kinase-P38-Mitogen-Activated Protein kinases signal transduction pathway has been demonstrated to be involved in HSP70 induction as evidenced by the attenuating effects of the specific tyrosine kinase inhibitor including the compound genistein and herbimycin A [143, 144]. HSP70 holds a therapeutical potential for treatment. However, several studies showing HSP70 involvement in ischemic stroke involved transgenic rodents, gene transfer or *in vitro* studies [145]. Hence more exploration on HSP70 should be done to provide more guidance that closely translates into clinical practice.

### 1.3 Sex differences in stroke incidence and mortality

Although the incidence of stroke has decreased significantly in high-earning countries in the last two decades, this observation was made in men [146, 147]. In midlife, between the ages of 35-44, there is a higher age-adjusted male: female incidence ratio for stroke [148]. However, increasing age results in a decline in the ratio with recent studies showing an upsurge in incidence rate amongst women, especially beyond the menopausal transition [148]. After menopause, women

between the ages 55 and 75 years have a 20% stroke risk increase compared to a 15% increase in men of similar ages, partly due to a higher life expectancy than men and also to physiological changes which diminish the neuroprotective mechanisms against ischemia [2, 149]. Again, women beyond 65 years have higher age-adjusted stroke-related death compared to men, are more likely to be hospitalized after stroke, are more liable to recurrent stroke within the first five years post-stroke and present with worse post-stroke prognosis [150-152]. Data from National Health and Nutrition Examination Survey (NHANES) - National Cardiovascular Disease Surveillance System between 1999 and 2020 further shows that women have a higher age-adjusted prevalence of stroke across most age groups and higher fold increases in stroke risk in the presence of modifiable risk factors including cigarette smoking, diabetes and heart disease compared to men [7].

### 1.3.1 Possible mechanisms underlying sex differences in stroke

To determine various strategies to identify the sex differences during stroke, it is important to investigate how both sexes differentially activate cell pro-survival and pro-apoptotic pathways in animal models. In *in vitro* and *in vivo* studies, the extent of cerebral injury following oxygen-glucose deprivation is a function of sex, compounded by age which is also an independent risk factor [153-155]. McCollough et al. showed that inhibition of neuronal nitric oxide synthase (nNOS), a component of the caspase-independent Poly-ADP ribose polymerase (PARP)-induced apoptosis cascade in adult male rats confers neuroprotection against stroke [156]. However, a similar inhibition in adult female rats worsens stroke outcomes [157]. When PARP-1 was inhibited by minocycline in a stroke model, young adult male mice had improved stroke outcomes while female rats did worse [158]. Differential responses have also been identified with the immune system activation following stroke. Female but not male mice express high IL-10-secreting $CD8^+$ cells after stroke, an immune response that worsens stroke [159, 160]. Further studies on caspase-

dependent cell death during stroke have also shown sexual differences. The extent of the injury was significantly attenuated when both neonatal and adult female mice were treated with the pan-caspase inhibitor, quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh)[155]. This neuroprotective effect was absent in male mice suggesting a sex-specific activation of caspase-dependent apoptotic pathways during stroke and a potential benefit of caspase inhibition in females [154, 155]. Nicotinamide Adenine Dinucleotide ($NAD^+$) has also been shown to differentially affect sex-dependent responses to cerebral ischemia. Male mice treated with $NAD^+$ showed improved stroke outcomes compared to wild-type female mice [161]. Other cell signaling cascades involving apoptosis-inducing factor (AIF) and angiotensin II receptor type 2 have also been shown to exhibit sexually dimorphic responses to cerebral ischemia [154, 157, 162-164]. Considering these sex differences, it is key to identify suitable and effective neuroprotective therapies that translate into bridging the gap between aging males and females regarding stroke in humans.

### 1.3.2 Menopause influence on stroke

Menopause is the age-associated absence of menstruation for 12 months or more. Ischemic stroke is low amongst premenopausal women as compared to men of similar age [165]. However, there is a sharp increase with increasing age during menopause and a two-fold increase in the risk within the second-decade post-menopause in addition to poorer prognosis post-stroke [166-170]. Neuroendocrine researchers attribute this observation to hormonal imbalances (Figure 1.4) coupled with other general age-related cardiovascular risk factors such as atherosclerosis. Female gonadal hormones (estrogen and progesterone) are neuroprotective against brain injury and neurodegenerative diseases [171, 172], and hence, their low circulating concentrations post-menopause have been implicated as one of the major culprits for increased ischemic stroke incidence [172]. Increased stroke risk after menopause in part, explains why the concept of

hormonal neuroprotection against cerebrovascular disease post-menopause has gained significant interest. However, hormone replacement therapy (HRT) has not substantially reduced overall stroke incidence. Interestingly, HRT was associated with enhanced stroke risk and severity in the Women Health Initiative and other meta-analyses [173-177]. For these reasons, there are intensified efforts to add to the limited preventative and clinical treatment options for ischemic stroke, especially in persons who may be ineligible for hormone therapy [178].

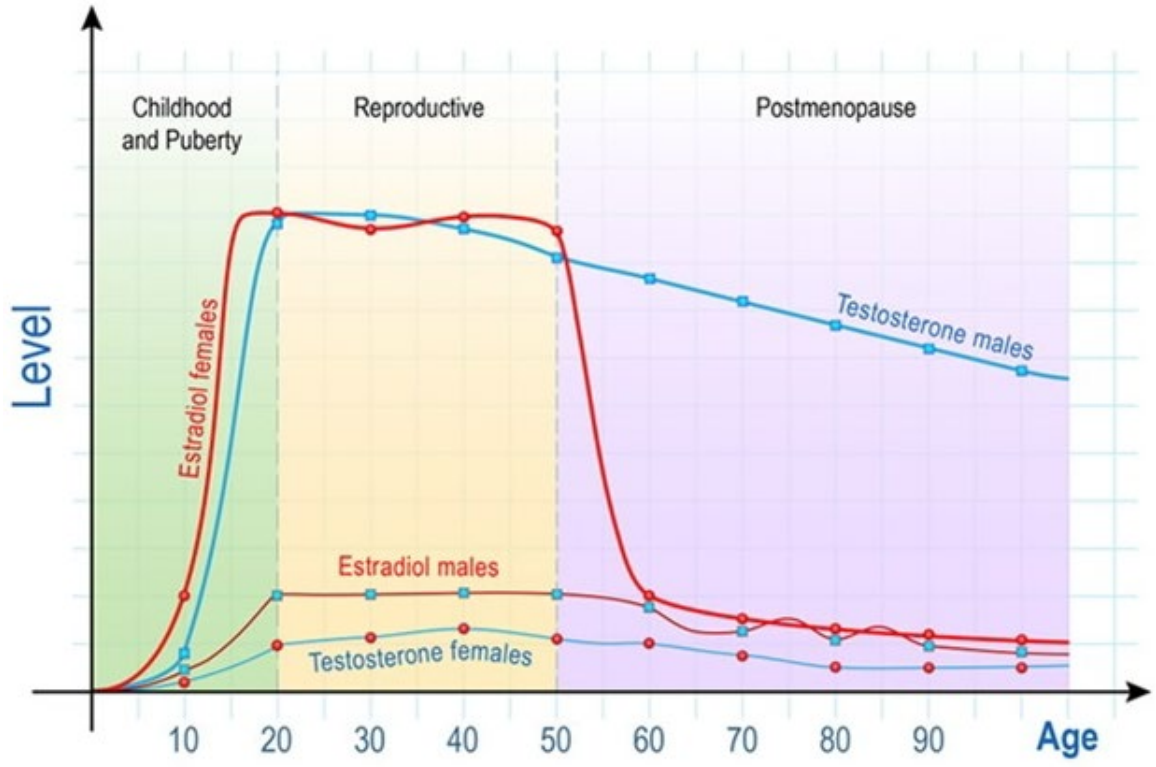


**Figure 1.4 Age-dependent time course of circulating estrogen and testosterone** (Adapted from Nature reviews | Genetics)

## 1.4 Neuroprotection concept

Under ischemic conditions, the ischemic core, the most vulnerable part of the brain parenchyma is almost unsalvageable when there is reduced blood flow for extended minutes into hours due to irreversible tissue neuronal damage. However, when blood flow is restored early or a pharmacologic agent is administered, progressive loss of neurons in a less susceptible region that

surrounds the core (the ischemic penumbra), can be protected from ischemic damage [179, 180]. As a consensus, efforts to protect the penumbra from the initial ischemic insult in the absence of an early restoration of blood flow can help maintain the basic functions for the sustenance of normal bodily activities, hence the concept of neuroprotection in the context of ischemic stroke [181]. Neuroprotection refers to therapeutic strategies that aim at preserving the morphology and functionality of brain cells under ischemic injury and other brain insults including trauma and toxins. [182-184]. Due to the numerous signaling pathways which are involved in ischemic stroke etiology, several neuroprotective agents have been investigated in animals, targeting one or more of such cascades for possible therapeutic use in the clinical setting. Notable among such neuroprotective strategies include nitric oxide production, inhibiting excitotoxic events, calcium influx inhibition, apoptosis inhibition and reactive oxygen species scavenging [185-187]. However, despite the advances made with understanding the molecular mechanism of ischemic cell death and the various promising neuroprotectants employed, they have not translated into clinical practice [184, 188]. Several reasons have been adduced to the failure of preclinical neuroprotective agents in human subjects [189]. First, the timing of administration of such agents is tightly regulated in the animal models unlikely in humans in the emergency room [190, 191]. In most animal studies, neuroprotectants are given shortly after induction of the stroke within the stipulated time window of treatment efficacy, contrary to the extended delays in real-world settings between the onset of stroke symptoms and treatment.

Another explanation for failure in clinical trials is that several, especially earlier studies, used young healthier animal models that do not necessarily reflect the pathophysiological state of the most susceptible middle-aged and old-aged populations who usually present with other comorbidities and lifestyles [190]. Again, the most popular ischemic stroke model employs the

middle cerebral artery in animal models. However human stroke encompasses a wide array of stroke types that include anterior, posterior and vertebrobasilar cerebral artery strokes whose manifestation and pathophysiology do not always mimic that of middle cerebral artery occlusions [192-194]. Pharmacokinetic limitations of the neuroprotective agents including inadequate dosing, the duration and frequency of administration among other human-specific changes of the neuroprotectants *vis-à-vis* that of rodents have also contributed to the failures of such agents in clinical trials and the clinical setting [195]. More recent findings of the anesthetic choices used and the duration of exposure in animal studies also add to the puzzle about why several neuroprotectants work pre-clinically but not in clinical trials [196]. Anesthetics such as the fluorinated ethers including isoflurane and sevoflurane have demonstrated neuroprotective effects in both *in vitro* and *in vivo* studies [197, 198]. Bicker et al. and others have shown that exposure of hippocampal slices to isoflurane provides protection against hypoxia [199, 200]. A combination of isoflurane and the caspase inhibitor z-VAD-fmk have achieved substantial cerebral infarction reduction as shown by Inoue et al [201]. These pieces of evidence show anesthetics used during stroke studies could confound the outcome, and hence, requires researchers to transparently report the conditions under which experimental procedures were conducted as suggested by The Stroke Therapy Academic Industry Roundtable (STAIR), regarding anesthesia use [202].

### 1.4.1 Stroke and neuroprotection with female sex hormones

Estrogen and progesterone, the principal female gonadal hormones have been extensively studied as neuroprotectants. There is ample evidence to show that both estrogen and progesterone are neuroprotective against ischemic insults and traumatic brain injury in preclinical studies [203-205]. This section focuses on the neuroprotective pathways of estrogen and progesterone and the challenges with hormone replacement therapy post-menopause.

#### 1.4.1.1 Estrogen-mediated neuroprotection

Estrogen is a multifunctional sex hormone in the body. It possesses several signaling mechanisms amongst which are the ones that confer protection on injured neurons in the brain [167]. Both classical estrogen receptor subtypes alpha (ERα) and beta (ERβ) and the membrane receptor G Protein-coupled Estrogen Receptor 1(GPER 1) have been described to mediate the neuroprotective effects of estrogen [206-210]. Estrogen, through nuclear signaling, increases the expression of brain-derived neurotrophic factor (BDNF) and the anti-apoptotic gene bcl-2 [211-213]. Estrogen is widely established as a preserver of mitochondrial function, preventer of oxidative insults and inhibitor of pro-apoptotic gene expression during an ischemia-reperfusion injury [214, 215]. Aside from the genomic influence of estrogen, estrogen is also known to possess extranuclear signaling which contributes to its neuroprotection. Estrogen has been shown in both *in vitro* and *in vivo* studies to protect the CA1 hippocampal and cortical neurons through the activation of extracellular signal-regulated kinases (ERK 1/2) and Akt involving its membrane receptor GPER 1 activation [216-219]. Insulin-like growth factor-1 (IGF-1) also plays an integral role in estrogen's neuroprotection. Studies have shown the capacity of estrogen to upregulate IGF-1 receptor mRNA and protein expression [220, 221]. Conversely, IGF-1 increases the expression of both estrogen receptor (ER) and IGF-1 receptor (IGF-1R) when given intracerebroventricularly, suggesting an interdependence of the signaling activities of ER and IGF-1R [222, 223]. Prominent amongst the convergent signaling cascades for ER and IGF-1R is the PI3K-Akt pathway. Both ER-alpha and IGF-1R activation can trigger the PI3K/Akt pathway which confers neuroprotection in the ovariectomized rat brain, further confirming the cooperative dependence of ER and IGF-1R [222, 224, 225]. These beneficial estrogenic effects partially explain the lower stroke incidence in women at reproductive age which may be lost after the menopause transition.

#### 1.4.1.2 Estrogen's neuroprotection in neurodegenerative diseases

Substantial evidence supports the neuroprotective role of estrogen against neurodegenerative diseases [226]. In humans, low circulating estrogens after menopause increase the risk of neurodegenerative diseases including Parkinson's Disease (PD), Stroke and Alzheimer's Disease (AD), effects of which are offset or reversed with estrogen replacement [227-230]. In ischemic stroke models, estrogen has been shown to adequately reduce ischemic injury in reproductively senescent female rats [231]. Similarly, estrogen treatment improves the symptoms of Parkinson's Disease and Alzheimer's disease in animal models, hence underscoring its neuroprotective benefits against neurodegeneration [232-235]. Amongst the mechanisms that mediate the protective effects of estrogen against neurodegeneration include apoptosis inhibition, beta-amyloid clearance and preservation of mitochondrial function [236, 237]. Estrogen reduces beta-amyloid secretion and accumulation in AD mouse models [233, 238-240] and ovariectomized mice [241] possibly through the inhibition of β-secretase 1 gene expression [242], regulation of amyloid precursor protein processing [243] and modulation of γ-secretase activity [244]. In rodent PD models, estrogen has been shown to reduce neurotoxin-induced dopamine depletion at the striatum in both mice and rats [232, 245], with ERα activation playing a critical role in the neuroprotective mechanism when estrogen is given before neurotoxicity induction [246, 247]. Even though the role of estrogen in improving symptoms of neurodegeneration and cognitive decline is not in contention, the parts played by estrogen receptor types are not fully established. ERβ knockout (KO), but not ERα KO mice showed spatial learning and memory deficits compared to wild-type animals [248, 249]. Furthermore, ERβ but not ERα agonist improved spatial learning performance in ovariectomized mice, depicting an integral role of ERβ in learning and memory [250]. On the contrary, ERα KO but not ERβ KO mice showed worse outcome after 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP)-induced PD compared to wild-type control, an observation which was attenuated by ERα but not ERβ agonist, suggesting the involvement of ERα in neuroprotection by estrogen [251, 252]. This evidence is corroborated by the differential expressions of ERα and ERβ in the brain with the former more predominant in the hypothalamus and amygdala while the latter is more abundant in the hippocampus and entorhinal cortex [253-255].

#### 1.4.1.3 Progesterone-mediated neuroprotection

Progesterone and allopregnanolone, its metabolite, exhibit neuroprotective effects through both genomic and non-genomic mechanisms involving the progesterone receptor (PR) [256, 257]. Increasing evidence from recent studies has shown that, like estrogen, progesterone is a potent neuroprotectant against ischemic injury [258-260]. In both male and female stroke models, progesterone reduces infarct sizes following transient focal cerebral ischemia [261, 262]. Genomic-dependent increased expression of BDNF following progesterone administration has been shown to play an integral role in progesterone-mediated neuroprotection [256, 263, 264]. As has been demonstrated with estrogen, Progesterone also confers neuroprotection to cortical explants through the activation of the phosphoinositide 3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAPK) pathways to increase BDNF expression [256]. Aside from the genomic effects of P4, it also works through its non-classical receptor, progesterone receptor membrane component-1 (Pgrmc-1) that can stimulate the release of BDNF against glutamate toxicity [256]. Pgrmc-1 is widespread in the brain with higher expression within regions including the hypothalamus, amygdala and hippocampus [265]. Pgrmc-1 serves as an adaptor protein, forms a complex with the membrane progesterone receptor type alpha (mPRα) to mediate progesterone-induced protection against apoptosis [266]. A more recent study by Sun et al demonstrated that Pgrmc-1 is involved in the release of BDNF to provide neuroprotection against cerebral ischemia

[267]. For these beneficial roles of Pgrmc-1 in progesterone's neuroprotective effects, it is key to identify agents that can increase its activity to improve stroke and other neurological disease outcomes. While progesterone has a promising potential for clinical application in ischemic stroke, having shown significant neuroprotection against ischemic brain injury in animal studies, its use is limited by a narrow therapeutic-time window [258, 268-270]. Further to that, progesterone's use is limited by its failure in the recent Progesterone for the Treatment of Traumatic Brain Injury (ProTECT) and Study of a Neuroprotective Agent, Progesterone, in Severe Traumatic Brain Injury (SyNAPSe) Phase III trials. The reasons for the negative results have been attributed to the complexity of traumatic brain injury, the quality of preclinical animal models and possible inappropriate clinical trial design [271]. For this, more robust clinical trial strategies moving forward would be relevant in identifying the brain's sensitivity to progesterone's neuroprotection.

#### 1.4.1.4 Effect of hormonal therapy on ischemic stroke incidence during menopause

Growing evidence from pre-clinical and human studies suggests that advancing age post-menopause plus long-term gonadal hormone deprivation significantly increases the loss of sensitivity of the neurons to estrogen's neuroprotection [208, 272-274]. It's observed that late initiation of oral conjugated equine estrogen (CEE) as either a single therapy or in combination with medroxyprogesterone acetate (MPA), 10 years post-menopause or beyond was associated with an increased incidence of both hemorrhagic and ischemic stroke[176]. Further evidence from the Women's Health Initiative also corroborated this pattern, where a late initiation of oral estrogen-only therapy was associated with an increased risk of stroke post-menopause[275]. Aside from the increased stroke risk, CEE plus MPA also increases the risk of developing breast cancer and myocardial infarction which explains the restrictions on HRT prescriptions for postmenopausal women[176]. A recent report, however, showed that early initiation of estrogen

therapy within 6 years but not beyond, reduced atherosclerosis in postmenopausal women expressed as carotid artery intima-media thickness[276]. Besides, a pooled analysis on the association between HRT initiation, type of HRT and risk of postmenopausal stroke showed that early detection of menopause and estrogen-only HRT initiation linearly related to a low incidence of stroke compared with never users[277]. Transdermal and vaginal routes of administration of HRT have also been reported to have a lower relative risk of ischemic stroke deaths compared to the oral route [278, 279]. Despite conflicting information on the safety and efficacy of HRT in the context of stroke, it is noteworthy to identify a suitable timing of initiation of HRT medication, type of HT, route of administration as well as dose [277] to meet the window of opportunity for HRT beneficial effects**.**

#### 1.4.1.5 Phytoestrogens and neuroprotection

Considering the limitations of estrogen therapy, several other agents are investigated as possible therapies for the management of menopause and its associated symptoms. Phytoestrogens are one set of compounds that have been studied extensively for their benefits post-menopause [280-287]. Phytoestrogens are plant-derived polyphenolic compounds unlike the endogenous steroid estrogen, but by their stereochemistry, can possess estrogenic as well as anti-estrogenic properties [288-290]. Soy isoflavones including genistein [291, 292] and daidzein represent a subclass of phytoestrogens that have demonstrated substantial estrogenic activity in several preclinical studies and further for menopausal therapy and cardiovascular diseases [293, 294]. Soy products rank among the most highly consumed food additives [295], especially in Asian regions where they are associated with lower risks of cardiovascular and cerebrovascular diseases, certain cancers and menopausal symptoms compared to the western world [296]. Genistein, daidzein and its metabolite equol consumption have been linked with a reduced risk of stroke and myocardial

infarction in countries where they are consumed in large quantities [297-299]. In animal studies, dietary genistein has shown potent neuroprotective effects against cerebral ischemia in both male and female stroke models. In rodents, a two-week pretreatment with genistein achieves an adequate reduction in infarct volume in young ovariectomized female mice and rats compared to the control [300-302]. Kindy et al. had previously shown that 30-minute pretreatment or 5-minutes post-treatment with genistein preserves hippocampal neuronal function during global cerebral ischemia, an effect which was not apparent when delayed beyond the 5 minutes [303]. Both acute and prophylactic treatments with genistein protect the brain against ischemic insults in both permanent and transient focal cerebral ischemia both *in vivo* and *in vitro* [300, 301, 304-307]. Like endogenous estrogen, genistein also confers its neuroprotection under ischemic conditions through the genomic and membrane estrogen receptors [301, 306, 308-310]. Nuclear factor E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) antioxidant defense system have also been identified to mediate the antioxidant effects of genistein under ischemic conditions (Figure 1.5) [311]. In global cerebral ischemia model, genistein increases heme oxygenase 1 and other antioxidant response elements through the activation of Nrf2/Keap1 pathway [311]. In addition to its estrogenic actions, genistein possesses other protective estrogen receptor-independent mechanisms such as inhibition of ROS production and activation of peroxisome proliferator-activated receptor subtype gamma (PPAR-γ) to decrease inflammation and atherosclerosis as well as improve vascular tone and integrity (Figure 1.5) [312-315]. Genistein is also a known protein tyrosine kinase inhibitor at high concentrations which is thought to offset the beneficial effects of some growth factors [316]. In other neurological disease studies including Alzheimer's disease, vascular dementia and traumatic brain injury, genistein exhibits neuroprotection through mechanisms involving inhibition of dopamine depletion as well as the reduction in beta-amyloid

accumulation [317-320]. Despite the protective effects of soy isoflavones in several studies, the effect of timing of their initiation and administration after hypogonadism in the aging population is less clear. However, given the pleiotropic properties of genistein and other soy isoflavones, they may still maintain their neuroprotective benefits against neurodegenerative diseases, and hence, stands a therapeutic potential for menopausal therapy [308, 311, 314, 315].

## 2 Specific Aims

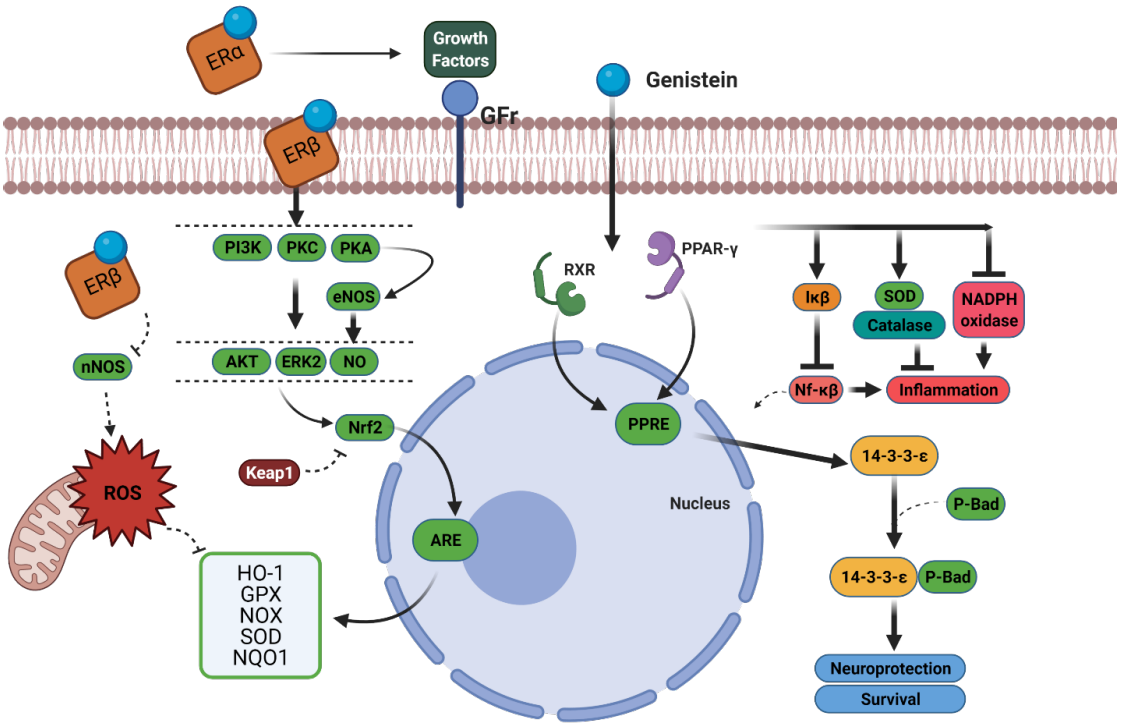


**Figure 1.5 Estrogen Receptor and Peroxisome-proliferator-activated receptor-mediated neuroprotection of Genistein** (Created with Biorender)

The overall aim of the dissertation was to test the impact of increasing age and length of hypogonadism on the neuroprotective effects of dietary genistein. The central hypothesis is that the neuroprotective effects of dietary genistein are less sensitive to the length of hypogonadism and aging. The transient intraluminal middle cerebral artery occlusion model of ischemic stroke

was used to investigate for motor and cognitive functions in young adult and middle-aged ovariectomized female Sprague-Dawley rats. Motor tests, cognitive assessments and poststroke inflammatory markers were used as measures of stroke recovery (Table 1).

**Specific Aim 1/ Chapter 2: To determine if the length of hypogonadism influences dietary genistein's neuroprotection in an experimental stroke model**. Rationale: The soy isoflavones genistein, daidzein, and its metabolite equol are neuroprotective against ischemic injury in young male and ovariectomized female rats with improved neurological function up to 7 days [300, 305, 306]. Approach: 3-months old ovariectomized female Sprague-Dawley rats were categorized into two lengths of hypogonadism following by treatment with genistein or Estrogen (E2) implants before middle cerebral artery occlusion and battery of motor and cognitive behavioral tasks.

**Specific Aim 2/ Chapter 3: To determine the impact of age on the neuroprotective effect of dietary genistein after focal cerebral ischemia cerebral ischemia in ovariectomized middle-aged rats**. Rationale: Aging and low E2 levels reduce synaptic density in cortical and hippocampal neurons [321, 322] with increased cognitive decline [323-325]. Genistein improves spatial performance by increasing the dendritic spine density in the hippocampus of middle-aged rats following eight weeks of hypogonadism [326]. Approach: 9-months old proven retired breeder rats were subjected to different lengths of hypogonadism followed by genistein or E2 treatment and middle cerebral artery occlusion. We followed the occlusion with series of behavioral test for locomotor and cognitive assessment.

**Specific Aim 3/ Chapter 4: To determine the effect of dietary genistein on chronic inflammation on the aging brain at different lengths of ovarian hormone deprivation following experimental stroke.** Rationale: Systemic inflammation with aging changes the brain's neuroinflammatory milieu as a contributor to the physiopathology of stroke and neuronal

necroptosis [327, 328]. Genistein reduces microglia expression in the CA1 hippocampal region under stress conditions [329]. Approach: Protein samples from young and middle-aged rats were extracted and probed for inflammatory markers as surrogate markers of microglial activation 3-weeks after focal cerebral ischemia.

**Table 1: Overall study design**

| | | | |
|---|---|---|---|
| **Species** | Female Sprague-Dawley rats | | |
| **Age** | Young | Middle age | |
| **Hypogonadism length** | Short-term | Long-term | |
| **Treatment** | Vehicle | Estrogen | Genistein |
| **Model** | Middle Cerebral Artery Occlusion | | |
| **Behavior** | Forelimb asymmetry | Motor function | Cognitive function |
| **Biochemistry** | Tissue loss | Poststroke inflammation | |

**Summary:** Data from this dissertation shows that different lengths of hypogonadism and ages at which they occur differentially impact the neuroprotective effects of dietary genistein after stroke. Additionally, dietary genistein reduces poststroke inflammation following short-term hypogonadism independent of age, an effect that is blunted with a long-term absence of estrogen. Further strategies, however, will be needed to identify the underlying temporal pathophysiological

changes that cause the differences in the effects of genistein during and around menopause. Such findings will provide the basis for genistein's sensitivity to changes in ovarian hormone depletion to allow the development of optimum treatment regimens to meet the clinical needs of postmenopausal women who may resort to its use.

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## CHAPTER 2

# LONG-TERM HYPOGONADISM DIMINISHES THE NEUROPROTECTIVE EFFECTS OF DIETARY GENISTEIN IN YOUNG ADULT OVARIECTOMIZED RATS AFTER TRANSIENT FOCAL CEREBRAL ISCHEMIA

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Running Title: Genistein loses neuroprotection after chronic hypogonadism

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**Abstract**

**Introduction:** Increasing age disproportionately increases the risk of stroke among women compared to men of similar age, especially after menopause. One of the reasons for this observation is a sharp drop in circulating estrogens. However, the timing of initiation of estrogen replacement after menopause is associated with mixed beneficial and detrimental effects, hence contributing to widespread mistrust of estrogen use. **Objective:** Agents including soy isoflavones are being assessed as viable alternatives to estrogen therapy. In this study, we hypothesized that the neuroprotective effects of genistein, a soy isoflavone are less sensitive to the length of hypogonadism in young adult ovariectomized rats following cerebral ischemia. We expected that long-term hypogonadism will worsen motor and cognitive function, increase post-stroke inflammation with no effect on the neuroprotection of genistein. **Method:** We compared the effect of treatment with dietary genistein (GEN) on short-term (2 weeks) and long-term hypogonadism (12 weeks) in young adult ovariectomized Sprague-Dawley rats on sensorimotor function, cognition and inflammation after focal cerebral ischemia. Dorsal Silastic implant of 17β-estradiol (E2) was used as a control for hormone therapy. **Results:** Long-term hypogonadism stroked rats performed worse than the short-term hypogonadism stroked rats on the motor and cognitive function tests. GEN did not improve neurological assessment and motor learning after either short-term or long-term hypogonadism. GEN improved cognitive flexibility after short-term hypogonadism but not after the long-term. Both GEN and E2 reduced tissue loss after short-term

hypogonadism and reduced GFAP expression at the contralateral side of ischemia after long-term hypogonadism. **Conclusion:** The length of hypogonadism may differentially influence the neuroprotective effects of both GEN and E2 on the motor and cognitive functions in young adult rats.

Key Words: Stroke, genistein, chronic hypogonadism, cognition

RRID:RGD_737903, RRID:AB_60418, RRID:AB_2224402, RRID:AB_2340593, RRID:SCR_001775, RRID:SCR_014199, RRID:AB_839506, RRID:AB_2273656, RRID:SCR_003070, RRID:SCR_010455

**Significance**

Starting at perimenopause and beyond menopause, many women elect to use soy isoflavones as hormone replacement therapy to control their menopausal symptoms. As with estrogen replacement therapy, the roles that age and timing of initiation play in a potential beneficial effect of soy isoflavones can have a significant effect on the desire to utilize soy products during the menopausal transition. Hence, understanding the basis for the time-dependent benefits of genistein will allow the development of optimal treatment regimens in the future for the improvement of menopausal symptoms and associated neurovascular disorders including stroke.

**Introduction**

Stroke is a leading cause of death and disability in the US and worldwide with ischemic stroke estimated to account for over 85% of all stroke incidence [330]. Women are substantially less prone to ischemic stroke during their reproductive years compared to men of similar age. However, with advancing age, this relative protection is lost with a corresponding reduced age-adjusted male-to-female ratio of stroke incidence after menopause [330, 331]. One of the likely reasons for this observation is a sharp decline in circulating sex hormone levels during menopause which has been associated with several age-related conditions including cardiovascular and cerebrovascular diseases [332, 333].

Estrogen has been used by postmenopausal women for decades as hormone replacement therapy (HRT) to mitigate the consequences of menopause including osteoporosis and vasomotor symptoms [334]. In several preclinical studies, estrogen has been shown to be a powerful neuroprotective agent in models of neurological diseases such as ischemic stroke, Alzheimer's disease, global ischemia and traumatic brain injury [335]. In rodent studies, physiological doses of estrogen have been shown to improve cerebral blood flow and guard against ischemic insults in both male and ovariectomized rats [336-342]. Acting through estrogen receptors in numerous cell types, estrogen has been shown to inhibit oxidative stress, inflammation and apoptosis associated with brain insults [167, 343-345].

Despite the potential benefits of estrogen, a review of premature termination of parts of the Women's Health Initiative study revealed that a delay in HRT initiation beyond the first decade after menopause increases the risk of stroke, myocardial infarction and dementia. This suggests a temporal beneficial effect of estrogen, considering that HRT initiated within 10 years of menopause provides cardiovascular benefits [346-350]. The timing of the switch from the beneficial to detrimental effects of estrogen is not fully understood, and off-target peripheral

effects of estrogen, including thromboembolism and breast cancer, were seen in early recipients [349, 351]. An increasing mistrust in the use of estrogen after menopause and an unclear time window of benefit for estrogen replacement has led to intensified efforts to find alternatives for postmenopausal women [352, 353]. The temporal window of benefit for estrogen has further been demonstrated in preclinical studies in which a 10-week delay in estrogen replacement after ovariectomy diminished its neuroprotective effects in young ovariectomized mice and rats undergoing cerebral ischemia [272, 354-357]. Due to this loss of effectiveness, several other agents have been investigated for their potential benefit on menopausal symptoms and chronic cerebrovascular diseases [358-362]. Among such compounds of interest are phytoestrogens, a group of biologically active plant-derived estrogenic compounds which have been extensively studied in the last three decades for their benefits during menopause [284-287]. Genistein, a phytoestrogen and soy isoflavone, is found at high levels in many soy products and other legumes, and it is consumed as both food and dietary supplement mainly for the management of menopausal symptoms [363, 364]. In short-term animal studies, dietary genistein is neuroprotective against both global and focal cerebral ischemia in male and female rodents [301, 303, 306, 310]. Our laboratory and others have previously shown that, following at least a two-week treatment, dietary genistein reduces infarct size in young ovariectomized female mice and rats [300-302]. The neuroprotective effects of dietary genistein are thought to be mediated by processes that include direct estrogen receptor activation, improved circulatory function and reduced oxidative stress [301, 305, 308, 310].

While the neuroprotective benefits of estrogen are lost after chronic hypogonadism, it is unclear whether the beneficial effects of genistein are also sensitive to the time of its initiation, especially when delayed for an extended period. Nonetheless, genistein's benefits through

estrogen-receptor independent pathways including direct activation of peroxisome proliferator-activated receptors and inhibition of specific receptor tyrosine kinases make it a potentially promising agent to maintain its neuroprotection [308, 311, 314, 315]. Hence, in this study, we sought to investigate whether the length of chronic hypogonadism following ovariectomy influences genistein's neuroprotection during experimental focal cerebral ischemia.

## Methods

### *2.1 Animals and treatment*

All procedures were approved by the Institutional Animal Care and Use Committee and performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. One hundred and thirty young adult female Sprague-Dawley rats (~3 months old) weighing 116-200g were purchased from Envigo (Strain Code: 002, RRID: RGD_737903) and housed under controlled temperature and humidity with 12-hour light/ dark cycle (lights on 07:00). Rats were fed with isoflavone-free diet *ad libitum* (Purina Test Diet, 5K96) and had free access to water.

A week after receipt, all animals were bilaterally ovariectomized under anesthesia with 2% isoflurane following the technique described by Jezierski et al [212]. Bilateral dorsal midline incisions were made in the region inferior to the rib cage and kidneys. The ovaries and surrounding tissues were clamped and removed, and the remaining tissue was ligated with a sterile absorbable suture. All animals were randomly assigned to one of two experimental hypogonadism periods of 2 weeks as short-term hypogonadism (STD) or 12 weeks as long-term hypogonadism (LTD). At the end of each time point, animals received an isoflavone-free diet (vehicle), or a custom-made genistein-containing diet (GEN) (500ppm genistein, LC Laboratories, added to Purina Advanced Protocol Verified Casein IF10 5K96) or dorsal Silastic implants (inner/outer diameter: 1.575/3.175

mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) of crystalline 17β-estradiol (E2, Sigma Chemical) and remained on the respective treatments for the remainder of the study [365, 366]. The dietary genistein concentration approximates 42mg/kg/day which produces a circulating genistein concentration of approximately 5μM [367] while the E2 implant provides an expected concentration of 50-70pg/ml. STD rats were divided into 6 groups: Sham (vehicle) (n= 10), sham+GEN (n= 10), sham+E2 (n= 10), stroke (vehicle) (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 10). LTD rats were divided into 5 groups: Sham (n= 10), sham+GEN (n=10), stroke (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 10) (Figure 1A).

**2.2 Transient intraluminal middle cerebral artery occlusion (tMCAO)**

Two weeks after treatment initiation, transient intraluminal middle cerebral artery occlusion (tMCAO) was performed as previously described [300] to model the typical manifestation of transient ischemic stroke in humans [368, 369]. Animals assigned to experimental stroke were anesthetized with 5% isoflurane and maintained on 1.5-2% isoflurane in oxygen. A midline incision was made in the neck region to expose the surgery area. A temporary ligation was made in the common carotid artery and the internal carotid artery after carefully separating them from the vagus nerve and adjoining tissues. The external carotid artery was severed, and a silicone suture (Doccol Corporation, Sharon MA) was inserted through it into the ICA and advanced to the origin of the middle cerebral artery (MCA). Heart rate and saturation pulse of oxygen were measured and maintained in real-time to ensure adequate oxygenation (≥97%) (Kent Scientific). Body temperature was maintained at 37±0.5 °C using an infrared homeothermic blanket. Following occlusion, animals were removed from anesthesia to assess occlusion success. Only animals that demonstrated right-sided hemiparesis during the 60-minutes ischemic phase were included in the study. After 60 minutes, animals were anesthetized, the suture was withdrawn, and

the ECA was ligated. All mortalities during and after stroke were documented and reported as stroke outcome. Animals in sham groups underwent all MCAO procedures except the passage of the silicone suture. The occlusion period of the study was limited to 60-minutes due to observed increased mortality in the long-term hypogonadism animals (~50% mortality) from our preliminary results following 90-minutes ischemia (Figures 2A and B).

**2.3 Behavioral Assessments** (Figure 1B)

***2.3.1 Neuroscore***

All behavioral assessments were carried out in dimly lit rooms with automated systems or by a treatment-blinded observer. Animals were assessed for neurological deficits after stroke on a 9-point modified Bederson scale [370]. This involved spontaneous circling (graded 0-3), contralateral forepaw flexion (graded 0-3) and contralateral hindlimb retraction (graded 0-3) and the sum of the scores was used as a measure of stroke severity on post-stroke day 1.

**2.3.2 Cylinder test**

All animals underwent the cylinder test as a measure of forelimb asymmetry to denote the severity of sensorimotor deficits [371]. Animals were placed in a vertically erect plexiglass cylinder (20 cm in diameter and 30 cm high) and allowed to explore for up to 10 minutes or 20 touches on the cylinder walls. Mirrors were positioned at either side of the cylinder to allow full coverage and capture of all wall touches. Behavior was digitally recorded and scored off-line by a treatment-blind observer. Forelimb asymmetry was determined by the contralateral paw use expressed as a percentage of the total wall touches. Subjects that did not rear nor touch the walls of the cylinder were included in the forelimb asymmetry analysis.

***2.3.3 Rotarod test***

The rotarod test was used to evaluate coordination and motor learning. Testing was performed using the AccuRotor instrument (Accuscan Instruments). Rats were placed on a rotating rod which accelerated from 0 to 75 rpm over 150 seconds. The time each animal spent on the rod was automatically determined once the animal fell. All the animals received two sessions daily separated by at least three hours rest with each session comprising four trials, at least 10 minutes apart with latency automatically recorded by the instrument. The average of the trials per session was determined and expressed as the latency to fall [372].

### *2.3.4 Morris Water Maze test*

The Morris water maze (MWM) was used to assess cognitive deficits after stroke, as previously described [373-375]. All animals were pre-trained on how to escape from the water tank by locating a hidden platform using a straight alley. Testing involved three phases: 1) a 4-day acquisition phase during which the rats were required to learn the position of a hidden platform based on maze external cues in the testing vicinity, 2) 1-day retention phase in which the rats were assessed on their capacity to recall the position of the platform after 72 hours and 3) a 2-day reversal phase which involved changing the platform's position from its original quadrant to its opposite quadrant and requiring the animals to learn and adapt to the new location. Each animal received a session-a-day for 7 days with each session comprising 3 trials, separated by 10 minutes. Data were expressed as the path length and path-independent swim speed. Probe trials were conducted to underscore the cognitive learning capacity of the rats and involved the removal of the platform before the fourth acquisition session (acquisition probe) and at the end of the second reversal session (reversal probe). Percent times spent in the platform quadrant, within 40- and 20 cm diameter annulus around the target site were also evaluated as a measure for spatial bias. All MWM behavior was automatically recorded and analyzed with AnyMaze software (Stoelting).

### *2.4 Biochemistry*

Twenty-one days after MCAO or sham MCAO, all the animals were euthanized by transcardial perfusion with normal saline after deep anesthesia with 5% isoflurane. Brains were quickly removed, and a 1 mm coronal slice was made at -1mm from bregma, snap-frozen and stored at -70° C for western blot analysis. The remaining brain tissues were post-fixed in 4% formaldehyde for 72 hours. Serial coronal sections (40 μm) were made using a vibratome, and sections were stored in a cryoprotectant at -20 C for immunohistochemistry [376].

#### *2.4.1 Immunohistochemistry*

Matched sections from each animal were incubated with glial fibrillary acidic protein (GFAP, 1:400, LabVision RB-087-A1; RRID:AB_60418) or ionized calcium-binding adaptor molecule 1 (Iba1, 1:1000, Abcam, ab5076; RRID:AB_2224402) as markers of astrogliosis and microgliosis, respectively as described by Yoon et al and Buscemi et al [377, 378]. The sections were further incubated with biotinylated secondary antibodies (Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit IgG, H+L, 1:400, Jackson ImmunoResearch 711-065-152; RRID: AB_2340593) and immunoreactivity was revealed with diaminobenzidine using a commercial kit (ImmPactDAB, Vector Labs) [379]. The sections were mounted on gelatin-coated slides, delipidated in alcohol and xylenes and coverslipped with DPX (Sigma-Aldrich) for imaging. Bright-field images were obtained with a 4x objective and charge-coupled device camera using the 2D Slide Scanning module of Neurolucida (MBF Bioscience; RRID: SCR_001775) and a motorized stage. Regions of interest were analyzed and quantified with Adobe Photoshop 2020 (Adobe; RRID: SCR_014199) in a treatment blinded manner (Full antibody description in Table 1).

### *2.4.2 Western blotting*

Protein was extracted from homogenates from both whole cerebral hemispheres (between Bregma 1.00mm and -2.80mm) with T-PER reagent (Pierce, Rockford, Illinois) containing HALT Protease Inhibitor Cocktail (Pierce). Protein concentrations were determined with a BCA protein assay kit (Pierce) and 30µg of protein was separated on 4-20% polyacrylamide gels (BioRad). Proteins were transferred to nitrocellulose membrane and blocked in 5% milk for 1 hour at room temperature. The membranes were incubated with GFAP (1:1000, LabVision RB-087-A1; RRID: AB_60418), Iba1 (1:1000, FUJIFILM Wako Shibayagi, 016-20001; RRID: AB_839506) and β-actin (1:5000, Thermo Fisher Scientific, MA1-91399; RRID: AB_2273656) overnight at 4ºC followed by Horseradish-peroxidase conjugated secondary antibody for 1 hour. Protein bands were visualized on SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). ImageJ (NIH, RRID: SCR_003070) software was used to analyze the protein bands in an unbiased manner using the plot lanes feature to determine GFAP and Iba1 expression (Full antibody description in Table 1).

### *2.5 Statistical Analysis*

Data are expressed as the mean ± standard deviation and were analyzed with Systat version 13.1 (Systat Software, Inc, San Jose, CA, USA; RRID: SCR_010455). Mortality data were analyzed with the Log-rank (Mantel-Cox) test. Neuroscore, inflammatory markers and comparisons within stroke groups were done using a two-way analysis of variance (ANOVA) using Duration (STD vs. LTD) and Treatment (vehicle vs. GEN vs E2) as the factors. Due to the difference in design for the STD and LTD, we chose to perform two-way ANOVA for STD with Surgery (Sham vs. Stroke) and Treatment (vehicle vs. GEN vs. E2) as the factors, and one-way ANOVA for LTD with Group (Sham vs. Stroke vs. Stroke+E2 vs. Stroke + GEN) as the

factor. Repeated measures analyses were performed on the rotarod test, acquisition and reversal phases of the Morris water maze test. Fisher's Least Significant Difference Test was used as a *post-hoc* test where there was a main effect of Duration, Treatment, Surgery or interactions of any of the factors. A *P*-value of 0.05 was set as a limit for significant difference. No outliers were removed from statistical analysis.

Initial group sizes were based on our previous studies of MWM performance in rats after tMCAO [373]. Interim power analyses were conducted approximately halfway through the study to estimate the sample sizes at an alpha of $P<.05$ with a power of 0.80 using the effect of treatment and length of hypogonadism on behavioral deficits in rotarod and MWM performance in both short-term and long-term cohorts. For the rotarod test in the short-term cohort, a total sample size of 36 (6 per group) was needed. For the MWM, a total sample size of 48 (8 per group) was required. In the long-term groups, a sample size of 40 (8 per cell) was required for the rotarod test, and a sample size of 54 (9 per cell) was required for the MWM.

## Results

### *3.1 Neurological assessment (Neuroscore)*

Neurological deficits were assessed on post-stroke day 1 using a modified Bederson scale and expressed as the Neuroscore (Figure 2). Neither GEN nor E2 improved Neuroscore compared to the vehicle after short-term hypogonadism, however, E2 but not GEN improved Neuroscore after long term hypogonadism. This was supported by an interaction between Treatment and Duration (Figure 2C, Two-way ANOVA, $F(2,65)=3.871$, $N=71$, $p=0.026$).

### *3.2 Forelimb asymmetry (Cylinder test)*

Forelimb asymmetry was assessed with the cylinder test on post-stroke days 3 and 7 (Figure 3). In the short-term hypogonadism, the stroke rats used their contralateral paws less than shams on both post-stroke days 3 (Figure 3A), $F(1,55)=23.393$, $N=61$, $p<0.001$) and 7 (Figure 3B) $F(1,59)=43.342$, $N=65$, $p<0.001$). There was no effect of treatment in either group. In the long-term hypogonadism cohort, stroke rats also showed deficiencies in the use of their contralateral paw on both post-stroke days 3 (Figure 3C, $F(4,51)=7.618$, $N=56$, $p<0.001$) and 7 (Figure 3D, $F(4,54)= 14.931$, $N=59$, $p<0.001$). There was no effect of treatment on day 3, but E2 improved forelimb asymmetry after long-term hypogonadism on post-stroke day 7 compared to the vehicle (Figure 3D).

### *3.3 Rotarod test*

Motor learning and coordination were determined with the rotarod test (Figure 4). The stroke rats fell faster than their sham counterparts in the short-term hypogonadism cohort (Figure 4A, $F(1,59) = 20.895$, $N=65$, $p<0.001$). Neither GEN nor E2 improved motor learning compared to the vehicle rats ($F(2,59) = 0.279$, $N=65$, $p = 0.738$). Sham-operated rats had higher average latency to fall compared to the stroke rats after long-term hypogonadism (Figure 4C, $F(4,56)= 4.007$, $N=59$, $p=0.006$). E2 but not GEN improved motor learning in the long-term hypogonadism animals though it did not reach statistical significance.

### *3.4 Morris Water Maze test*

Cognitive effects of stroke were assessed with the Morris water maze test (Figures 5, 6 and 7, statistics in Table 2 and summary in Table 3). All the groups showed an overall decrease in path length over the sessions in the acquisition (sessions 1-4) and reversal phase (sessions 6-7). In the short-term hypogonadism, the sham operated rats traveled shorter distances to locate the hidden

platform in the reversal phase (Figure 5A). GEN-treated rats had shorter path lengths to the hidden platform on the second day of the reversal phase after short-term hypogonadism compared to the vehicle and E2-treated rats. There was no difference between the distances covered by the sham operated rats and stroke rats after long-term hypogonadism (Figure 5C). Neither E2 nor GEN reduced the path length traversed to find the hidden platform after long-term hypogonadism (Figure 5C). These findings were supported by the interaction between Session, Treatment and Surgery in the reversal phase after short-term hypogonadism and within stroke interaction between Treatment and Duration of hypogonadism in the reversal phase. Both vehicle and GEN-treated sham operated rats swam faster than the E2 sham operated rats after short-term hypogonadism (Figure 6A). After long-term hypogonadism, sham operated rats had higher swim speeds compared to the stroke rats (Figure 6C) and neither GEN nor E2 improved swim speed. Within stroke comparisons revealed effects of Duration and Treatment on the retention phase and reversal phase respectively for swim speed. There was no effect of Treatment on the time spent in the acquisition probe target quadrant for both lengths of hypogonadism and on annulus 40cm of short-term hypogonadism (Figures 7A, B and C). GEN improved spatial bias after long-term hypogonadism where GEN-treated stroke rats spent more time in the Annulus 40cm of the acquisition probe compared to the vehicle and E2-treated rats (Figure 7D).

### *3.5 Infarct size*

Percent tissue loss was determined following GFAP stain (Figure 8) [377, 378]. Chronic brain injury resulting from experimental stroke is not fully revealed by Nissl staining nor standard 2,3,5-triphenyltetrazolium under chronic conditions, and particularly perilesional areas [377, 380]. Several protein markers peak within the first week, but GFAP can serve as a persistent marker of regions surrounding the core of the infarct and perilesional regions [377, 380]. Both E2 and GEN

reduced post-stroke tissue loss compared to the vehicle after short-term hypogonadism (Figure 8C). Neither E2 nor GEN reduced percent tissue loss in the long-term hypogonadism cohort compared to vehicle. Two-way ANOVA revealed main effects of Treatment ($F(2,50)=3.411$, $N=56$, $p=0.041$) and interaction between Treatment and Duration of hypogonadism ($F(2,50)=7.65$, $N=56$, $p=0.001$)

### 3.6 *GFAP expression*

E2 but not GEN increased GFAP expression at the ipsilateral side compared to the vehicle after short-term hypogonadism (Figure 9C). Neither E2 nor GEN reduced GFAP expression at the ipsilateral side after long-term hypogonadism (Figure 9C). vehicle rats had higher expression of GFAP than both E2 and GEN at the contralateral side after short-term hypogonadism (Figure 9F). Both E2 and GEN reduced GFAP expression at the contralateral side after long-term hypogonadism (Figure 9F) compared to vehicle. These were supported by an interaction between Treatment and Duration on GFAP expression at both the ipsilateral ($F(2,44)=5.139$, $N=54$, $p=0.01$) and contralateral sides ($F(2,48)=5.101$, $N=54$, $p=0.01$).

### *3.7 Iba1 expression*

Neither E2 nor GEN reduced Iba1 expression at the ipsilateral side of ischemia in either hypogonadism lengths (Figure 10C). Both E2 and GEN reduced Iba1 expression at the contralateral side after long-term hypogonadism (Figure 10F) but not short-term hypogonadism (Figure 10C) compared to the vehicle rats. Long-term hypogonadism rats had higher Iba1 expression compared to the short-term hypogonadism rats at the contralateral side ($F(2,48)=4.098$, $N=54$, $p=0.049$).

## Discussion

Chronic reductions in circulating estrogen are associated with increased cerebrovascular diseases including stroke and cognitive decline [333, 346]. Preclinical studies with E2 have shown dramatic neuroprotection in rodent models under ischemic conditions [337-340]. However, the use of exogenous estrogens as hormone therapy and for chronic prevention of cardiovascular and cerebrovascular diseases is limited by its adverse side effects including increased risk for breast cancer and thromboembolism [351, 381]. This study is the first effort to investigate the temporal effects of dietary genistein on experimental stroke at different durations of hypogonadism following ovariectomy. Our results showed two key findings; first, E2 treatment but not dietary genistein (GEN) improved forelimb motor deficits after stroke in long-term estrogen deprived animals but not after short-term hypogonadism; second, dietary genistein but not E2 improved spatial learning after short-term estrogen deprivation but not long-term hypogonadism. Both E2 and dietary genistein further reduced post-stroke tissue loss after short-term hypogonadism, not long-term hypogonadism. Both GFAP and Iba1 expressions at the contralateral side of ischemic injury were also reduced by dietary genistein and E2 after long-term hypogonadism. Our results suggest that treatment with dietary genistein at a concentration of 500 ppm following hypogonadism and 60 minutes of transient focal cerebral ischemia does not alter sensorimotor deficits but may be beneficial for maintaining some cognitive function.

Previous results from our laboratory and others have shown that dietary genistein, a soy isoflavone, is neuroprotective against ischemic brain damage after short-term hypogonadism [300, 302]. In this study, we found that dietary genistein reduces infarct size after short-term hypogonadism but did not reduce sensorimotor deficits, independent of the duration of hypogonadism. The studies which had reported protective effects of dietary genistein had maintained the occlusion phase at 90 minutes followed by reperfusion up to 7 days after stroke

[300, 302, 304, 305]. In the current study, the duration of focal cerebral ischemia was limited to 60 minutes due to a high mortality rate in the long-term hypogonadism group after 90-minute ischemia. The increase in mortality after long-term hypogonadism further supports the importance of endogenous estrogen in limiting stroke injury. This suggests that the 60-min occlusion period might not be long enough to obtain substantial cortical involvement which could have impacted the sensory and motor functions [300, 301, 309, 382]. Furthermore, the extent of tissue injury in the present study was performed 21 days after experimental stroke. Because stroke injury evolves over the chronic phase of recovery, this time-point may better represent the final extent of injury than shorter post-ischemic measurements. Additionally, even though genistein treatment has been shown to improve vasomotor menopausal symptoms in some human studies [285, 286, 360], fewer reports are available on the benefits of genistein on motor functions in preclinical neurological disease studies. In a Parkinson's disease study with 6-hydroxydopamine injection, Kyuhou et al. showed an improvement of forelimb deficits with acute pretreatment with genistein (10 mg/kg, intraperitoneally) in young ovariectomized rats [383]. Chronic treatment with genistein (150mg/kg/day, intragastrically) has further been shown to improve fear-related locomotor activity in a streptozotocin-induced sporadic Alzheimer's disease model [384]. In contrast, Lu et al. reported that chronic pretreatment with genistein had no effect on open field locomotor tasks in a sporadic scopolamine-induced amnesia model [385]. Arbabi et al. also reported differential responses to genistein treatment in a 6-hydroxydopamine hydrochloride-induced Parkinson disease model where genistein improved cognition but had no effect on kinetic endpoints such as the rotarod test [386]. In line with previous reports, genistein had no effect on the motor function tests involving forelimb asymmetry assessment and motor learning in the current study. Unlike genistein, E2 improved fine limb asymmetry deficits in the long-term hypogonadal but not the

short-term hypogonadal animals. Previous studies of E2 have reported the absence of benefit or worsened behavioral performance after long-term hypogonadism [355-357]. However, a critical appraisal of the preclinical information on E2 following extended absence of circulating estrogen shows the loss of benefit closely related to worse cognitive performance and an enhanced inflammatory response with a paucity of negative effect on motor tasks during stroke. This evidence suggests that the E2's deleterious effect may be confined to the cognitive domains but not on motor domains of the brain. Furthermore, E2 has been shown to promote neurogenesis within the subventricular zone and other brain regions [387, 388] as well as interact with insulin-like growth factor-1 system to improve motor function in 6-OHDA Parkinson's Disease model [389]. Hence, the observed benefit of E2 on locomotor performance could be as a result of an improved tissue repair poststroke in the motor domains, translating into its benefit in the current study.

In the present study, 2-week pretreatment with dietary genistein but not 17β estradiol improved spatial learning on the reversal phase of the Morris water maze task following short-term hypogonadism, supporting previously established neuroprotective benefits of genistein in model neurological diseases. This also supports other studies that have shown beneficial effects of genistein but not estrogen in behavioral studies for learning and memory functions [323-326]. For example, in beta-amyloid lesioning models of Alzheimer's disease, genistein pretreatment reduces hippocampal tissue injury and improves spatial learning deficits following intrahippocampal lesioning [390, 391]. Genistein pretreatment also improves memory deficits in scopolamine-induced amnesia and Huntington's disease models on Morris water maze and passive avoidance tasks respectively [385, 392]. The benefit of genistein on the reversal task compared to the absence of effect by E2 suggests that genistein could be affecting other domains that mediate cognitive

flexibility, one that may not be apparent with E2 under the current experimental conditions [393]. Extra-hippocampal cognitive regions that may be implicated in the differential spatial flexibility include the prefrontal cortex. This is because of the disproportionately higher expression of estrogen receptor-beta (ERβ) compared to estrogen receptor-alpha (ERα) within the prefrontal cortex. Considering that genistein has a very high binding affinity for ERβ [394-399], it may serve as the molecular underlying factor for improved cognitive flexibility with Genistein treatment but not E2, with ERβ more indicated in estrogen-mediated cognition [400]. Unlike the short-term estrogen-deprived animals, genistein failed to reverse memory deficits following chronic hypogonadism, even though it improved spatial bias on the probe trials. Previous long-term estrogen deprivation studies in rodents have shown that extended low circulating E2 results in downregulation of brain ER with an accompanied reduced estrogen-mediated activity [401]. This observation could be due to a possible time-dependent benefit of dietary genistein as has been reported with estrogen therapy [323-325]. Therefore, delaying the treatment with genistein up to 12-weeks in this study suggests a possible diminished ER activity that may have blunted the cognitive benefits [321, 322, 357].

Previous studies with genistein and high soy diets have demonstrated substantial anti-inflammatory effects through microglial inhibition under ischemic conditions [402, 403]. In the present study, E2 treatment but not genistein was associated with elevated GFAP activation in the short-term hypogonadism group compared to the vehicle subjects. Whilst several studies have reported E2's capacity to reduce neuroinflammatory response during the acute phase post-stroke [272, 404, 405], our finding suggests that E2's anti-inflammatory effects may not persist into the chronic phase at the site of ischemic injury. Both genistein and E2 treatment reduced GFAP activation and Iba1 expression at the contralateral side of focal cerebral ischemia in the long-term

hypogonadism group but not at the ipsilateral side. This underscores the anti-inflammatory effects of both genistein and E2, an effect that may not be overt at the ipsilateral hemisphere under short occlusion periods of focal cerebral ischemia compared to the vehicle subjects. The subtle differences between dietary genistein and E2 after short-term and chronic hypogonadal states on sensorimotor and cognitive functions may further support the observation that early recipients of menopausal hormone therapy may stand a greater benefit from it compared to subjects who receive it late. Moreover, our findings have shown that chronic hypogonadism alone exacerbates experimental stroke outcomes irrespective of the type of treatment. This means menopause is an independent risk factor for a compromised neuroprotective response against brain insult. Thus, the worsening of stroke relates to the length of estrogen and progesterone absence regardless of aging considering that young adult animals were used in the current study. Moving forward, a combined administration of genistein and E2 could be investigated for possible beneficial effects for both locomotor and cognitive functions as observed in this study. In non-ischemic studies, genistein has been combined with E2 for possible role of genistein on the off-target detrimental effects of E2 [406-408]. Even though, the detriments of genistein or E2 on functional female organs including the endometrium and mammary glands, the combined therapy for the two compounds can equally be explored for their safety under conditions of ischemia.

**Limitations**

The occlusion period of this study was limited to 60-minutes due to observed increased mortality in the long-term E2 deprived animals from our preliminary results following 90-minutes ischemia. This allowed extended survival studies in the chronic hypogonadal states beyond the acute phase. On the other hand, it possibly may also explain the reduced cortical impact of the stroke model used, and hence a minimal differential treatment effect of genistein on cortically

involved behavioral tasks compared to the soy-free fed animals. Again, young adult animals were used in the study. Unlike middle-aged and old animals in whom the predisposing factors for ischemic stroke may already be in place, the young adults might still have residual protection against ischemic insults, hence the observed minimal differences between genistein-fed and soy-free fed animals.

**Conclusion**

Following 60-mins focal cerebral ischemia, dietary genistein had no effect on sensorimotor deficits following both short-term and long-term hypogonadism when compared to the vehicle and estrogen-treated animals. Dietary genistein improved spatial memory function when initiated two weeks after ovariectomy but its effects were not adequate when treatment was delayed up to 12 weeks post-ovariectomy in young rats. This suggests that the benefits of dietary genistein, like E2, have a window of effectiveness, and that estrogen receptors are at least partially involved in the beneficial effects of genistein. Estrogen treatment but not dietary genistein improved certain aspects of motor function but failed to improve memory function following chronic hypogonadism. Overall, our findings suggest different brain region responses to E2 and dietary genistein following different lengths of hypogonadism and ischemia. However, like estrogen, when dietary genistein treatment is delayed well in advance of the start of hypogonadism, the benefits seen with short-term hypogonadism may diminish. It's noteworthy that, further studies are required to investigate the molecular changes and differences which may account for the varied responses between dietary genistein and E2 treatment. Altogether, the further evidence may provide a paradigm for use of genistein in and around menopause for managing and associated cardiovascular profile.

**Acknowledgments**:

The authors wish to acknowledge the assistance of E. Michael Cuellar and Callie Fort in blood and tissue processing and behavioral analysis, Dr. Fen Sun in stroke surgery and Ella Anle Kasanga in euthanasia.

**Disclosure Statement:**

The authors have no conflicts of interest to declare.

**Funding Sources:**

This project was funded by the JES Edwards Foundation (DAS), Sigma Xi Grant-in-Aid of Research (AO) and T32 AG020494 training grant (AO).

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**Data availability statement**

The authors assert that the supporting data for the study are available in the article and from the corresponding author upon reasonable request.

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## Figure Legends

**Figure 1:** (A) Study design. After 7 days of acclimation, the animals in short-term hypogonadism (STD) study were randomly assigned to the groups: Sham (vehicle) (n= 10), sham+GEN (n= 10), sham+E2 (n= 10), stroke (vehicle) (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 10). Animals in long-term hypogonadism (LTD) study were assigned to the groups: Sham (n= 10), sham+GEN (n=10), stroke (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 10). After the hypogonadism period and pretreatment with vehicle diet, GEN diet or E2 implant, animals underwent MCAO or sham surgery followed by behavioral tests (B) and euthanasia.

**Figure 2:** (A and B) Survival Data. Logrank (Mantel-Cox) test showed no difference between the treatment groups after 90-minutes (Chi-square test=7.364, p=0.061) and 60-minutes (Chi-square test=5.701, p=0.336). Occlusion period of the study was however reduced due to the high mortality after 90 minutes to maximize survival. Neuroscore. Assessment of neurological deficits 24 hours after MCAO. (C) Neither genistein (GEN) nor 17β-estradiol (E2) improved Neuroscore compared to isoflavone-free diet-fed animals. E2 but not GEN improved Neuroscore after long-term hypogonadism. Analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean± SD, n=10-14, *p<0.05 compared with vehicle animals.

**Figure 3:** Cylinder Test. Assessment of forelimb asymmetry. (A and B) Main effect of stroke was observed after short-term hypogonadism. Neither GEN nor E2 improved forelimb asymmetry compared to the isoflavone-free diet-fed animals. (C and D) Sham-operated animals had better forelimb symmetry compared to stroke subjects after long-term hypogonadism. E2 but not GEN improved forelimb asymmetry on post-stroke day 7. Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SD, n=9-14, *p<0.05.

**Figure 4:** Rotarod. Assessment of motor learning and coordination. (A and B) Main effect of stroke was observed in short-term hypogonadism animals. Neither GEN nor E2 improved the time spent on the rotating rod compared to vehicle animals. (C and D) E2 but not GEN improved motor learning compared to isoflavone free group. Two-way ANOVA and one-way ANOVA followed

by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SD, n=10-14, *p<0.05. (AUC)=Area under the curve.

**Figure 5:** Water Maze. Assessment of spatial learning and cognitive performance. (A and B) Main effect of stroke was observed in the reversal phase of the short-term hypogonadism animals. GEN but not E2 improved spatial learning in the reversal phase compared to the isoflavone free group. No effect of treatment was observed after long-term hypogonadism. Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SD, n=10-12, *p<0.05 compared to vehicle sham animals, @P<0.05 compared to vehicle stroke animals. (AUC)=Area under the curve.

**Figure 6:** Water Maze. Assessment of path-independent swim speed. (A and B) Sham subjects swam faster to find the hidden platform compared to treatment-matched stroke subjects. Both vehicle and GEN shams swam faster than E2 shams but neither GEN nor E2 improved swim speed compared with vehicle stroke rats. (C and D) Neither GEN nor E2 improved spatial learning after long-term hypogonadism. Sham subjects had improved path-independent swim speed compared to the stroked subjects after long-term hypogonadism. Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SD, n=10-12, *p<0.05 compared to vehicle sham animals, @P<0.05 compared to vehicle stroke animals. (AUC)=Area under the curve.

**Figure 7:** Water Maze. Probe trials. Neither GEN nor E2 improved the time spent in target quadrant after both short and long-term hypogonadism (A and C). GEN but not E2 improved time spent in Annulus 40cm of acquisition probe trial after long-term hypogonadism (D) but not short-term (B). Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Dashed lines represent the probability that the time spent in the respective regions is due to chance. Data are expressed as mean ± SD, n=9-12, *$p<0.05$.

**Figure 8:** Percent tissue loss. Determination of extent of tissue injury. (A) Both E2 and GEN reduced tissue after short term hypogonadism animals. (B) Neither GEN nor E2 reduced the extent of brain injury after long-term hypogonadism. (C and D) Immunohistochemical stains for GFAP from brain slices after stroke. Analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean± SD, n=8-10, *$p<0.05$.

**Figure 9:** GFAP expression. (C) E2 but not GEN increased GFAP expression at the ipsilateral side after 21 days of reperfusion post-stroke after short-term hypogonadism. Neither E2 nor GEN reduced GFAP at the ipsilateral side of the long-term hypogonadism cohort. (F) GEN but not E2 reduced GFAP expression at the contralateral side after short-term hypogonadism animals. Both GEN and E2 reduced GFAP expression at the contralateral side in the long-term hypogonadism animals. (A, B, D and E) Representative western blot images for GFAP stain of stroked subjects. Analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean± SD, n=7-11, *$p<0.05$.

**Figure 10:** Iba1 expression. (C) Neither E2 nor GEN reduced Iba1 expression at the ipsilateral side after 21 days of reperfusion post-stroke in both short-term and long-term hypogonadism. (F) Both GEN and E2 reduced Iba1 expression at the contralateral side in the long-term hypogonadism animals but not in the short-term hypogonadism group. (A, B, D and E) Representative western blot images for GFAP stain of stroked subjects Analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SD, n=7-11, *$p<0.05$.

**Table 2:** Statistics for Morris Water Maze test. Two-way ANOVA was used for the short-term hypogonadism cohort with Surgery and Treatment as the independent factors. The long-term hypogonadism cohort was analyzed with one-way ANOVA with subgroups as the factors. Within stroke comparisons were done with a two-way ANOVA with Duration of hypogonadism and Treatment as factors. Fisher's LSD test was used as a *posthoc* test for main effects observed. Columns in the table describe the variables investigated in the Water Maze test with the rows descriptive of the study cohorts and independent variables. The F-ratios and p-values show the statistical outcome describing the main effects of Surgery, Treatment or Duration of Hypogonadism.

TABLE 1. Additional information about antibodies used in the study

| | **Antibody** | **RRID** | **Manufacturer** | **Catalogue number** | **Raised in** |
|---|---|---|---|---|---|
| 1 | Anti-Glial Fibrillary Acidic Protein (GFAP) Ab-4 Polyclonal Antibody | RRID:AB_60418 | Lab Vision | RB-087-A1 | Rabbit |
| 2 | Iba1 Antibody | RRID:AB_2224402 | Abcam | Ab5076 | Rabbit |
| 3 | Anti-Iba-1 Polyclonal Antibody, Unconjugated | RRID:AB_839506 | FUJIFILM Wako Shibayagi | 016-20001 | Rabbit |
| 4 | Beta Actin Monoclonal Antibody (AC-15) | RRID:AB_2273656 | Thermo Fisher Scientific | MA1-91399 | Mouse |
| 5 | Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit IgG, H+L | RRID:AB_2340593 | Jackson ImmunoResearch | 711-065-152 | Donkey |

TABLE 2: Statistics for Morris Water Maze test showing the effect of treatment, surgery and duration of hypogonadism on path length, swim speed and probe trials.

| Duration of Hypogonadism | | | Pathlength | | | | | | Swim speed | | | | | | Target Quadrant | | | | Annulus 40cm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Acquisition | | Retention | | Reversal | | Acquisition | | Retention | | Reversal | | Acquisition | | Reversal | | Acquisition | | Reversal | |
| | Effect | df | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* |
| Short-term | | | | | | | | | | | | | | | | | | | | | | |
| N=64 | Treatment | 2 | 1.626 | 0.206 | 2.156 | 0.125 | 1.334 | 0.271 | 4.122 | 0.021 | 8.7 | 0.001 | 9.073 | 0 | 0.863 | 0.427 | 1.721 | 0.188 | 0.288 | 0.751 | 0.991 | 0.378 |
| | Surgery | 1 | 2.699 | 0.106 | 0.028 | 0.868 | 4.863 | 0.031 | 0.72 | 0.4 | 0.562 | 0.457 | 7.742 | 0.007 | 0.046 | 0.832 | 2.127 | 0.15 | 0.861 | 0.358 | 2.192 | 0.144 |
| | (Treatment*Surgery) | 2 | 0.391 | 0.678 | 0.427 | 0.655 | 1.613 | 0.208 | 1.047 | 0.358 | 0.793 | 0.458 | 0.671 | 0.515 | 0.582 | 0.562 | 0.64 | 0.531 | 0.453 | 0.638 | 1.98 | 0.148 |
| Long-term | | | | | | | | | | | | | | | | | | | | | | |
| N=54 | Group | 4 | 2.135 | 0.091 | 0.297 | 0.879 | 1.541 | 0.205 | 2.662 | 0.043 | 4.655 | 0.003 | 3.405 | 0.016 | 1.408 | 0.245 | 2.692 | 0.042 | 3.85 | 0.008 | 1.79 | 0.146 |
| Within stroke | | | | | | | | | | | | | | | | | | | | | | |
| | Treatment | 2 | 1.613 | 0.208 | 0.378 | 0.687 | 0.215 | 0.807 | 0.964 | 0.387 | 1.397 | 0.255 | 3.962 | 0.024 | 0.482 | 0.62 | 2.559 | 0.086 | 3.184 | 0.049 | 3.467 | 0.037 |
| | Duration | 1 | 0.784 | 0.379 | 1.626 | 0.207 | 0.443 | 0.508 | 3.371 | 0.071 | 5.242 | 0.026 | 0.823 | 0.368 | 3.651 | 0.061 | 1.714 | 0.195 | 0.518 | 0.474 | 1.823 | 0.182 |
| | (Treatment*Duration) | 2 | 2.798 | 0.069 | 0.721 | 0.491 | 3.239 | 0.046 | 0.331 | 0.719 | 1.258 | 0.291 | 0.469 | 0.628 | 2.38 | 0.101 | 3.206 | 0.047 | 2.442 | 0.096 | 1.713 | 0.189 |

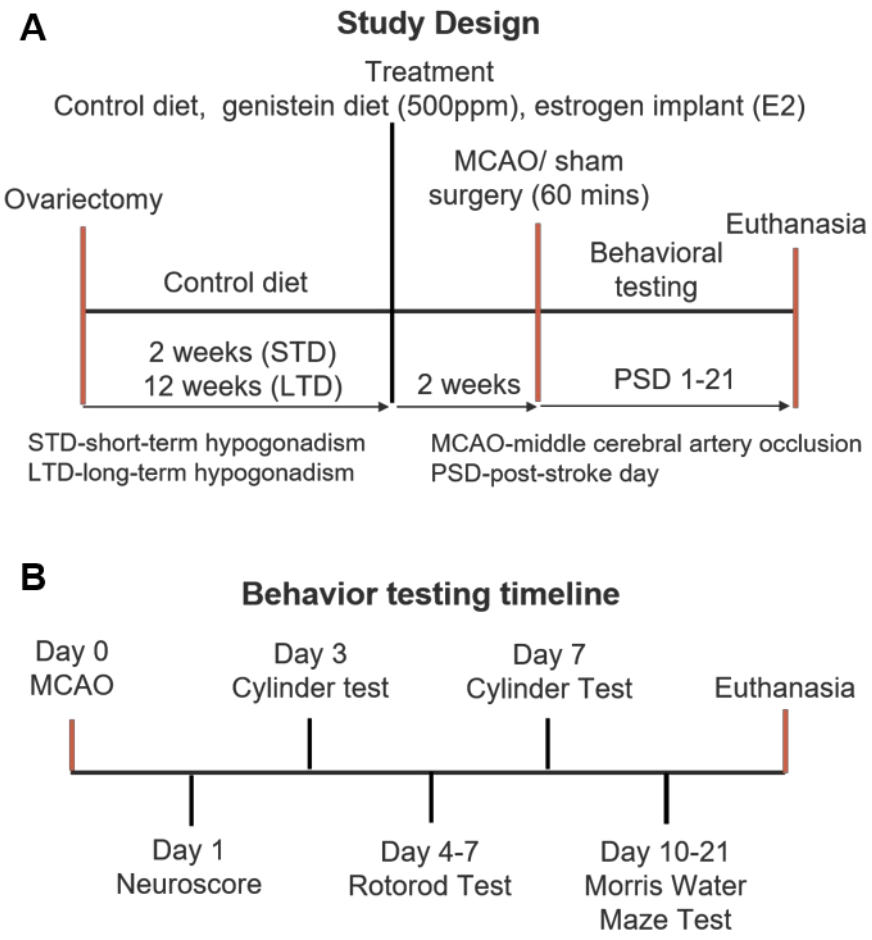
A
Study Design
Treatment
Control diet, genistein diet (500ppm), estrogen implant (E2)
Ovariectomy
MCAO/ sham surgery (60 mins)
Euthanasia
Behavioral testing
Control diet
2 weeks (STD)
12 weeks (LTD)
2 weeks
PSD 1-21
STD-short-term hypogonadism
LTD-long-term hypogonadism
MCAO-middle cerebral artery occlusion
PSD-post-stroke day
B
Behavior testing timeline
Day 0
MCAO
Day 3
Cylinder test
Day 7
Cylinder Test
Euthanasia
Day 1
Neuroscore
Day 4-7
Rotorod Test
Day 10-21
Morris Water
Maze Test

**Figure 1**

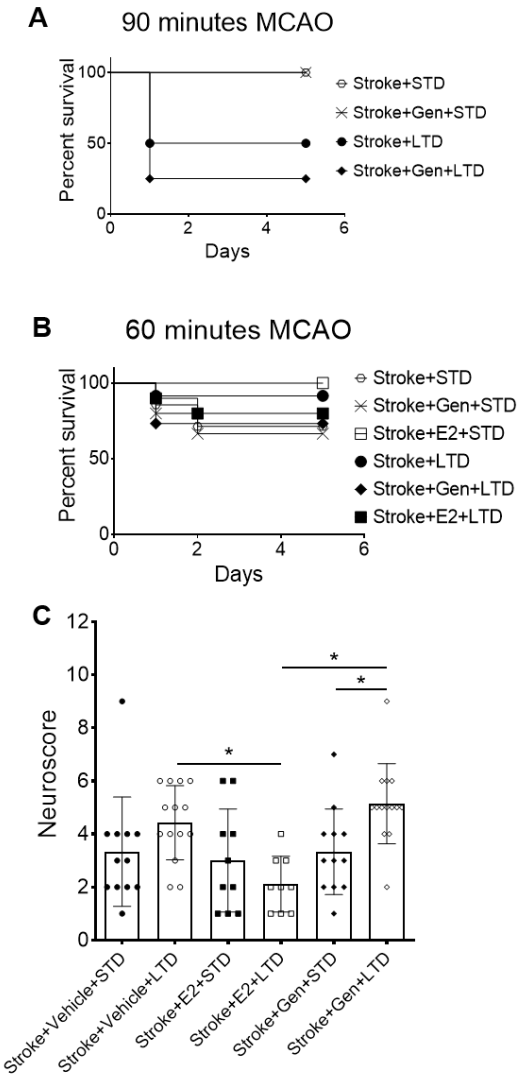
A
90 minutes MCAO
Percent survival
100
50
0
0
2
4
6
Days
Stroke+STD
Stroke+Gen+STD
Stroke+LTD
Stroke+Gen+LTD
B
60 minutes MCAO
Percent survival
100
50
0
0
2
4
6
Days
Stroke+STD
Stroke+Gen+STD
Stroke+E2+STD
Stroke+LTD
Stroke+Gen+LTD
Stroke+E2+LTD
C
Neuroscore
12
10
8
6
4
2
0
*
*
*
Stroke+Vehicle+STD
Stroke+Vehicle+LTD
Stroke+E2+STD
Stroke+E2+LTD
Stroke+Gen+STD
Stroke+Gen+LTD

**Figure 2**

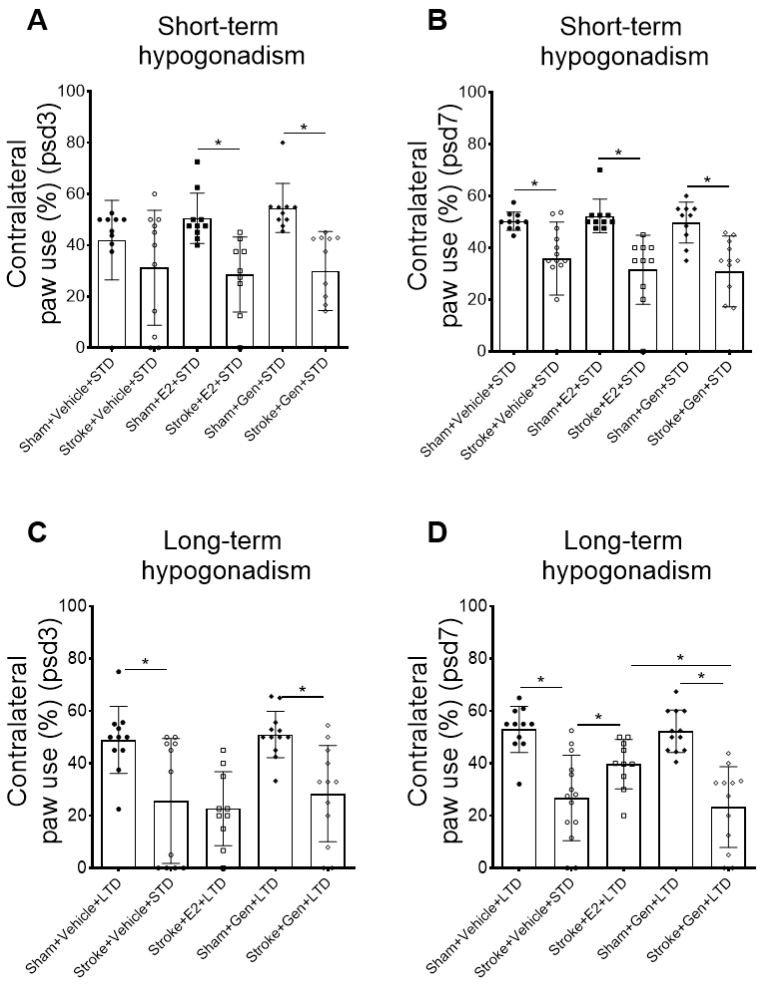
A
Short-term hypogonadism
Contralateral paw use (%) (psd3)
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
B
Short-term hypogonadism
Contralateral paw use (%) (psd7)
C
Long-term hypogonadism
Contralateral paw use (%) (psd3)
Sham+Vehicle+LTD
Stroke+Vehicle+STD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
D
Long-term hypogonadism
Contralateral paw use (%) (psd7)

**Figure 3**

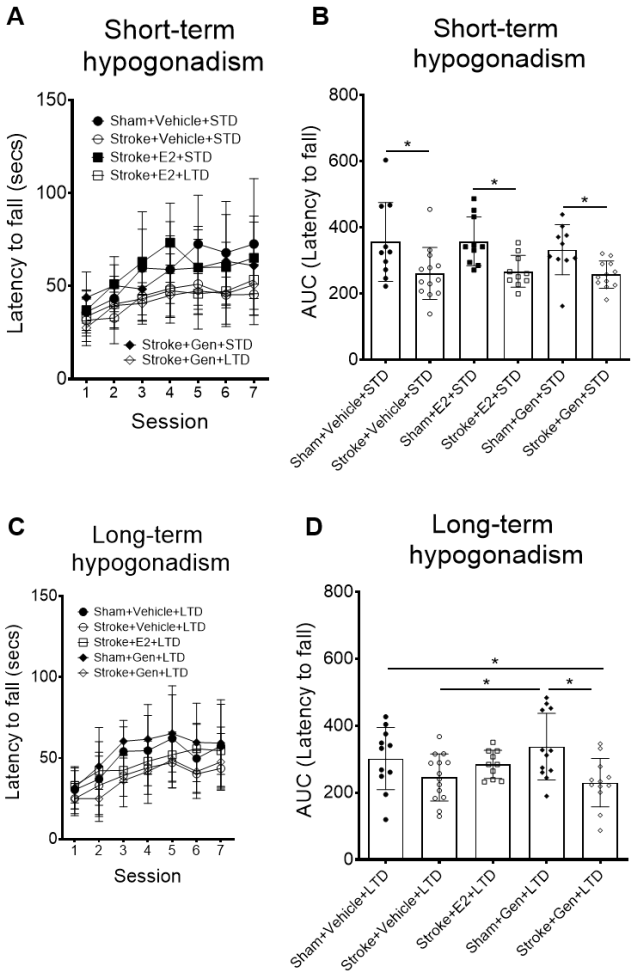
A
Short-term hypogonadism
Latency to fall (secs)
Session
Sham+Vehicle+STD
Stroke+Vehicle+STD
Stroke+E2+STD
Stroke+E2+LTD
Stroke+Gen+STD
Stroke+Gen+LTD
B
Short-term hypogonadism
AUC (Latency to fall)
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
C
Long-term hypogonadism
Latency to fall (secs)
Session
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
D
Long-term hypogonadism
AUC (Latency to fall)
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD

**Figure 4**

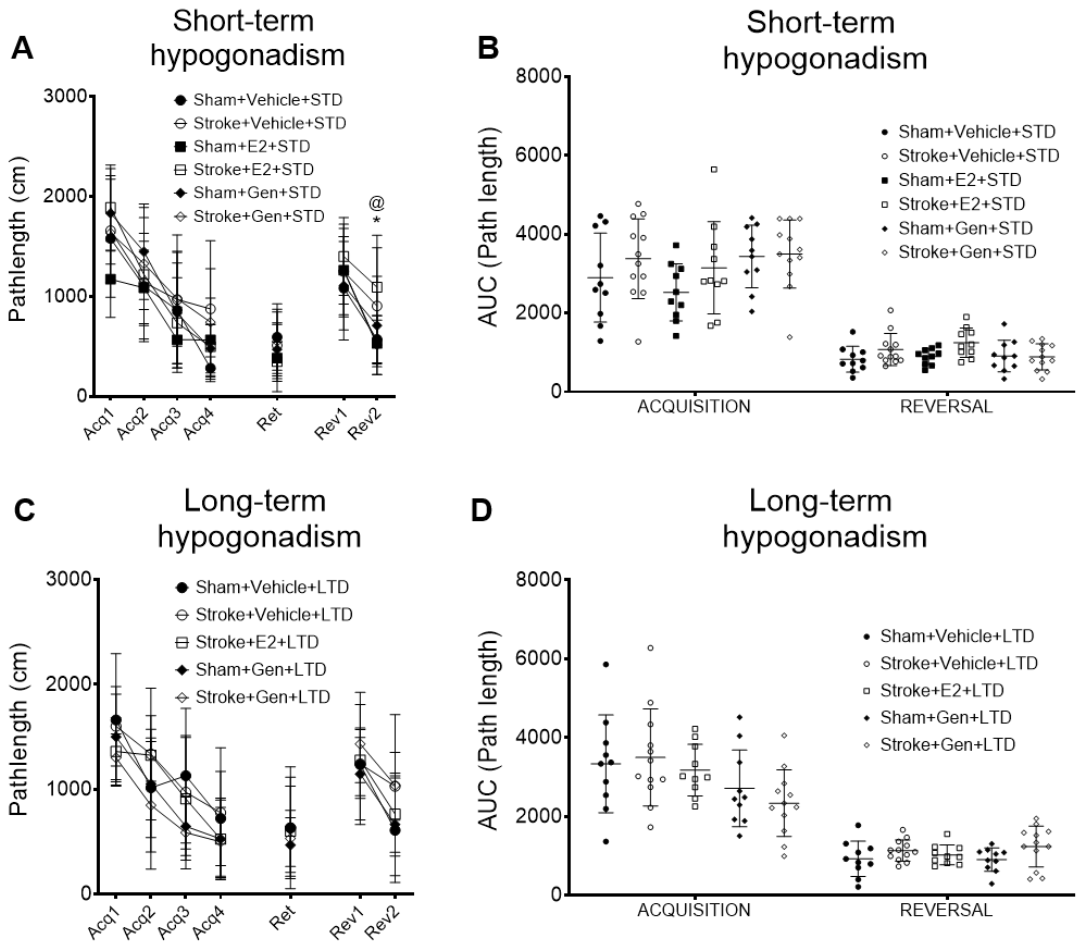
A
Short-term hypogonadism
Pathlength (cm)
3000
2000
1000
0
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
@
*
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
B
Short-term hypogonadism
AUC (Path length)
8000
6000
4000
2000
0
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
ACQUISITION
REVERSAL
C
Long-term hypogonadism
Pathlength (cm)
3000
2000
1000
0
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
D
Long-term hypogonadism
AUC (Path length)
8000
6000
4000
2000
0
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
ACQUISITION
REVERSAL

**Figure 5**

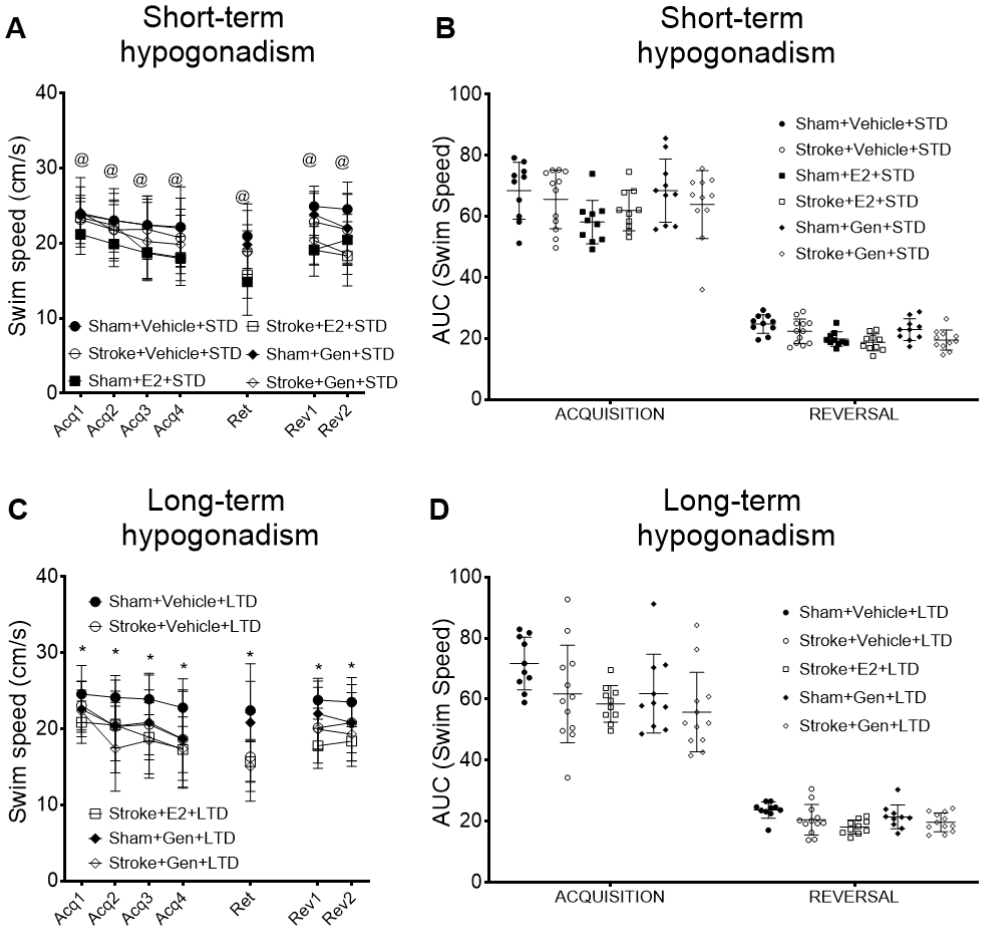
A
Short-term hypogonadism
Swim speed (cm/s)
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
B
Short-term hypogonadism
AUC (Swim Speed)
ACQUISITION
REVERSAL
C
Long-term hypogonadism
Swim speed (cm/s)
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
D
Long-term hypogonadism
AUC (Swim Speed)

**Figure 6**

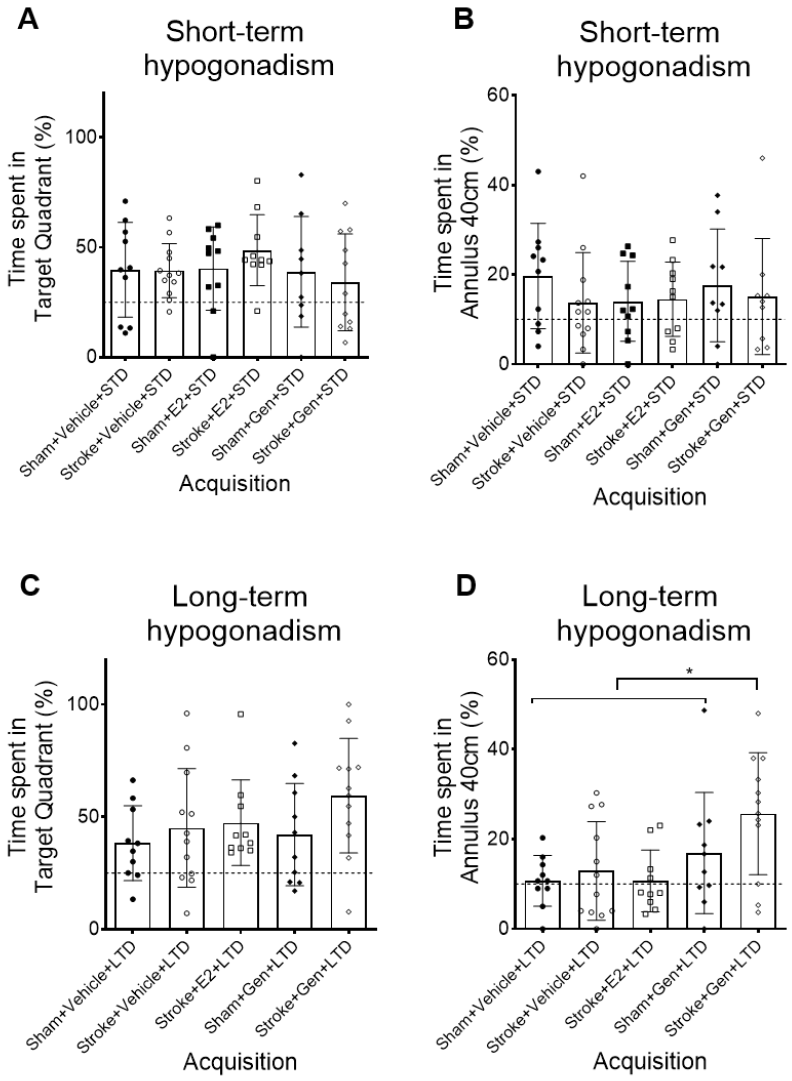
A
Short-term hypogonadism
Time spent in Target Quadrant (%)
100
50
0
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acquisition
B
Short-term hypogonadism
Time spent in Annulus 40cm (%)
60
40
20
0
Sham+Vehicle+STD
Stroke+Vehicle+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acquisition
C
Long-term hypogonadism
Time spent in Target Quadrant (%)
100
50
0
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
Acquisition
D
Long-term hypogonadism
*
Time spent in Annulus 40cm (%)
60
40
20
0
Sham+Vehicle+LTD
Stroke+Vehicle+LTD
Stroke+E2+LTD
Sham+Gen+LTD
Stroke+Gen+LTD
Acquisition

**Figure 7**

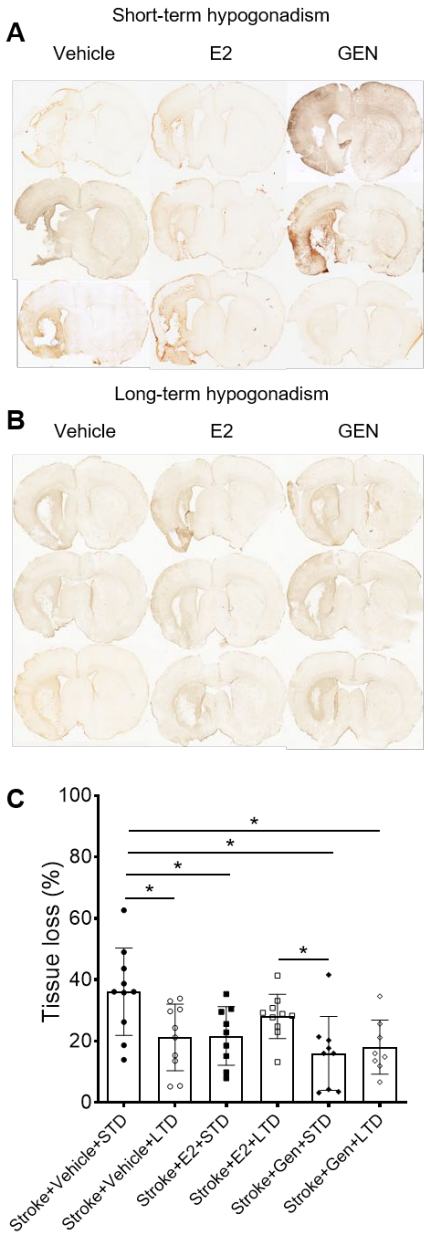
A
Short-term hypogonadism
Vehicle
E2
GEN
B
Long-term hypogonadism
Vehicle
E2
GEN
C
Tissue loss (%)
100
80
60
40
20
0
*
*
*
*
*
Stroke+Vehicle+STD
Stroke+Vehicle+LTD
Stroke+E2+STD
Stroke+E2+LTD
Stroke+Gen+STD
Stroke+Gen+LTD

**Figure 8**

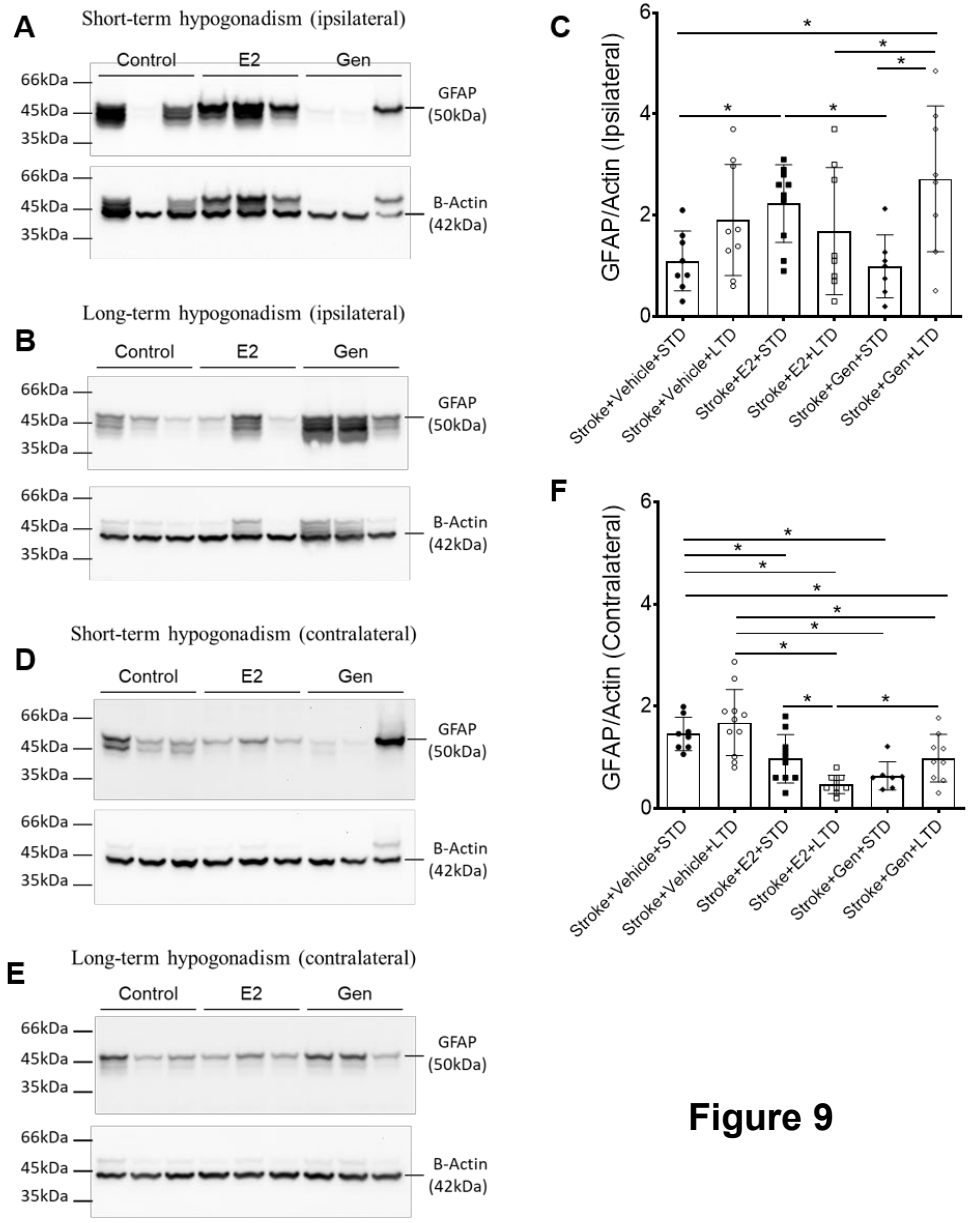
A
Short-term hypogonadism (ipsilateral)
Control
E2
Gen
66kDa
45kDa
35kDa
GFAP
(50kDa)
B-Actin
(42kDa)
B
Long-term hypogonadism (ipsilateral)
D
Short-term hypogonadism (contralateral)
E
Long-term hypogonadism (contralateral)
C
GFAP/Actin (Ipsilateral)
F
GFAP/Actin (Contralateral)
Stroke+Vehicle+STD
Stroke+Vehicle+LTD
Stroke+E2+STD
Stroke+E2+LTD
Stroke+Gen+STD
Stroke+Gen+LTD

**Figure 9**

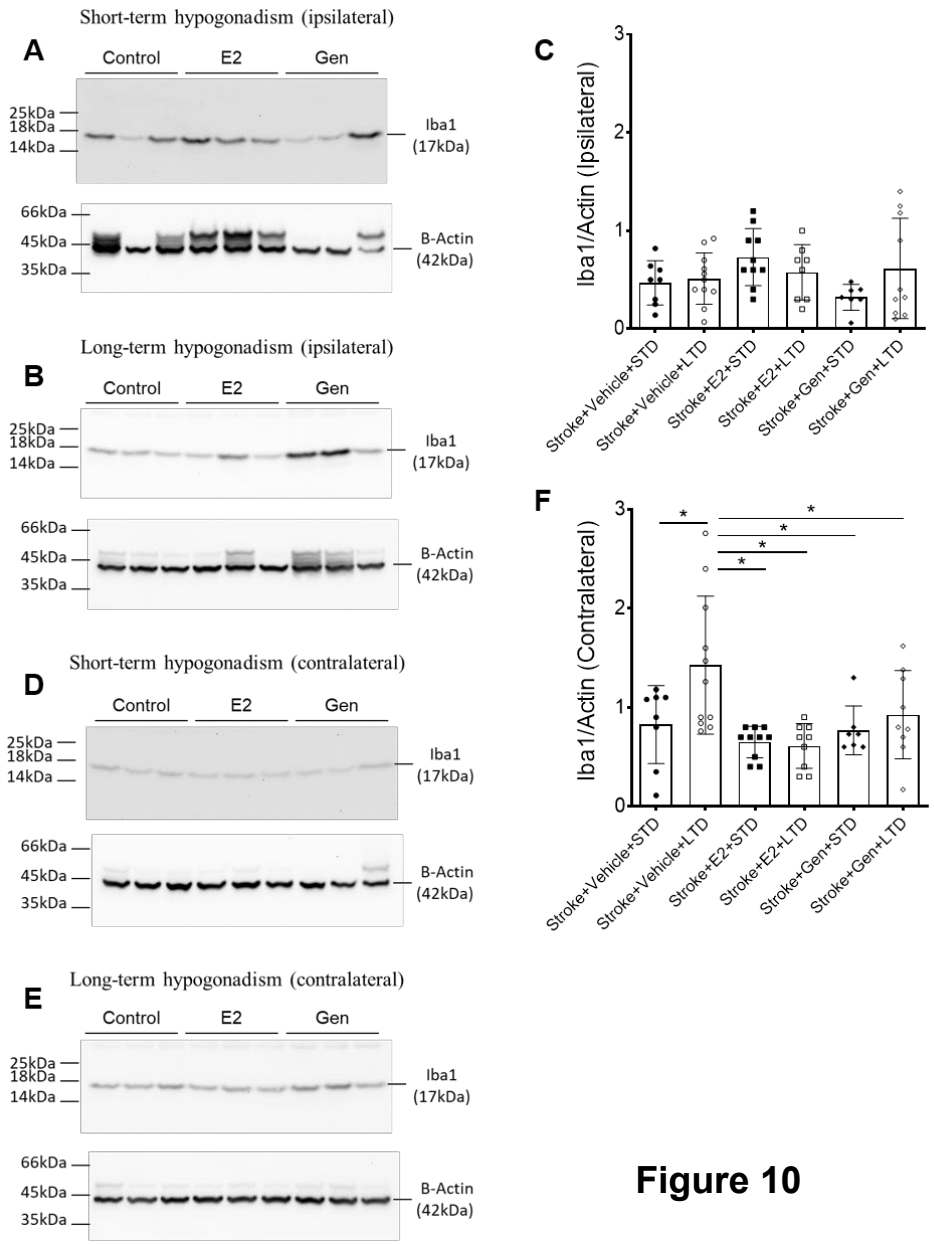
Short-term hypogonadism (ipsilateral)
A
Control
E2
Gen
25kDa
18kDa
14kDa
Iba1
(17kDa)
66kDa
45kDa
35kDa
B-Actin
(42kDa)
Long-term hypogonadism (ipsilateral)
B
Short-term hypogonadism (contralateral)
D
Long-term hypogonadism (contralateral)
E
C
Iba1/Actin (Ipsilateral)
F
Iba1/Actin (Contralateral)
Stroke+Vehicle+STD
Stroke+Vehicle+LTD
Stroke+E2+STD
Stroke+E2+LTD
Stroke+Gen+STD
Stroke+Gen+LTD

**Figure 10**

TABLE3: Summary of behavioral performance and extent of injury

| Variable | Treatment | Estrogen | | Genistein | |
|---|---|---|---|---|---|
| | Age | Young | | Young | |
| | Hypogonadism length | STD | LTD | STD | LTD |
| Neuroscore | | ↔ | ↑ | ↔ | ↔ |
| Locomotor Symmetry | | ↔ | ↑ | ↔ | ↔ |
| Motor learning | | ↔ | ↔ | ↔ | ↔ |
| Path length | Acquisition learning | | | | |
| | Retention | ↔ | ↔ | ↔ | ↔ |
| | Reversal Learning | ↔ | ↔ | ↑ | ↔ |
| | | | | | |
| Swim speed | Acquisition learning | ↔ | ↔ | ↔ | ↔ |
| | Retention | ↓ | ↔ | ↑ | ↔ |
| | Reversal Learning | ↔ | ↔ | ↔ | ↔ |
| | | | | | |
| Probe | Acquisition | ↔ | ↔ | ↔ | ↑ |
| | Reversal | ↔ | ↔ | ↔ | ↔ |
| Extent of Injury | | ↑ | ↔ | ↑ | ↔ |

↑ Benefit

↔ No effect

↓ Worse effect

# CHAPTER 3

# DIETARY GENISTEIN DIFFERENTIALLY INFLUENCES LOCOMOTOR AND COGNITIVE FUNCTIONS FOLLOWING TRANSIENT FOCAL CEREBRAL ISCHEMIA IN MIDDLE-AGED OVARIECTOMIZED RATS AT DIFFERENT LENGTHS OF HYPOGONADISM

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Running Title: Length of hypogonadism influences the neuroprotective effect of dietary genistein

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**Counts in the manuscript:**

Figures: 10

Tables: 1

Abstract: 222 words

Significance Statement: 99 words

Introduction: 561 words

Discussion: 958 words

**Acknowledgments**:

The authors acknowledge the assistance of Dr. Fen Sun in stroke surgery and Ella Anle Kasanga in euthanasia and tissue collection.

**Disclosure Statement:**

The authors have no conflicts of interest to declare.

**Funding Sources:**

This work was funded by the JES Edwards Foundation (DAS), Sigma Xi Grant-in-Aid of Research (AO) and T32 AG020494 training grant (AO).

**Notes on contributors**:

**Anthony Oppong-Gyebi**- PhD candidate at the University of North Texas Health Science Center; performed animal studies including treatments, surgery, stroke and behavioral testing, data analysis, western blotting, immunohistochemistry and led manuscript preparation.

**Daniel Metzger** – Lab manager at University of North Texas Health Science Center; assisted with sample preparation, western blotting.

**Philip H. Vann** – Lab manager at University of North Texas Health Science Center; assisted with animal behavior training and data analysis.

**Nathalie Sumien** – Associate Professor at University of North Texas Health Science Center; directed behavioral studies and analysis of behavioral data and assisted with manuscript preparation.

**Derek A. Schreihofer** – Associate Professor at the University of North Texas Health Science Center; led the overall study design, directed the study, provided funding and co-led manuscript preparation.

***Submitted to eNEURO***

**Abstract**

Ischemic stroke disproportionately affects women beyond the menopause transition compared to men of similar age. Several observational studies had shown that estrogen therapy among postmenopausal women was associated with reductions in cardiovascular events including coronary heart diseases. However, reports from more recent prospective clinical trials not only failed to show cardiovascular benefits, but also revealed an increased risk for cardiovascular disease in late postmenopausal women, hence accounting for mistrust in estrogen use. For this discrepancy, other agents, including the soy isoflavone genistein, have been investigated as possible alternatives for hormone therapy post-menopause. In the current study, we investigated the neuroprotective effects of dietary genistein at varied lengths of hypogonadism in middle-aged ovariectomized Sprague-Dawley rats under ischemic conditions. Two weeks of treatment with dietary genistein at 42 mg/kg improved cognitive flexibility (Morris water maze test) after short-term hypogonadism (2-weeks) but not long-term hypogonadism (12-weeks). Dietary genistein improved locomotor asymmetry (cylinder test) after long-term but not short-term hypogonadism. Dietary genistein did not reduce infarct size after either short-term or long-term hypogonadism. Genistein, however, reduced ionized calcium-binding adaptor molecule-1 (Iba1) expression, a marker of brain inflammation, at the ipsilateral side of stroke injury after short-term but not long-term hypogonadism. This study suggests that the neuroprotective effects of dietary genistein on motor and cognitive functions are distinctly influenced by the length of hypogonadism following focal cerebral ischemia.

**Significance**

There is an increasing postmenopausal population opting for homeopathic medicines for the management of menopausal symptoms due to the perceived distrust in estrogen use as hormone

replacement. Basic and clinical studies support the notion that early, but not delayed, hormone replacement after menopause is beneficial. Furthermore, evidence suggests that delaying hormone replacement augments the detrimental, rather than the beneficial, effects of estrogens. Because soy isoflavones such as genistein are used as alternatives to estrogen replacement, it is necessary to understand whether this popular alternative treatment also loses efficacy when treatment is delayed after the loss of endogenous ovarian hormones. **Keywords:** Ischemic stroke, genistein, chronic hypogonadism, cognition, locomotor activity

## 1.1 Introduction

Stroke remains a leading cause of death and long-term disability [2]. From human studies, it is evident that young adult and premenopausal women have substantially lower stroke incidence and prevalence than men of similar ages [332, 333]. However, beyond the menopause transition, there is a narrowing of the age-dependent sex difference such that there is a higher prevalence of stroke in women than men with women accounting for the majority of cerebrovascular disease-related mortalities [330, 332, 333, 409, 410]. Besides, postmenopausal women with stroke are more likely to be hospitalized, have worse stroke prognoses, and have a much higher stroke recurrence within 5 years of the first stroke compared to men of similar ages [411, 412]. The reduction in circulating female sex hormones estrogen and progesterone is thought to underlie the change in stroke risk and outcomes in women after menopause [413-416]. Because both estrogen and progesterone demonstrate neuroprotection against ischemic insults in both male and female rodent preclinical studies [417, 418], several clinical trials have been conducted using estrogens and progestins to investigate the effect of the female sex hormones on long-term cardiovascular risk and management of menopausal symptoms. However, detrimental outcomes from several studies

including the Women's Health Initiative (WHI) study [417] and the Nurse's Health Study [275] led to clinical recommendations to restrict the use of estrogens and/or progesterone as a measure for the prevention of chronic cardiovascular diseases [419, 420]. The leading hypothesis for this observed detriment is the timing of initiation of hormone replacement therapy in which the average age of participating recipients (63 years) was well above the median age of menopause (~51years) [176, 275, 421]. This suggests that delaying hormone replacement therapy up to 10 years after menopause may diminish the benefits of E2. This conclusion was further confirmed in some preclinical studies in which there were increased vasoconstriction and constrictor-prostanoid activity in 12-month old rat middle cerebral artery, disruption of mitogen-activated protein kinase signaling in 18-month old rats and reduction in cortical and hippocampal spinal density after 10-week hypogonadism in rats [321, 322, 422, 423]. Hence, the effectiveness of estrogen therapy may depend on the timing of treatment [176, 177, 272, 424-426]. Because of these limitations, there are intensified efforts to identify other agents as potential alternatives to estrogen replacement for managing menopausal symptoms and for cerebrovascular risk reduction [358-362]. Such agents include the plant-derived estrogens, or phytoestrogens, that have been investigated for their potential benefits during menopause [284-287]. Among the well-studied phytoestrogens is the compound Genistein that belongs to a larger subclass collectively called isoflavones. Previous studies have shown the neuroprotective benefits of genistein through its constitutive antioxidant properties, estrogen receptor-dependent pathways and activation of receptors peroxisome proliferator-activated receptor-gamma (PPARγ), as well as the inhibition of specific tyrosine kinases [314, 427]. These established actions by genistein attenuate inflammatory responses and increase neurotrophic factors including brain-derived neurotrophic factors to confer neuroprotection [211-213, 428, 429]. After long-term hormone deprivation, genistein increases

dendritic spine density in the hippocampus to improve spatial performance in middle-aged rats [326]. Given this evidence and a substantially lower prevalence of stroke in regions where there is a higher consumption of soy isoflavones [296], genistein has the potential to preserve cognitive function while maintaining motor function under extended hormone depletion following menopause. In the current study, we investigated the effects of different lengths of hormone depletion on the neuroprotective benefits of dietary genistein in middle-aged female Sprague-Dawley rats during focal cerebral ischemia.

## Materials and Methods

### 2.1 Animals and treatment groups

Middle-aged retired breeder female Sprague-Dawley rats (266-356g; Envigo) were housed in pairs under a 12-h light-dark cycle throughout the study. All animals received food and water ad libitum and all experimental procedures were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

All rats underwent bilateral ovariectomy under 2% isoflurane anesthesia [212, 430]. The region between the ribcage and kidneys was thoroughly cleaned of the fur and a dorsal midline incision was made through the skin into the abdominal cavity. The ovaries and their contiguous tissues including the fallopian tube were located, tightly clamped at the junction between the ovaries and funnel of the fallopian tube with a suitable hemostat. The ovaries were carefully removed, and an absorbable suture was used to seal the end of the severed tissue to minimize bleeding post-surgery. The cuts made were closed with sterile absorbable sutures and sanitized with antibiotic ointment

to avoid post-surgical infection. All animals were grouped into two timepoints of ovarian hormone depletion, short-term hypogonadism (2 weeks deprivation, STD) and long-term hypogonadism (12-weeks deprivation, LTD), periods during which they were all fed with certified isoflavone-free diet (Purina Test Diet, 5K96). Rats were recategorized into treatment groups to continue on isoflavone free diet (Vehicle), switched to customized genistein-diet from 5K96 at a concentration of 500 ppm of pure genistein (Gen, LC Laboratories) or received Silastic implant (inner/outer diameter: 1.575/3.175 mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) of 17β-estradiol (E2, Sigma Chemical). Based on previous studies, the genistein formulation and Silastic implants of estrogen were expected to provide plasma concentrations of 5 μM and 50-70 pg/ml respectively [300, 365-367]. Short-term hormone deprived rats were divided into 6 groups: Sham (n= 10), sham+GEN (n= 10), sham+E2 (n= 10), stroke (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 15). Long-term hypogonadal animals were divided into 4 groups: Sham (n= 10), stroke (n= 15), stroke+GEN (n= 15) and stroke+E2 (n= 15) (Figure 1).

**2.2 Ischemic stroke model**

All rats underwent reversible middle cerebral artery occlusion (MCAO) or sham surgery as previously described [300]. Rats were anesthetized using 2% isoflurane and an incision is made at the ventral side of the neck to expose the right common carotid artery (CCA). A temporary ligation is made in the CCA and the internal carotid artery (ICA). The external carotid artery is then severed, a suitable silicone suture (Doccol Corporation, Sharon MA) with a diameter range of 0.37-0.41 mm was inserted into the ICA, advanced ~19 mm to the origin of the middle cerebral artery and left in place for 60-minutes before withdrawal. The appropriate suture size was selected based on the bodyweight of the animal to achieve consistent occlusion. A rectal temperature probe and automated heating blanket were used to maintain the body temperature of the animals at

37±0.5 °C. Pulse oximetry (Kent Scientific) was used to monitor and maintain an adequate saturation oxygen pulse ≥ 97%. After suture insertion, the incision was temporarily closed, and animals were removed from anesthesia. Only subjects who showed signs of right-sided paresis during the occlusion period were included in the study. The sham animals were subjected to the same procedures as the stroked animals except for the insertion of suture into the ICA. Rats were re-anesthetized, and the suture was withdrawn after a 60 min occlusion phase.

**2.3 Behavioral testing**

All animals underwent a battery of behavioral assessments as measures of stroke severity over 21 days in the following order: Neuroscore, cylinder test, rotarod and Morris water maze. On day one after stroke, rats underwent neurological assessment involving spontaneous circling, contralateral forelimb flexion and hindlimb retraction all on 9-point modified Bederson scale [300, 431].

The cylinder test was used to assess locomotor asymmetry between the forelimbs as a measure of sensorimotor deficits on post-stroke days (PSD) 3 and 7 as previously described [371, 430]. Forelimb asymmetry was calculated as the ratio of all contralateral/right forelimb touches to the total wall touches and expressed as the percent contralateral paw use.

Motor learning was evaluated with the rotarod test on the motor-driven AccuRotor instrument (Accuscan Instruments). Rats were required to adjust to a rotating beam over a speed range of 0 - 75 rpm up to 150 secs. Each animal received two sessions of four trials per session that were separated by a resting period of at least 10 minutes after every trial. A minimum of three hours was allowed in between the sessions. Motor learning was expressed as the average latency to fall off the rod over the four trials.

The Morris water maze (MWM) was employed to assess spatial learning and memory as previously described [373-375]. Rats underwent pretraining on a straight alley under a visual cue-independent manner MWM test day 1. Subsequent testing involved four days of acquisition to locate the hidden platform based on visual cues, a one-day retention test after a 48-h break and two days of reversal test involving adjustment to the hidden platform positional change. Data from the seven phases were expressed as the path length in cm and the path-independent swim speed in cm/s. Thirty-second probe trials were conducted before the last session of the acquisition phase and after the last session of the reversal phase to determine spatial bias for the platform location amongst the rats. Results from probe trials were expressed as the percentage of time spent in the target quadrant and the imaginary annulus 40cm ring around the platform location. All data used in spatial learning and memory analysis were obtained with a computerized tracking system (AnyMaze software, Stoelting).

**2.4 Biochemistry**

Twenty-one days after stroke, all rats were humanely euthanized by transcardial perfusion with 0.9% saline solution under 5% isoflurane as previously described [212]. After perfusion, a 1 mm coronal section from ~ 0.0 to -1.0 mm Bregma was made, snap-frozen and stored at -70° C for future western blot experiments. The rest of the brain was post-fixed in 4% paraformaldehyde after which 40µm coronal sections were obtained and stored in a suitable cryoprotectant for immunohistochemical analysis [376].

Brain slices between Bregma -1.00mm and -2.80mm were stained for glial fibrillary acidic protein (GFAP, 1:400, Santa Cruz, SC-58766) and ionized calcium-binding adaptor molecule 1 (IBA1, 1:1000, Abcam, ab5076) as measures of the extent of tissue loss poststroke. A matched representative section for poststroke damage was evaluated per animal to recapitulate previous

studies that examined the effects of genistein over shorter time points [300]. Sections were washed with phosphate-buffered saline with 0.1% Triton X-100 (PBS-T), blocked in 10% horse serum and incubated overnight at 4° C with the above-mentioned primary antibodies. The slices were rinsed with PBS-T incubated with a suitable secondary antibody conjugated with horseradish peroxidase (Jackson Immuno) for an hour followed by 45 minutes incubation with avidin-biotin complex before visualizing with a commercial diaminobenzidine product (ImmPactDAB, Vector Labs) [379]. Stained slices were mounted on gelatin-coated slides, air-dried, delipidated and coverslipped with DPX mounting medium (Sigma-Aldrich). Images were obtained at 4x magnification using Neurolucida 2D slide scanning microscope (MBF Bioscience) and the amount of tissue lost was expressed as the percent difference between the contralateral and ipsilateral sides relative to the contralateral side after analysis with Adobe Photoshop 2020.

The 1 mm snap-frozen coronal slices were homogenized in T-PER reagent (Thermo-Pierce, Rockford, Illinois) containing HALT Protease Inhibitor Cocktail (Thermo-Pierce). A BCA protein assay (BioRad) was used to determine the protein concentrations per supernatant obtained after centrifugation. Equal sample concentrations (30µg) were loaded on 4-20% sodium dodecyl sulfate-polyacrylamide precast gels (BioRad) to separate proteins. Proteins were transferred to a nitrocellulose membrane overnight at 4° C. Non-specific binding was blocked with 5% milk for an hour at room temperature and membranes were incubated with the primary antibodies GFAP (1:1000, Santa Cruz, SC-58766), Iba1 (1:1000, Wako, 016-20001) and β-actin (1:5000, Thermo Scientific, MA1-91399) overnight. Membranes were washed in PBS with Tween-20 and further incubated with Horseradish-peroxidase conjugated secondary antibody for 1 hour at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used to visualize reactive bands and the densities of the bands were quantified with the ImageJ software

(NIH, Bethesda, MD, USA) after which protein levels were determined relative to the densities of β-actin (housekeeping protein).

### 2.5 Statistical Analysis

All data are expressed as mean ±S EM. Within stroke comparisons were done with two-way analysis of variance (ANOVA) with Duration (STD vs. LTD) and Treatment (Vehicle vs. E2 vs. Gen) as the dependent variables. Data from the cylinder, rotorod and MWM from the short-term hypogonadism cohort were analyzed with two-way ANOVA using Type of Surgery (Sham vs. Stroke) and Treatment (Vehicle vs. E2 vs. Gen) as the factors. Behavioral data from the long-term hypogonadism group were analyzed with a one-way ANOVA. Immunohistochemical and Western blot data were analyzed using a two-way ANOVA with Treatment and Duration as the independent factors. Where main effects were seen, Fisher's Least Significance Difference (LSD) was employed as the *posthoc* test. An alpha value of 0.05 was set for significance level and all data were analyzed using Systat Version 13.1 (Systat Software, Inc, San Jose, CA, USA).

## Results

### 3.1 Effect of Gen and E2 treatment on neurological deficits

A modified Bederson scale was used to assess neurological deficits expressed as the Neuroscore. No statistical difference was observed among the Gen-fed and E2-treated animals compared to the vehicle (Figure 2). The short-term hypogonadism animals, however, had higher Neuroscore compared to the long-term hypogonadism cohort. This was revealed by the effect of Duration following two-way ANOVA ($F(1,75) = 8.46$, $p = 0.005$).

### 3.2 Effect of Gen and E2 treatment on locomotor asymmetry

Locomotor deficits between the right and forelimbs were determined with the cylinder test. After short-term hypogonadism, the stroke rats showed impairment in use of the contralateral forelimb on both post-stroke days 3 (Figure 3A, $F(1,55) = 56.813$, $p<0.001$) and 7 (Figure 3B, $F(1,54) = 25.466$, $p<0.001$). Neither Gen nor E2 pretreatment improved the symmetry between the forelimbs compared to the vehicle. However, in the long-term group, both Gen and E2 improved contralateral paw usage on poststroke days 3 for both treatments (Figure 3C, $F(3,47) = 8.371$, $p<0.001$) and 7 for E2 (Figure 3D, $F(3,49) = 10.26$, $p<0.001$). Within stroke comparisons revealed main effect of treatment on post-stroke day 7 in which E2 but not Gen showed improvement in forelimb asymmetry compared to the vehicle ($F(2,68) = 4.527$, $p = 0.014$).

### 3.3 Effect of Gen and E2 treatment on motor learning and coordination

The rotarod apparatus was used to assess motor learning and coordination after stroke. The stroke subjects spent less time on the rotating rod compared to their sham counterparts after short-term hypogonadism (Figures 4A and B, $F(2,48) = 30.788$, $p<0.001$). This was supported by the interaction between the Surgery and sessions of the rotorod test ($F(6,288) = 3.724$, $p = 0.001$). The sham group in the long-term cohort also performed better than the stroke groups (Figures 4C and D, $F(3,49) = 5.96$, $p = 0.002$). There was no effect of Gen and E2 on motor learning compared to the vehicle despite what appears to be an increased latency to fall in the Gen-treated group after long-term hypogonadism.

### 3.4 Effect of Gen and E2 treatment on water maze performance

The Morris water maze was used to evaluate spatial and reversal learning and memory after stroke (see Table 1 for detailed statistics on MWM). All the groups demonstrated a reduction in path length during the acquisition phase (sessions 1 to 4) in both the short-term and long-term cohorts.

There were no difference between the groups in the acquisition or retention phases. However, the sham rats in the short-term hypogonadism cohort traversed shorter distances to find the hidden platform compared to their treatment-matched stroke animals in the reversal phase. Further, Gen-treated but not E2-treated stroke animals had a shorter path length than the vehicle (Figures 5A and B). These observations were supported by the main effects of Treatment and Surgery in the reversal phase. There was no difference in the path length traveled by the groups in the long-term hypogonadism (Figures 5C and D). Within stroke comparisons showed an interaction between Duration and Session in the acquisition phase but neither Gen nor E2 had an effect compared to the vehicle. The sham animals swam much faster than the treatment-matched stroke subject in the retention phase after short-term hypogonadism and no effect of treatment was observed (Figures 6A and B). In the reversal phase, an effect of Treatment was observed with vehicle and Gen-treated shams swimming faster than E2-treated shams. After long-term hypogonadism, the vehicle stroke subjects showed improved swim speed in the reversal phase compared to the Gen and E2-treated stroke animals (Figures 6C and D). A comparison of the stroke subjects revealed main effects of Treatment in the acquisition and reversal phases and Duration in the retention phase. No effect of treatment was observed on the times spent in the target quadrant (Figures 7A and C) and Annulus 40cm (Figures 7B and D) around the hidden platform in the acquisition and reversal phases after both short-term and long-term hypogonadism. Within stroke comparisons, on the other hand, revealed main effect of Duration on the time spent in the target quadrant during the acquisition probe.

### 3.5 Effect of Gen and E2 treatment on tissue loss, Iba1 and GFAP expression

No significant difference was seen on percent tissue loss between the treatment groups and lengths of hypogonadism (Figures 8A-D, $F(2,60) = 0.157$, $p = 0.855$). Both Gen and E2 reduced Iba1

expression at the stroke side after short-term hypogonadism. Long-term hypogonadism cohorts also showed reduced Iba1 expression compared to short-term hypogonadism. These differences were observed as an interaction between the Treatment and Duration (Figures 9A-D, $F(2,50) = 5.524$, $p = 0.007$). At the contralateral side, a main effect of Duration was seen in which the rats in long-term hypogonadism cohorts had lower expression of Iba1 compared to the short-term hypogonadism animals (Figures 9E-H, $F(2,51) = 6.277$, $p = 0.015$). Neither genistein nor E2-treated stroke animals reduced GFAP expression after both short-term and long-term hypogonadism (Figures 10A-H, $F(2,50) = 1.173$, $p = 0.318$).

**Discussion**

Genistein, a selective estrogen receptor modulator has been studied for its neuroprotective role following ischemic injury. In animal studies, genistein crosses the blood-brain barrier to confer neuroprotection without toxicity [432]. Even though genistein has shown promising outcomes after ischemia in animal models, its role against ischemia after extended hormone depletion has not been elucidated. In the current study, we investigated the temporal effects of hypogonadism on the neuroprotective properties of dietary genistein. Our results demonstrated that a two-week pretreatment with dietary genistein at a dose of 500ppm (~42mg/kg body weight) before stroke improves cognitive flexibility after short-term hypogonadism and improves locomotor deficits after long-term hypogonadism as observed in the reversal phase of the Morris water maze test and the cylinder test, respectively. Dietary genistein further reduces Iba1 expression at the ipsilateral side of the ischemic brain after short-term hypogonadism. The selected dose translates into an approximate routine consumption of genistein in a typical adult Asian diet associated with a low risk of ischemic stroke [433].

Although a handful of studies have reported on the role of genistein on locomotor deficits in neurological disease models, little is known about the effects of genistein on locomotor function after stroke. In the current stroke model, both E2 and dietary genistein improved forelimb asymmetry after long-term hypogonadism. This finding is in line with previously reported evidence including the 6-hydroxydopamine Parkinson's disease model with where intraperitoneal administration of genistein improved forelimb deficits [383]. Additionally, fear-related locomotor activity in a streptozotocin-induced sporadic Alzheimer's disease model was improved following an intragastric administration of genistein in support of our observation [384]. In contrast, other studies have reported no effect of genistein on locomotor deficits including open field and rotarod tests in 6-hydroxydopamine hydrochloride-induced Parkinson's disease and sporadic scopolamine-induced amnesia models respectively [385, 386]. Further, dietary genistein showed no effect on motor learning after either short-term or long-term hypogonadism even though genistein appeared to improve performance on the rotarod compared to the vehicle after long-term hypogonadism. Here, contrasting responses to genistein's effect on rotarod performance have been reported where dietary genistein improved the average latency to fall in a SOD1-G93A transgenic mouse model of amyotrophic lateral sclerosis whereas no effect was observed by Arbabi et al [386].

Dietary genistein but not E2 improved performance on the reversal phase of the Morris water maze test after short-term hypogonadism, suggesting genistein's effect on cognitive flexibility after spatial learning. This finding is supported by the previously observed cognitive benefit of genistein in young ovariectomized rats after short-term hypogonadism and as reported by Wang et al. in which genistein reduced the latency to the hidden platform in a global ischemia model [311]. Other neurological studies including the 3-NPA-induced Huntington disease model and beta-amyloid of Alzheimer's disease in rats have also reported improved spatial performance of genistein on the

water maze, all of which support the beneficial role of genistein in improving certain aspects of cognition [385, 390-392]. On the other hand, neither dietary genistein nor E2 improved memory impairments after long-term hypogonadism. Previous studies have reported a reduced beneficial effect of E2 following an extended delay in its administration. The lack of effect of genistein and E2 after long-term hypogonadism observed in the current study could be due to diminished estrogen-receptor mediated benefit for both treatments.

We observed no difference in tissue loss among the treatment groups after either short-term or long-term hypogonadism. This finding runs contrary to our previous observations in young ovariectomized rats and data previously published by other laboratories in which genistein substantially reduced infarct size in both ovariectomized rats and mice. This suggests that increasing age blunts the neuroprotective effects of genistein, unlike in the young subjects, especially following 21-day chronic reperfusion poststroke used in the current study as opposed to acute infarct size quantifications [300, 434]. Both dietary genistein and E2 reduced Iba1 expression after short-term hypogonadism at the ipsilateral side of stroke injury but not at the contralateral side. However, the long-term hypogonadism animals had lower Iba1 expression at both the ipsilateral and contralateral sides. These observations corroborate studies that have reported the anti-inflammatory properties of both genistein and E2 following focal cerebral ischemia [402-405]. A reduced Iba1 expression after long-term hypogonadism than short-term hypogonadism could be due to temporal changes in inflammatory responses over a long period as the endpoint for the Iba1 was determined 21 days after stroke.

**Limitations**

Our results partly contrast with previous studies showing that dietary genistein reduces ischemic infarct size. The current study was limited to a 60-minute occlusion period followed by 21-day

reperfusion in ovariectomized middle-aged rats. Conversely, studies that reported a reduction in infarct size employed 90-minute occlusion with shorter reperfusion endpoints up to 7 days poststroke. The reduced occlusion period aimed at maximizing survival in the long-term hypogonadism and hence could explain reduced cortical involvement on motor function in this study. Again, the current study is the first attempt to evaluate the neuroprotective effects of dietary genistein under ischemic conditions in ovariectomized middle-aged rats as far forward as 21-days poststroke. Hence the observed differential effects of genistein may reflect the actual prevailing experimental state under which the various tests were performed.

**Conclusion**

In sum, our findings demonstrate that dietary genistein confers differential effects on motor and cognitive functions in the ovariectomized middle-aged retired breeder Sprague-Dawley rats under ischemia following different ovarian hormone depletion timepoints. Like E2, genistein also exhibits temporal effects of hypogonadism on its neuroprotective potential. Nevertheless, the benefits of dietary genistein after long-term hypogonadism suggest that genistein administration after chronic hypogonadism may not necessarily be detrimental but may distinctly affect different brain regions to yield varied responses compared to E2. Further studies are, therefore, required to delineate the underlying molecular mechanisms for which the different responses between the short-term and long-term hypogonadism cohorts were seen.

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## Figure Legends

**Figure 1:** Study design. Animals underwent bilateral ovariectomy after 7 days of receipt and assigned to short-term hypogonadism (2-week hormone deprivation, STD) or long-term hypogonadism (2-week hormone deprivation) cohort. STD cohort had the subgroups Sham (vehicle) (n= 10), sham+GEN (n= 10), sham+E2 (n= 10), stroke (vehicle) (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 10). LTD cohort were categorized as Sham (n= 10), stroke (n= 15), stroke+GEN (n= 15), stroke+E2 (n= 15). Based on assigned group, animals received soy-free diet (vehicle), genistein diet (GEN) or silastic implant of Estrogen (E2) for two weeks prior to middle cerebral artery occlusion or sham surgery and till the end of the study (A). Experimental stroke was followed by behavioral tests (B) and euthanasia.

**Figure 2:** Neuroscore. Assessment of neurological deficits 24 hours after MCAO on modified Bederson scale. Main effect of length of hypogonadism was observed. LTD cohort had lower neuroscores compared to STD group. No effect of treatment by GEN or E2 was seen on the neurological deficits. The analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=10-14, *$p<0.05$.

**Figure 3:** Cylinder Test. Locomotor asymmetry. Main effect of stroke was observed after short-term hypogonadism. Neither GEN nor E2 improved forelimb asymmetry compared to the vehicle (A and B). Sham-operated animals had better forelimb symmetry compared to stroke subjects after long-term hypogonadism. Both E2 and GEN improved locomotor asymmetry on post-stroke day 3 (C). On day 7, E2 but not GEN improved locomotor deficits compared to the vehicle (D). Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-

term and long-term hypogonadism respectively. Data are expressed as mean ± SEM, n=9-13, *p<0.05.

**Figure 4:** Rotorod. Motor learning and coordination. Sham animals performed better than the stroke animals after short-term hypogonadism. Neither GEN nor E2 improved the latency to fall off the rotating rod (A and B). After long-term hypogonadism, sham animals spent more time on the rotating rod compared to the stroke subjects. No effect of GEN or E2 treatment was seen after long-term hypogonadism (C and D). short-term and long-term hypogonadism data were analyzed with two-way ANOVA and one-way ANOVA respectively followed by Fisher's LSD test. Data are expressed as mean ± SEM, n=9-14, *p<0.05. (AUC)=Area under the curve.

**Figure 5:** Path length. Spatial learning and cognitive performance. (A and B) No effect of stroke or treatment was observed on path length in the acquisition and retention phases (A). Main effect of Treatment was observed in the reversal phase of the short-term hypogonadism animals. Main effects of Treatment and Surgery were seen in the reversal phase. Sham animals traveled shorter distances to find the platform. GEN but not E2 treated animals also showed improved reversal learning compared to vehicle (A and B). Neither GEN nor E2 improved the path length after long-term hypogonadism (C and D). Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SEM, n=9-14, *p<0.05 compared to vehicle sham animals, @P<0.05 compared to vehicle stroke animals. (AUC)=Area under the curve.

**Figure 6:** Swim speed. Sham animals showed improved swim speed to find the platform compared to the stroke subjects. Vehicle and GEN treated animals showed better swim speed than E2-treated animals (A and B). Vehicle stroke animals swam faster than E2 and Gen-treated animals on the reversal phase after long-term hypogonadism (C and D). Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Data are expressed as mean ± SEM, n=9-14, *$p<0.05$ compared to vehicle sham animals, @$P<0.05$ compared to vehicle stroke animals. (AUC)=Area under the curve.

**Figure 7:** Water Maze. Probe trials. Neither GEN nor E2 improved the time spent in the target quadrant and the Annulus 40cm ring after both short and long-term hypogonadism (A-D). Two-way ANOVA and one-way ANOVA followed by Fisher's LSD test were performed for the short-term and long-term hypogonadism respectively. Dashed lines represent the probability that the time spent in the respective regions is due to chance. Data are expressed as mean ± SEM, n=9-14, *$p<0.05$.

**Figure 8:** Tissue injury. Neither GEN nor E2 reduced the extent of brain injury after both short-term and long-term hypogonadism (C). Representative images of immunohistochemistry for GFAP from 40microns brain slices after stroke (A and B). The analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=8-13, *$p<0.05$.

**Figure 9:** Iba1 expression. Both E2 and GEN reduced Iba1 expression at the ipsilateral side of stroke after short-term but not long-term hypogonadism (C). Long-term hormone deprived animals showed reduced Iba1 expression compared to the short-term hormone deprived animals at the contralateral (F).

Immunoblot images for Iba1 stain of stroked subjects (A, B, D and E). The analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=7-10, *$p<0.05$.

**Figure 10:** GFAP expression. Neither GEN nor E2 reduced GFAP expression at the ipsilateral and contralateral sides (C and F). Representative immunoblot images for GFAP (A, B, D and E). The analysis was performed with two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=7-11, *$p<0.05$.

**Table1:** Statistics for Morris Water Maze test. Two-way ANOVA was used for the short-term hypogonadism cohort with Surgery and Treatment as the independent factors. The long-term hypogonadism cohort was analyzed with one-way ANOVA with subgroups as the factors. Within stroke comparisons were done with a two-way ANOVA with Duration of hypogonadism and Treatment as factors. Fisher's LSD test was used as a *posthoc* test for main effects observed.

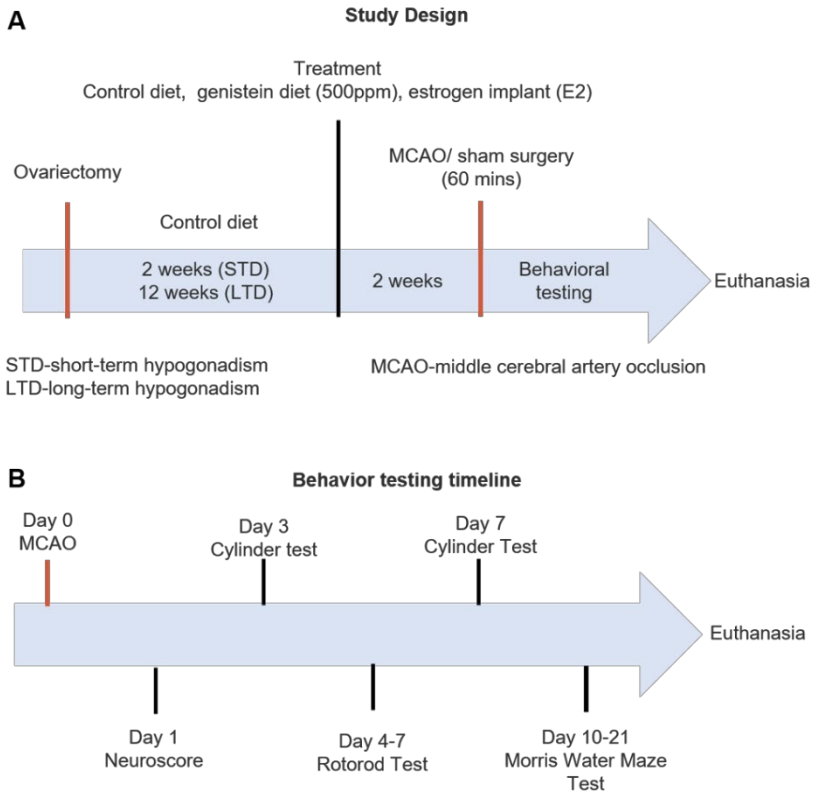
A
Study Design
Treatment
Control diet, genistein diet (500ppm), estrogen implant (E2)
Ovariectomy
MCAO/ sham surgery
(60 mins)
Control diet
2 weeks (STD)
12 weeks (LTD)
2 weeks
Behavioral
testing
Euthanasia
STD-short-term hypogonadism
LTD-long-term hypogonadism
MCAO-middle cerebral artery occlusion
B
Behavior testing timeline
Day 0
MCAO
Day 3
Cylinder test
Day 7
Cylinder Test
Euthanasia
Day 1
Neuroscore
Day 4-7
Rotorod Test
Day 10-21
Morris Water Maze
Test

**Figure 1**

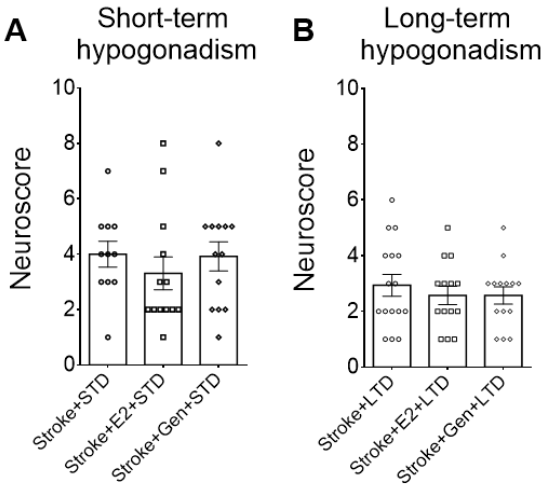
A
Short-term hypogonadism
Neuroscore
10
8
6
4
2
0
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
B
Long-term hypogonadism
Neuroscore
10
8
6
4
2
0
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 2**

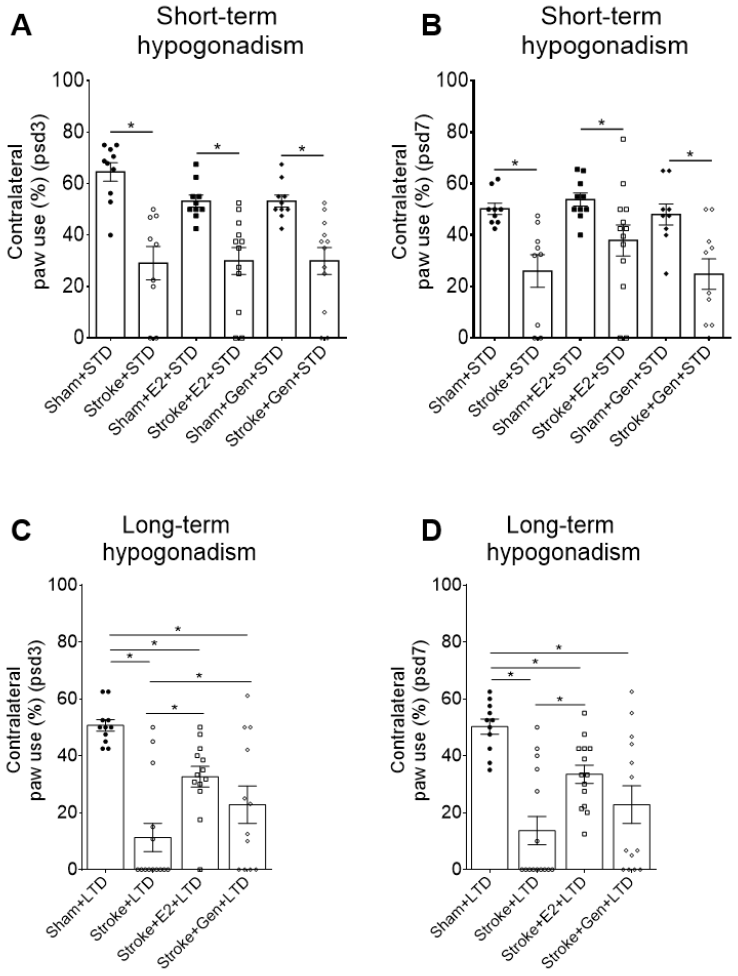
A
Short-term hypogonadism
Contralateral paw use (%) (psd3)
100
80
60
40
20
0
*
*
*
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
B
Short-term hypogonadism
Contralateral paw use (%) (psd7)
100
80
60
40
20
0
*
*
*
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
C
Long-term hypogonadism
Contralateral paw use (%) (psd3)
100
80
60
40
20
0
*
*
*
*
*
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
D
Long-term hypogonadism
Contralateral paw use (%) (psd7)
100
80
60
40
20
0
*
*
*
*
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 3**

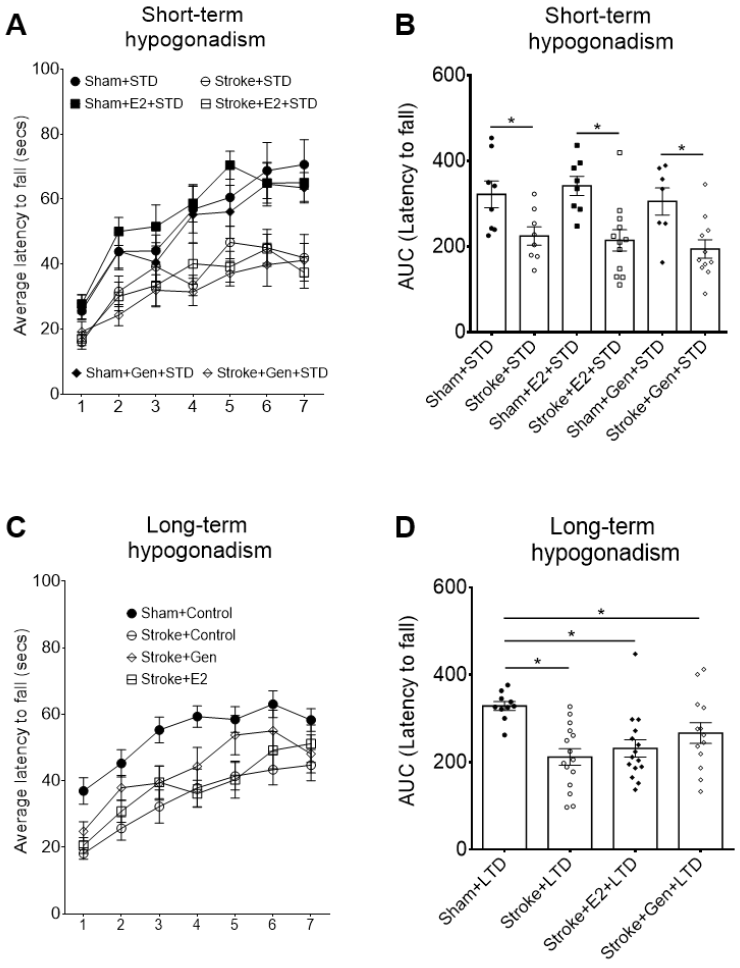
A
Short-term hypogonadism
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Average latency to fall (secs)
B
Short-term hypogonadism
AUC (Latency to fall)
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
C
Long-term hypogonadism
Sham+Control
Stroke+Control
Stroke+Gen
Stroke+E2
Average latency to fall (secs)
D
Long-term hypogonadism
AUC (Latency to fall)
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 4**

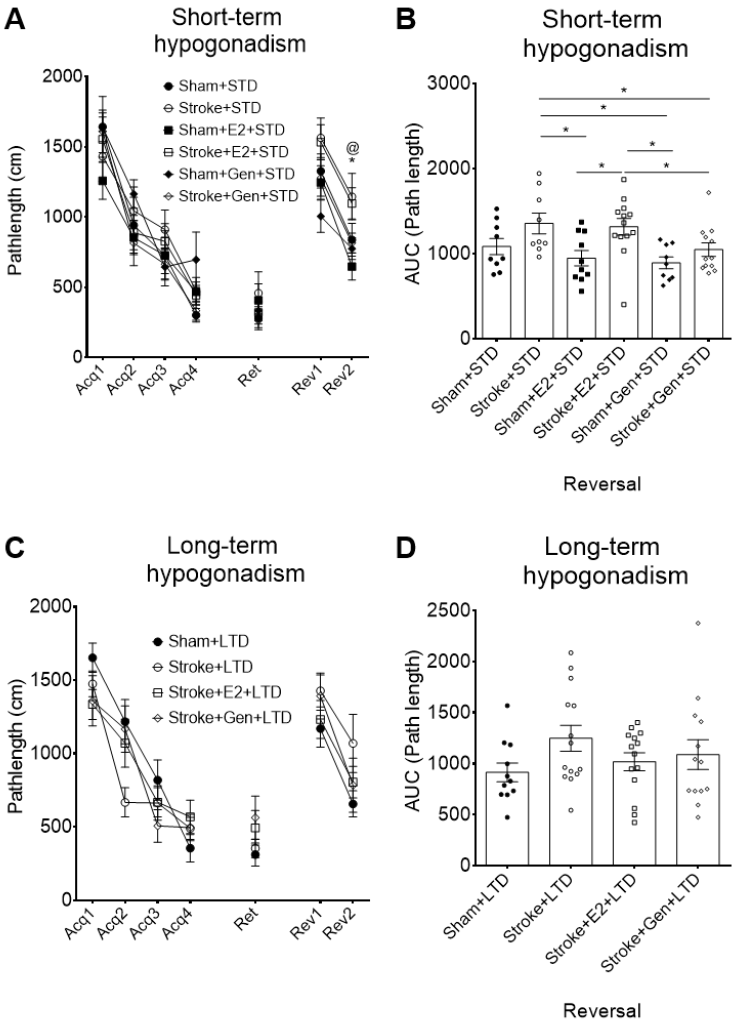
A
Short-term hypogonadism
Pathlength (cm)
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
B
Short-term hypogonadism
AUC (Path length)
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Reversal
C
Long-term hypogonadism
Pathlength (cm)
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
D
Long-term hypogonadism
AUC (Path length)
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Reversal

**Figure 5**

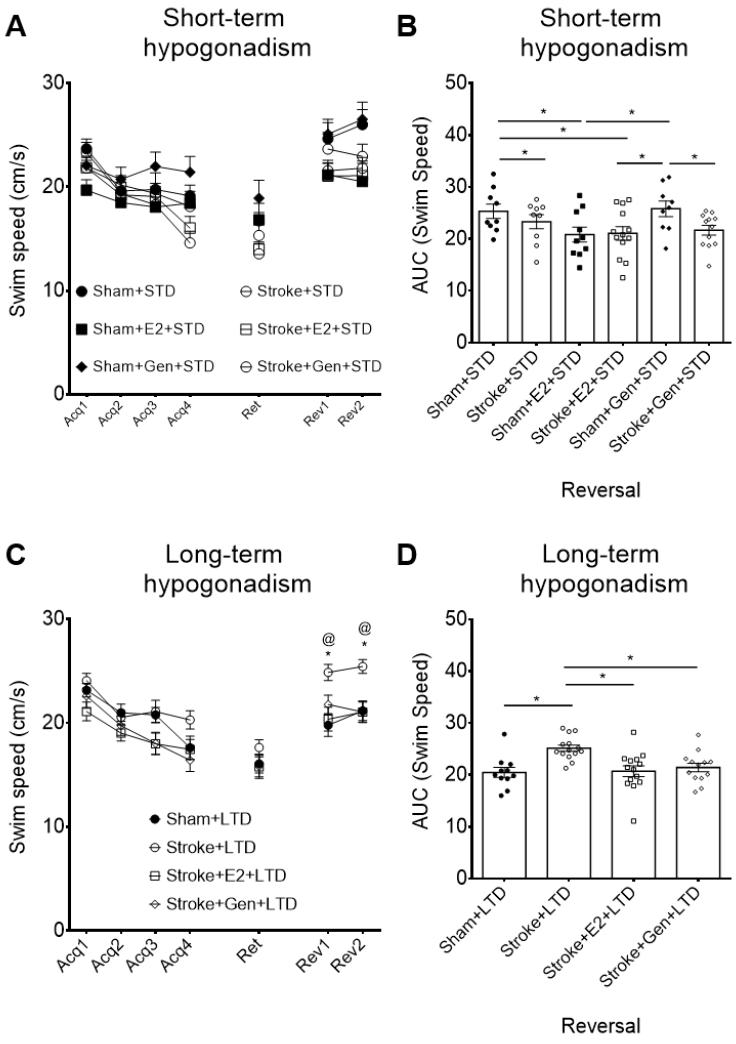
A
Short-term hypogonadism
Swim speed (cm/s)
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
B
Short-term hypogonadism
AUC (Swim Speed)
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Reversal
C
Long-term hypogonadism
Swim speed (cm/s)
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Acq1
Acq2
Acq3
Acq4
Ret
Rev1
Rev2
D
Long-term hypogonadism
AUC (Swim Speed)
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Reversal

**Figure 6**

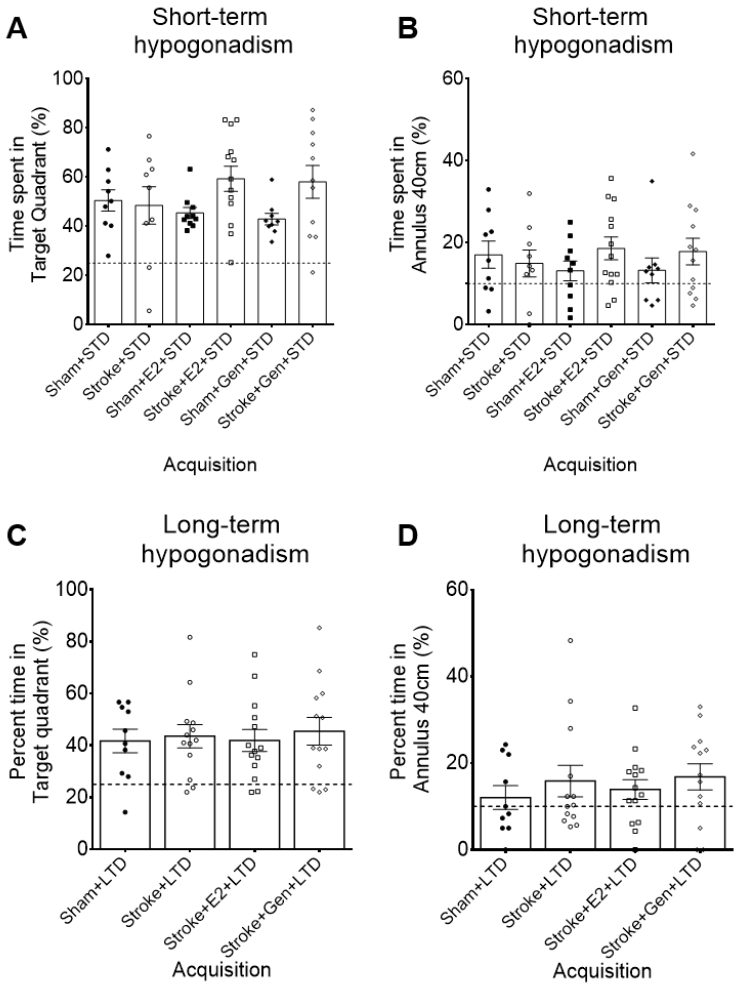
A
Short-term hypogonadism
Time spent in Target Quadrant (%)
100
80
60
40
20
0
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acquisition
B
Short-term hypogonadism
Time spent in Annulus 40cm (%)
60
40
20
0
Sham+STD
Stroke+STD
Sham+E2+STD
Stroke+E2+STD
Sham+Gen+STD
Stroke+Gen+STD
Acquisition
C
Long-term hypogonadism
Percent time in Target quadrant (%)
100
80
60
40
20
0
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Acquisition
D
Long-term hypogonadism
Percent time in Annulus 40cm (%)
60
40
20
0
Sham+LTD
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
Acquisition

**Figure 7**

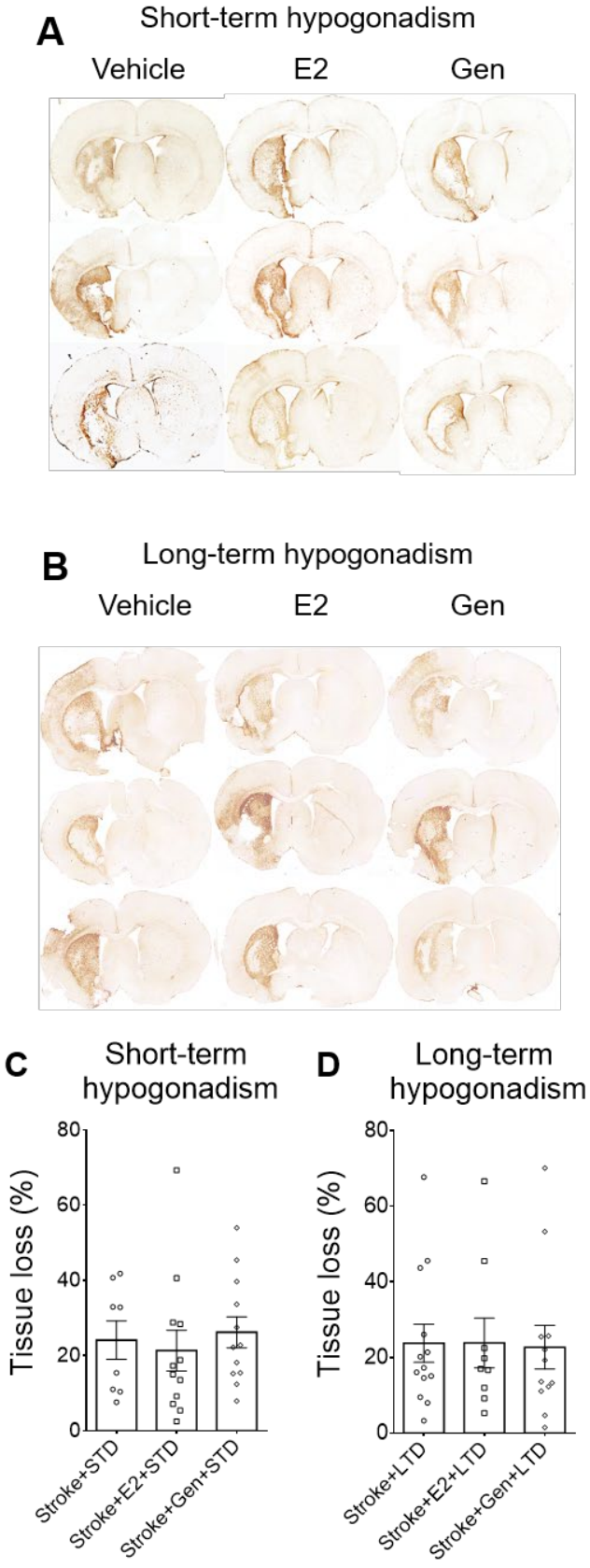
A
Short-term hypogonadism
Vehicle
E2
Gen
B
Long-term hypogonadism
Vehicle
E2
Gen
C
Short-term hypogonadism
Tissue loss (%)
80
60
40
20
0
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
D
Long-term hypogonadism
Tissue loss (%)
80
60
40
20
0
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 8**

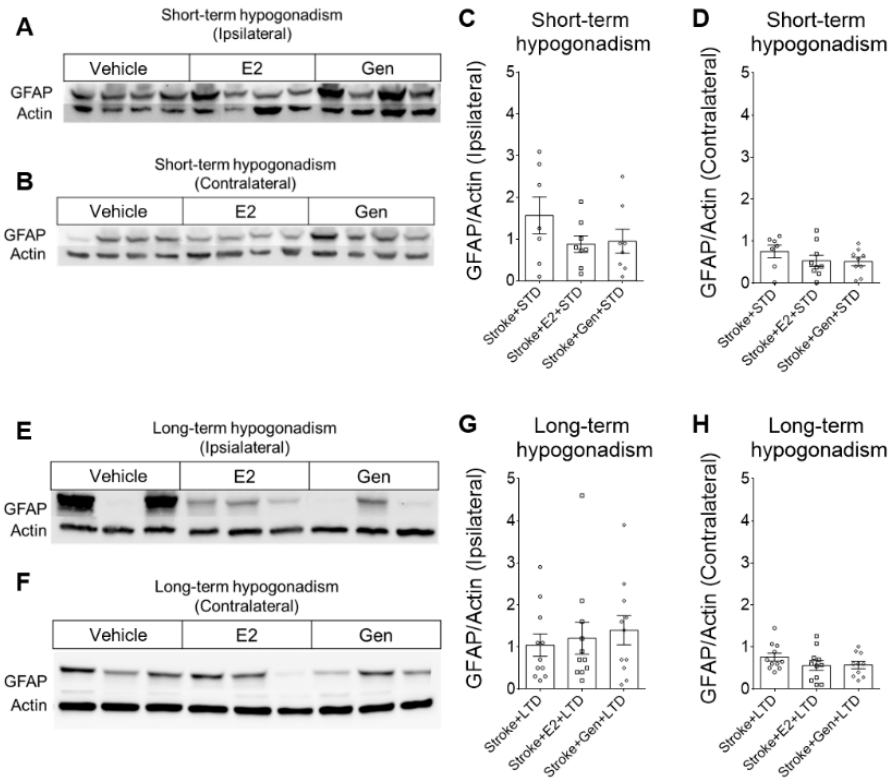
A
Short-term hypogonadism
(Ipsilateral)
Vehicle
E2
Gen
GFAP
Actin
B
Short-term hypogonadism
(Contralateral)
Vehicle
E2
Gen
GFAP
Actin
C
Short-term
hypogonadism
GFAP/Actin (Ipsilateral)
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
D
Short-term
hypogonadism
GFAP/Actin (Contralateral)
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
E
Long-term hypogonadism
(Ipsialateral)
Vehicle
E2
Gen
GFAP
Actin
F
Long-term hypogonadism
(Contralateral)
Vehicle
E2
Gen
GFAP
Actin
G
Long-term
hypogonadism
GFAP/Actin (Ipsilateral)
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
H
Long-term
hypogonadism
GFAP/Actin (Contralateral)
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 9**

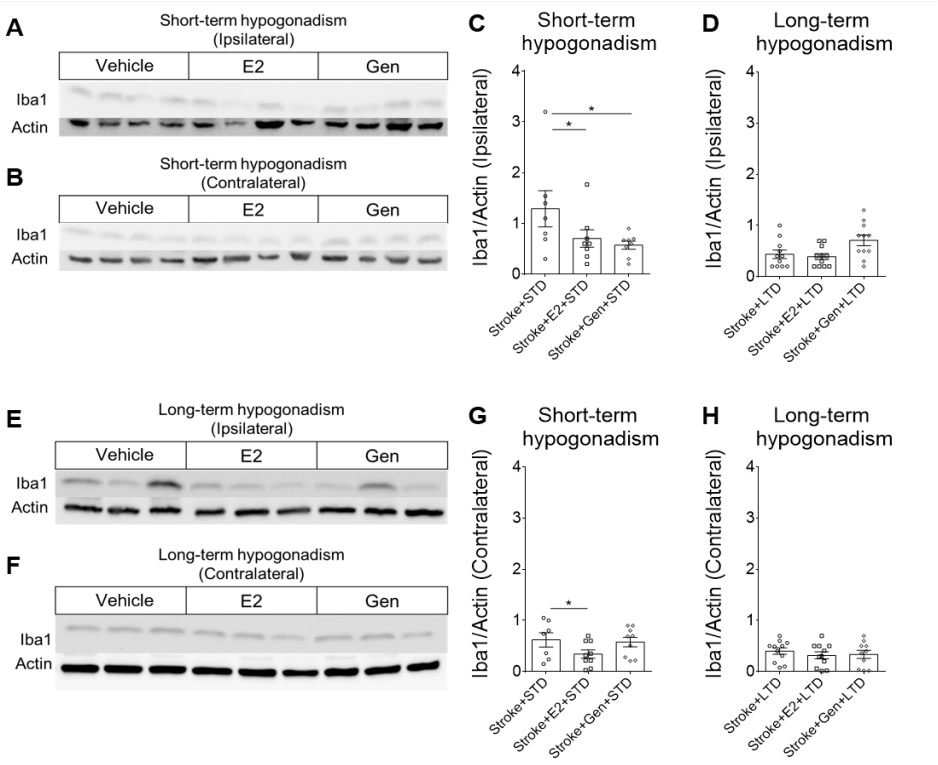
A
Short-term hypogonadism
(Ipsilateral)
Vehicle
E2
Gen
Iba1
Actin
B
Short-term hypogonadism
(Contralateral)
Vehicle
E2
Gen
Iba1
Actin
C
Short-term
hypogonadism
Iba1/Actin (Ipsilateral)
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
D
Long-term
hypogonadism
Iba1/Actin (Ipsilateral)
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD
E
Long-term hypogonadism
(Ipsilateral)
Vehicle
E2
Gen
Iba1
Actin
F
Long-term hypogonadism
(Contralateral)
Vehicle
E2
Gen
Iba1
Actin
G
Short-term
hypogonadism
Iba1/Actin (Contralateral)
Stroke+STD
Stroke+E2+STD
Stroke+Gen+STD
H
Long-term
hypogonadism
Iba1/Actin (Contralateral)
Stroke+LTD
Stroke+E2+LTD
Stroke+Gen+LTD

**Figure 10**

TABLE1: Statistics for Morris Water Maze test showing the effect of treatment, surgery and duration of hypogonadism on path length, swim speed and probe trials

| Duration of Hypogonadism | | | Pathlength | | | | | | Swim speed | | | | | | Target Quadrant | | | | Annulus 40cm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Acquisition | | Retention | | Reversal | | Acquisition | | Retention | | Reversal | | Acquisition | | Reversal | | Acquisition | | Reversal | |
| | Effect | df | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* | F ratio | *P-value* |
| Short-term | | | | | | | | | | | | | | | | | | | | | | |
| N=56 | Treatment | 2 | 0.062 | 0.94 | 0.362 | 0.698 | 3.443 | 0.039 | 1.07 | 0.35 | 0.287 | 0.751 | 3.8 | 0.028 | 0.17 | 0.844 | 0.408 | 0.667 | 0.011 | 0.989 | 0.498 | 0.611 |
| | Surgery | 1 | 0.032 | 0.858 | 0.008 | 0.929 | 11.723 | 0.001 | 3.746 | 0.058 | 11.125 | 0.002 | 3.278 | 0.076 | 4.314 | 0.042 | 5.537 | 0.022 | 1.124 | 0.294 | 3.046 | 0.086 |
| | (Treatment*Surgery) | 2 | 2.201 | 0.12 | 1.189 | 0.312 | 0.66 | 0.521 | 2.648 | 0.08 | 1.392 | 0.257 | 1.425 | 0.249 | 1.532 | 0.225 | 0.871 | 0.424 | 0.869 | 0.425 | 0.428 | 0.654 |
| Long-term | | | | | | | | | | | | | | | | | | | | | | |
| N=48 | Group | 4 | 0.926 | 0.435 | 1.15 | 0.339 | 1.15 | 0.339 | 2.994 | 0.04 | 0.919 | 0.439 | 6.585 | 0.001 | 0.136 | 0.938 | 1.335 | 0.275 | 0.469 | 0.705 | 0.628 | 0.601 |
| Within stroke | | | | | | | | | | | | | | | | | | | | | | |
| | Treatment | 2 | 0.421 | 0.658 | 0.002 | 0.998 | 1.994 | 0.144 | 4.009 | 0.023 | 1.955 | 0.149 | 5.687 | 0.005 | 0.554 | 0.577 | 1.59 | 0.211 | 0.192 | 0.825 | 0.476 | 0.624 |
| | Duration | 1 | 0.021 | 0.885 | 2.002 | 0.162 | 1.644 | 0.204 | 1.159 | 0.285 | 7.17 | 0.009 | 0.242 | 0.625 | 6.814 | 0.011 | 2.804 | 0.099 | 0.412 | 0.523 | 2.062 | 0.156 |
| | (Treatment*Duration) | 2 | 1.638 | 0.202 | 1.787 | 0.175 | 1.158 | 0.32 | 0.358 | 0.701 | 0.046 | 0.955 | 0.697 | 0.501 | 0.658 | 0.521 | 0.621 | 0.54 | 0.435 | 0.649 | 0.419 | 0.659 |

TABLE2: Summary of behavioral performance and extent of injury

| Variable | Treatment | Estrogen | | Genistein | |
|---|---|---|---|---|---|
| | Age | Mid-Age | | Mid-Age | |
| | Hypogonadism length | STD | LTD | STD | LTD |
| Neuroscore | | ↔ | ↔ | ↔ | ↔ |
| Locomotor Symmetry | | ↔ | ↑ | ↔ | ↑ |
| Motor learning | | ↔ | ↔ | ↔ | ↔ |
| Path length | Acquisition learning | | | | |
| | Retention | ↔ | ↔ | ↔ | ↔ |
| | Reversal Learning | ↔ | ↔ | ↑ | ↔ |
| | | | | | |
| Swim speed | Acquisition learning | ↔ | ↔ | ↔ | ↔ |
| | Retention | ↔ | ↔ | ↔ | ↔ |
| | Reversal Learning | ↔ | ↔ | ↔ | ↔ |
| | | | | | |
| Probe | Acquisition | ↔ | ↔ | ↔ | ↔ |
| | Reversal | ↔ | ↔ | ↔ | ↔ |
| Extent of Injury | | ↔ | ↔ | ↔ | ↔ |

↑ Benefit

↔ No effect

↓ Worse effect

# CHAPTER 4

# DIFFERENT EFFECTS OF DIETARY GENISTEIN ON CHRONIC POSTSTROKE INFLAMMATION AT DIFFERENT LENGTHS OF HYPOGONADISM IN FEMALE SPRAGUE-DAWLEY RAT

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Running Title: Dietary genistein confers distinct inflammatory marker expression after stroke

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****To be formatted and submitted to eNeuro****

**Abstract**

Poststroke inflammation plays a critical role in the pathophysiology of ischemic stroke. Genistein, a well-studied soy isoflavone has been shown to confer neuroprotection in several preclinical ischemic injury studies. However, little is known about the sensitivity of genistein's neuroprotection under different conditions of hypogonadism and age. In this study, we investigated the effects of dietary genistein (Gen) on chronic poststroke inflammation and the probable mechanisms underlying its inflammatory responses after hypogonadism. We used young adult and proven retired breeder middle-aged Sprague-Dawley rats in the study. Rats were ovariectomized and assigned to different durations of hypogonadism (2 weeks and 12 weeks) followed by a two-week pretreatment with Gen or an 17β-estradiol (E2) implant before inducing 60 minutes middle cerebral artery occlusion. Twenty-one days after reperfusion, brains were harvested and processed for immunohistochemical analysis and immunoblotting to determine the effect of treatment on the Interleukin-6 (IL-6) and Transforming Growth Factor (TGF-β1) as markers of M1 and M2 activation respectively. We further investigated GAP43 and HSP70 as markers of functional recovery. Gen treatment reduced IL-6 expression in the contralateral hemisphere in the young cohort while E2 had no effect on IL-6 across lengths of hypogonadism in the young cohort. Following short-term hypogonadism, Gen increased TGF-β1 expression in the contralateral hemisphere in middle-aged rats while E2 increased TGF-β1 in the contralateral hemisphere in the young rats. E2 but not Gen increased HSP70 induction at the ipsilateral side in young cohorts regardless of hypogonadism length and at the contralateral side following short-term hypogonadism. Gen but not E2 increased GAP43 in the contralateral hemisphere of the young following short-term hypogonadism. Our results show that Gen reduces contralesional M1

activation in young ovariectomized rat brain independent of the length of hypogonadism after focal cerebral ischemia. Again, both Gen and E2 differently promote M2 activation as a function of age.

Keywords: middle cerebral artery occlusion, hypogonadism, neuroinflammation, genistein

## Introduction

Neuroinflammation is a constitutive mechanism to restore normal neuronal function following oxidative stress and ischemic injury [435]. However, when the induced inflammatory response is not properly regulated, brain tissue injury is protracted due to impaired repair [6, 436-439]. Reactive oxygen and nitrogen species are generated under ischemic and stress conditions and trigger inflammatory cascades involving the resident immune cells, microglia, as well as peripherally recruited macrophages [87, 438, 440, 441]. Microglia acts as the primary source of pro-inflammatory cytokines including interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor (TNF) that can worsen stroke and impede recovery [96, 442, 443]. There is ample evidence to show that there is a bimodal transformation of resident microglia under stress conditions. During ischemia, microglia can transform from a pro-restorative M2 phenotype to a pro-inflammatory M1 phenotype that influences the extent of injury and disease progression [86-91]. The involvement of microglial phenotype activation in poststroke recovery has further been shown in stroke. A deficiency in the pro-M1 cytokine IL-6 results in substantial reduction in inflammatory response in astrocytes and microglia following ischemic injury in mice [96]. This suggests microglial activation during the pathophysiology of stroke involves IL-6. Additionally, Shin et al showed that an increase in M2 microglia by noggin, an inhibitor of bone morphogenetic proteins, was associated with an increase in GAP43 to improve performance on wire suspension test after stroke [120]. We have previously shown that dietary genistein reduces ionized calcium-binding adaptor molecule 1 (Iba1) expression as a marker of microglial activation after stroke

following short-term hypogonadism [430]. It is, however, unclear what prevailing microglial phenotype is involved during stroke recovery following a 21-day reperfusion [88, 444], even though there is evidence to show the impact of genistein on microglial action. *In vitro*, genistein has been shown to reduce BV2 microglial activation and inflammation in microglial cell lines under stress conditions [329, 445, 446]. Genistein, through attenuation of microglia activation, has also been shown to reduce the expression of pro-inflammatory cytokines including IL-6 and TNF-alpha while increasing TGF-β1 [447]. Hence, in the current study, we investigated the expression of two surrogate markers of the M1 and M2 phenotypes that have been shown to persist for weeks after the initial injury, namely IL-6 and TGF-β1 [104]. We further measured HSP70 and GAP43 expression in both the whole ipsilateral and contralateral hemispheres as markers of axonal regeneration and functional recovery under the different conditions of hormone depletion and age. This is based on previous studies that showed that genistein improves functional recovery following peripheral nerve injury with the involvement of GAP43 expression [448, 449]. In PC12 cells, genistein treatment inhibits erythropoietin-induced GAP43 dephosphorylation to increase the activity and immunoreactivity of GAP43 [450]. These pieces of evidence together with the preliminary results on genistein's capacity to reduce microglial activation suggest that genistein can increase the expression of GAP43 under conditions of neuronal damage to improve dendritic spine plasticity and axonal regeneration [451]. On the other hand, tyrosine kinase signaling is involved in HSP70 induction, an observation that was confirmed with genistein studies. Das et al showed that genistein abolishes HSP70 expression in heart cells [143]. This evidence suggests that tyrosine kinase signaling influences HSP70 induction under stress and ischemic conditions. Hence, in the current study, we expected that a reduction in microglial activation by genistein will be accompanied by reduced HSP70 expression after ischemia.

## Materials and methods

**Animals:** All experiments and procedures were approved by the Institutional Animal Care and Use Committee of UNT Health Science Center**.** Young adult (3-4-month-old) and proven retired breeders (9 months old) female Sprague-Dawley rats were obtained from Envigo. Upon purpose, rats were housed in pairs and had free access to water and a soy-free diet under a 12-h reverse light-dark cycle. All animals underwent bilateral ovariectomy as previously described [212, 430], treated with a soy-free diet (Purina Test Diet, 5K96) for two weeks or 12-weeks as lengths of hypogonadism, followed by two weeks treatment of soy-free diet, custom-made genistein diet (Gen, LC Laboratories) or E2 implant (inner/outer diameter: 1.575/3.175 mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) before middle cerebral artery occlusion. Sliced brain tissues and protein samples were obtained 21 days poststroke to probe for chronic inflammatory markers.

**Western blot**: Western blotting analysis was performed as previously described [430]. Protein samples from tissue slices taken from both hemispheres (between Bregma 0 to +1.0 mm) were used for the immunoblotting. Tissues were lysed in T-PER reagent (ThermoFisher, Pierce, Rockford, Illinois) and HALT protease inhibitor cocktail (ThermoFisher, Pierce, Rockford, Illinois). Preprocessed samples were incubated for 10 min on ice, and centrifuged at 10,000Xg for 5 min at 4°C. The concentrations of the proteins within the supernatant were determined using the Bicinchoninic Acid Protein Assay (ThermoFisher, Pierce, Rockford, Illinois). 30 µg of protein per sample was separated on 4-20% SDS–polyacrylamide pre-cast gels (BioRad). Proteins were transferred overnight to nitrocellulose membranes at 4°C. Before blocking, membranes were stained with ponceau solution to highlight transferred protein bands which were captured and subsequently quantified as the loading control. Membranes were blocked in 1% milk in

Phosphate-Buffered Saline Solution and 0.1% Tween-20 (PBST) for 1 hour and incubated with primary antibodies overnight at appropriate concentrations (Table 1) followed by 2-hour room temperature incubation with a suitable secondary antibody conjugated to horseradish peroxidase (Table 3). Following washing, the protein bands were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). To determine the relative densities of the visualized bands, FIJI ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the bands and expressed as a ratio to the total proteins as determined by the ponceau.

**Double Immunohistochemistry:** Immunohistochemistry was performed on every $12^{th}$ section of 40-micron brain slices (between Bregma -1.00mm and -2.80mm). Triplicate washing of free-floating sections was done with tris-buffered saline with 0.1% Triton (TBST). Slices were then incubated in 10% horse serum with 0.3% Triton X100 to block non-specific binding followed by another triplicate wash for 15 mins at 5 minutes intervals. Slices incubated with the first primary antibody of interest overnight at 4°C in TBS + 1% horse serum + 0.1% Triton X 100 followed by incubation with biotin-SP-conjugated secondary antibodies (Jackson Immunochemicals) in 1% horse serum for 1 hour at room temperature. Slices were rinsed with TBST and incubated again with Alexa 488 conjugated to Strepavidin for 1 hour at room temperature. The slices were incubated with the second primary antibody overnight at 4°C after which they were incubated with Cy3-conjugated secondary antibodies for immunofluorescence detection. Slides were washed, mounted on uncoated slides and allowed to dry in a dark environment. Dried slides were then cover-slipped with Krystalon (EMD Millipore, Burlington, MA, USA) and dried before fluorescent microscopy using an Olympus BX61 microscope to acquire 2D scanned images at 40x magnification. Acquired images were analyzed with the Adobe Photoshop CC 2020 package to determine the expression of the probed proteins.

## Statistics

All data collected were expressed as mean±SEM. A Three-way Analysis of Variance was used to analyze both the young cohorts and middle-aged cohorts together. Treatment, Duration of Hypogonadism and Age of cohort were used as the independent variable/factors and the probed proteins were analyzed as the dependent variables. Fisher's Least Significance Test was used as a *post-hoc* test where main effects were seen. For correlational analysis, Pearson's correlation coefficient was employed followed by Dunn-Sidak's probabilities. Data were analyzed with Systat Software Version 13.1 (Systat Software, Inc, San Jose, CA, USA) with significance level set at $p < 0.05$.

## Results

### IL-6 expression

Neither Gen nor E2 reduced IL-6 expression in the ipsilateral hemisphere across groups (Figures 1A-D, $F(2,98) = 0.625$, $p = 0.538$). IL-6 in the contralateral side, however, was significantly reduced by Gen in the young cohorts after both short-term and long-term hypogonadism (Figures 1E and F). No effect of treatment was observed in the middle-aged cohorts (Figures 1G and H). These findings were evident in the main effects of Treatment ($F(2,98) = 5.516$, $p = 0.005$), Age ($F(1,98) = 10.713$, $p = 0.001$) and interaction between Treatment and Age ($F(2,98) = 4.714$, $p = 0.011$).

### TGF-β1 expression

No difference was observed on TGF-β1 expression at the ipsilateral side (Figures 2A-D, $F(2,98) = 1.801$, $p = 0.171$). Interaction amongst Treatment, Duration of Hypogonadism and Age were seen at the contralateral side ($F(2,98) = 6.635$, $p = 0.002$). E2 but not Gen increased TGF-β1 expression in the young cohort after short-term hypogonadism (Figure 2E). Both Gen and E2

reduced TGF-β1 after long-term hypogonadism in the young cohort (Figure 2G). Gen but not E2 increased TGF-β1 expression in the middle-aged cohort after short-term hypogonadism compared to vehicle (Figure 2G). Neither E2 nor Gen increased TGF-β1 after long-term hypogonadism in the middle-aged cohort (Figure 2H)

**HSP70 expression**

Main effects of Treatment ($F(2,98) = 8.413$, $p < 0.001$), Duration ($F(1,98) = 8.662$, $p = 0.004$), Age ($F(1,98) = 4.357$, $p = 0.039$), interaction between Treatment and Age ($F(2,98) = 6.012$, $p = 0.003$) and interaction between Duration and Age ($F(2,98) = 4.065$, $p = 0.047$) were observed at the ipsilateral side. E2 but not Gen increased HSP70 expression after long-term hypogonadism (Figure 3B). Neither Gen nor E2 affected HSP70 in the middle-aged cohorts (Figures 3C and D). Main effect of Age was observed on HSP70 at the contralateral side ($F(2,98) = 9.513$, $p = 0.003$). E2 but not Gen increased HSP70 after short-term hypogonadism in the young cohort (Figure 3E) but not long-term hypogonadism (Figure 3F). Neither Gen nor E2 increased HSP70 in the middle-aged group (Figures 3G and H).

**GAP43 expression**

GAP43 expression was similar in the ipsilateral hemisphere among the treatment groups and across lengths of hypogonadism and age (Figures 4A-D, $F(2,98) = 1.42$, $p = 0.247$). In the contralateral side, there was elevated expression of GAP43 by Gen following short-term hypogonadism in the young cohort (Figure 4E) but not in the middle-aged cohort (Figure 4G). Neither Gen nor E2 affected GAP43 levels after long-term hypogonadism at the contralateral side (Figures 4F and H). These findings were supported by main effects of Age ($F(2,98) = 8.988$, $p = 0.003$), interaction between Treatment and Age ($F(2,98) = 4.419$, $p = 0.015$), interaction between Treatment and

Duration (F(2,98) = 4.4206, $p$ = 0.018), and interaction between Duration and Age (F(1,98) = 10.219, $p$ = 0.002) in the contralateral side.

## Discussion

The main findings are that neither Gen nor E2 affected IL-6, TGF- β1 or GAP43 in the ipsilateral side of ischemic stroke across ages and lengths of hypogonadism. However, E2, but not Gen, increased HSP70 expression at the ipsilateral side following short-term hypogonadism in the young adult cohort. On the contralateral side, Gen reduced IL-6 expression after both short-term and long-term hypogonadism in the young adult cohort and increased TGF-β1 in the middle-aged cohort after short-term hypogonadism. In the contralesional hemisphere after short-term hypogonadism in the young cohort, Gen increased GAP43 while E2 increased HSP70 induction. These data suggest that Gen may promote the repression of contralesional M1 activation in young subjects after shorter hypogonadal lengths while promoting M2 activation in middle-aged subjects. Further, E2 may promote contralesional M2 activation in young subjects after shorter hypogonadal length.

During stroke, IL-6 concentration is significantly elevated in the cerebrospinal fluid, an increase accounted for by increased production in dying neurons and activated microglia [452]. In humans, IL-6 positively correlates with infarct size [453, 454]. Genistein possesses anti-inflammatory properties against lipopolysaccharide-induced BV2 microglial cell activation through the reduction in inducible nitric oxide synthase production of IL-6 expression [446]. In this study, Gen reduced IL-6 expression in the young cohorts after both short-term and long-term hypogonadism. This reduction follows the already observed reduction of Iba1 expression after short-term hypogonadism [430]. As has been reported by Beridze et al, microglia are a source of IL-6 under

ischemic conditions [454] and hence, reducing microglial activation by Gen likely reduces the expression of IL-6 and M1 phenotype.

Positive effects of genistein on TGF-β1 have been previously demonstrated both *in vivo* and *in vitro*. *In vitro* experiments with human mammary epithelial cells showed that incubating genistein with epidermal growth factor resulted in a 5-fold increase in TGF-β1 [455, 456]. In humans, genistein consumption for up to 14 days significantly reduced chronic bleeding in Hereditary Hemorrhagic Telangiectasia in a manner that mimics increased TGF-β1 downstream signaling [457]. In the contralateral hemisphere, TGF-β1 was increased by Gen after short-term hypogonadism in the middle-aged cohort, while E2 increased it in the young cohort. This is supportive of previous findings that established the interplay of genistein as well as E2 with TGF-β1 signaling [447, 458, 459]. In the other cohorts, no effect of Gen nor E2 was observed on TGF-β1. However, an overall effect of interaction among Treatment, Duration and Age on TGF-β1 depicts that Gen and E2's effects under ischemic conditions are in part influenced by the aforementioned factors.

Genistein has been shown in previous studies to attenuate stress response through the reduction of HSP70 levels [143, 144]. This stems from the direct inhibition of tyrosine kinases which are involved in the signaling of HSP70 induction. In a study by Das et al, genistein reduced HSP70 expression in cardiac myocytes with further evidence by Kiang (2003) showing that genistein blunts HSP70 induction by Herbimycin-A [144]. In the current study, we see that Gen reduced HSP70 expression in the young cohort after short-term hypogonadism while E2 increased HSP70. This is in line with previous evidence that decreased microglial activation is commensurate with decreased HSP70. Hence, it was confirmatory that, while Gen reduced microglial activation as was detected by Iba1 expression in the ipsilateral hemisphere, it would further reduce HSP70

induction [141-144]. The lack of effect of Gen after long-term hypogonadism across the age groups suggests that the advanced absence of estrogens and age may confer resistance to the repression of HSP70 induction by Gen within the peri-infarct area.

In animal models of ischemic stroke, the levels of GAP43 within the brain have been attributed to one of two main outcomes: 1) an increase due to reparative processes to correct the ischemic injury or 2) a reduction as a result of non-functional axonal activity [117, 118, 460]. The increase in GAP43 by Gen at the contralateral side in the young likely reflects the regenerative capacity of genistein as it has been shown to increase axonal sprouting in nerve injury [448, 449]. What was, however, intriguing was that the effect was not seen at the ipsilateral side in either young long-term estrogen-deprived group or middle-aged cohorts across lengths of hypogonadism. This suggests that the axonal regeneration properties of genistein may be limited by the extent of axonal damage within the site of injury. Previous evidence has shown that the absence of circulating estrogens following ovariectomy results in reduced GAP43 mRNA, an observation that is reversed by estrogen administration [461]. However, an extended absence of circulating estrogen and age shows a diminished intrinsic neuroprotective mechanism underlying ischemic stroke recovery, as evident in reduced spine density and increased stroke volumes in animal models [321, 322, 422, 423]. Hence, it is likely that the length of hypogonadism and increasing age could have contributed to the lack of Gen and E2's effect as previously reported [430, 462].

Several associations, mostly weak and moderate were observed among the probed proteins as well as behavioral performances previously recorded. We see from the middle-aged cohort, a moderate negative correlation between GAP43 and Iba1 expression within the contralateral hemisphere ($r = -0.50$, $p = 0.001$). This may be supported by Cobianchi et al's work where the authors saw a decreased microglial activation with an accompanying increased GAP43 following treadmill

exercise in peripheral nerve injured rodents [121]. Furthermore, GAP43 positively correlated with TGF-β1 in the middle-aged across lengths of hypogonadism ($r = 0.56$, $p < 0.001$) and after long-term hypogonadism across ages ($r = 0.66$, $p < 0.001$). Evidence from White et al which showed that intrathecal administration of TGF-β1 promotes axonal growth manifested by elevated GAP43 in C57BL/6 mice [463]. Hence, it is possible that in the current study, the release of GAP-43 is influenced by TGF-β1. In the young cohorts, a negative correlation between GAP43 and HSP70 was observed ($r = -0.50$, $p = 0.033$). Demyanenko et al showed that GAP43 is elevated mainly due to increased axonal damage and regeneration after stroke [464]. Welsh et al also showed that the extent of the ischemic stroke determines the expression of HSP70 where protracted hypoxic events attenuate HSP70 induction [130]. Considering the opposing expressions of GAP43 and HSP70 under similar stroke injury, it is likely that the extent of neuroinflammation was higher in the young cohorts, hence the negative correlation. After short-term hypogonadism across ages, IL-6 expression showed a weak negative correlation with the acquisition path length ($r = 0.41$, $p = 0.176$). In a 90-min MCAO study by Zhang et al, cognitive performance was improved in treadmill exercised rats through the decrease in inflammatory markers including IL-6 and TNF-alpha [465]. Khoshnam et al also showed that reduction of IL-6 and other pro-inflammatory markers was associated with improved spatial performance on the Morris Water Maze [466]. What these suggest is that the cognitive learning capacity of stroked rodents may be inversely related to IL-6 expression after stroke. Our current study further supports the inverse relationship between cognitive performance and IL-6 expression as was observed in decreasing pathlength with increasing IL-6 levels. This study was limited by the fleeting nature of more specific cytokines after stroke [467, 468]. Hence, we only made use of the ones which have been established to remain elevated for several weeks even after the initial acute injury. Overall, we observed a minimal effect

of both Gen and E2 in the ipsilateral hemisphere with their effects majorly seen in the contralateral hemisphere. While this occurrence is uncommon, some rodent studies on stroke have reported similar observations. In such studies, axonal sprouting and corticospinal neuron projections were elevated in the contralateral hemisphere and distant regions from the lesion following large strokes [469-471]. Other clinical and experimental studies have also shown that poststroke inflammation is not limited to only the ischemic hemisphere. Rather the inflammatory response extends to the contralateral side where there is induced morphological and functional changes including dysfunctional blood brain barrier, altered blood flow and gliosis [472-476]. This evidence supports the findings from the current study and could serve as a possible explanation for the observed changes in the contralateral hemisphere that were either subverted or potentiated after treatment.

## Conclusion

We report that dietary genistein confers differing effects on select surrogate markers of M1 and M2 along with functional recovery. Genistein had no effect in the ipsilateral hemisphere aside from the reduction in HSP70 in the young. In the contralateral side, genistein shows disparate patterns in cytokine expression in the different age groups and at different lengths of hypogonadism. The beneficial effects of genistein as previously seen in the young and middle-aged, therefore, could have the distinct expression of the probed inflammatory markers as contributing factors. The increased expression of TGF-β1 and GAP43 further suggest the more likelihood of M2 microglia in genistein's actions. Moving forward, immunohistochemical stains of brains previously collected will be carried out to confirm the presence of specific microglial phenotype identifying proteins or otherwise as revealed by the correlations from the immunoblotting.

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## Figure Legends

**Figure 1. Effect of Gen and E2 on IL-6 expression.** (A-D). No difference was observed on IL-6 expression at the ipsilateral side ($F(2,98) = 0.625$, $p = 0.538$). (E-H) Main effects of treatment ($F(2,98) = 5.516$, $p = 0.005$), age ($F(1,98) = 10.713$, $p = 0.001$) and interaction between treatment and age ($F(2,98) = 4.714$, $p = 0.011$) were observed at the contralateral side. *Post-hoc* test revealed that Gen reduced IL-6 in the young cohort after both short-term ((E), vs stroke, $p = 0.001$; vs stroke+E2, $p = 0.001$) and long-term hypogonadism ((F), vs stroke, $p = 0.034$). All data expressed as mean±SEM. The analysis was performed with three-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. n=6-12, $*p < 0.05$.

**Figure 2. Effect of Gen and E2 on TGF-β1 expression.** (A-D). No difference was observed on TGF-β1 expression at the ipsilateral side ($F(2,98) = 1.801$, $p = 0.171$). (E-H) Interaction amongst Treatment, Duration of Hypogonadism and Age were seen at the contralateral side ($F(2,98) = 6.635$, $p = 0.002$). *Post-hoc* analysis revealed that E2 increased TGF-β1 expression in the young cohort after short-term hypogonadism ((E), vs stroke, $p = 0.003$; vs stroke+Gen, $p = 0.018$). Both Gen and E2 reduced TGF-β1 after long-term hypogonadism in the young cohort ((F), stroke vs

stroke+E2, $p = 0.008$; stroke vs stroke+Gen, $p = 0.001$). Gen increased TGF-β1 expression in the middle-aged cohort after short-term hypogonadism ((G), vs stroke, $p = 0.01$). The analysis was performed with three-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=6-12, $*p < 0.05$.

**Figure 3. Effect of Gen and E2 on HSP70 expression.** (A-D). Main effects of Treatment ($F(2,98) = 8.413$, $p < 0.001$), Duration ($F(1,98) = 8.662$, $p = 0.004$), Age ($F(1,98) = 4.357$, $p = 0.039$), interaction between Treatment and Age ($F(2,98) = 6.012$, $p = 0.003$) and interaction between Duration and Age ($F(2,98) = 4.065$, $p = 0.047$) were observed at the ipsilateral side. (A) Gen reduced HSP70 compared to young E2-treated animals after short-term hypogonadism ($p = 0.023$). (B) E2 increased HSP70 induction after long-term hypogonadism (vs stroke, $p = 0.004$; vs stroke+Gen, $p < 0.001$). (E-F) Main effect of Age was observed on HSP70 at the contralateral side ($F(2,98) = 9.513$, $p = 0.003$)). E2 increased HSP70 after short-term hypogonadism in the young cohort ((E), vs stroke, $p = 0.013$). Analysis was performed with three-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=6-12, $*p < 0.05$.

**Figure 4. Effect of Gen and E2 on GAP43 expression.** (A-D). No difference was observed on IL-6 expression at the ipsilateral side ($F(2,98) = 1.42$, $p = 0.247$). (E-F) Main effects of Age ($F(2,98) = 8.988$, $p = 0.003$), interaction between Treatment and Age ($F(2,98) = 4.419$, $p = 0.015$), interaction between Treatment and Duration ($F(2,98) = 4.4206$, $p = 0.018$), and interaction between Duration and Age ($F(1,98) = 10.219$, $p = 0.002$) were observed at the contralateral side. (E) Gen increased GAP43 in the young cohort after short-term hypogonadism (vs stroke, p=0.034; vs stroke+E2, $p < 0.001$). Analysis was done with three-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All data expressed as mean±SEM, n=6-12, $*p < 0.05$.

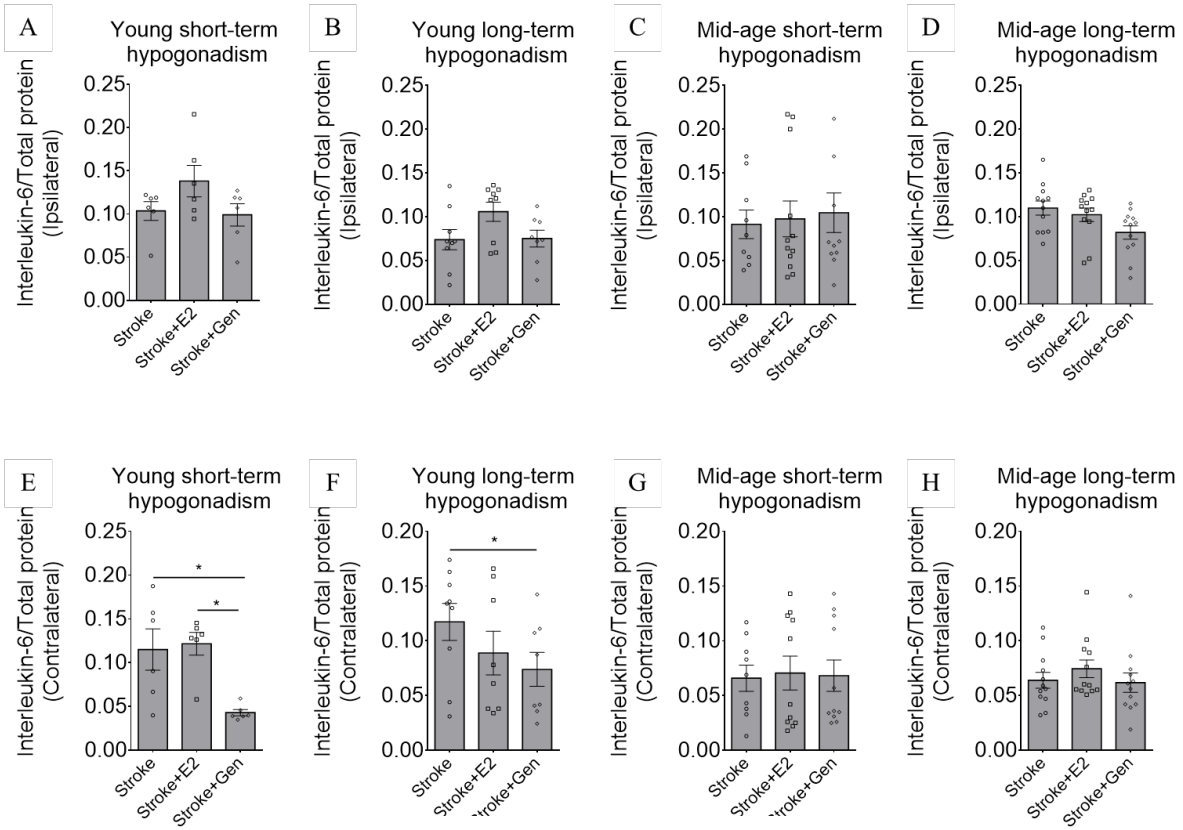

A
Young short-term hypogonadism
B
Young long-term hypogonadism
C
Mid-age short-term hypogonadism
D
Mid-age long-term hypogonadism
E
Young short-term hypogonadism
F
Young long-term hypogonadism
G
Mid-age short-term hypogonadism
H
Mid-age long-term hypogonadism
Interleukin-6/Total protein (Ipsilateral)
Interleukin-6/Total protein (Contralateral)
Stroke
Stroke+E2
Stroke+Gen
*

Figure 1

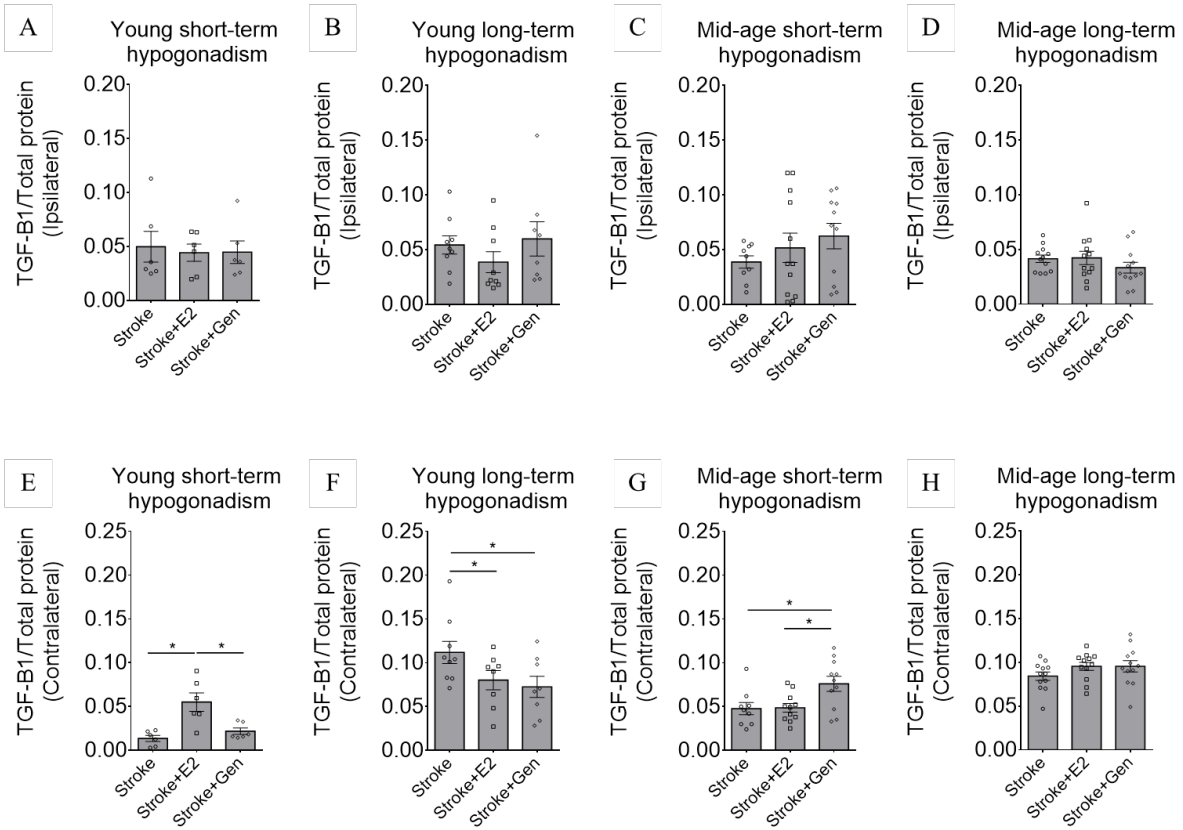

A
Young short-term hypogonadism
B
Young long-term hypogonadism
C
Mid-age short-term hypogonadism
D
Mid-age long-term hypogonadism
E
Young short-term hypogonadism
F
Young long-term hypogonadism
G
Mid-age short-term hypogonadism
H
Mid-age long-term hypogonadism
TGF-B1/Total protein (Ipsilateral)
TGF-B1/Total protein (Contralateral)
0.00
0.05
0.10
0.15
0.20
0.25
Stroke
Stroke+E2
Stroke+Gen
*

Figure 2

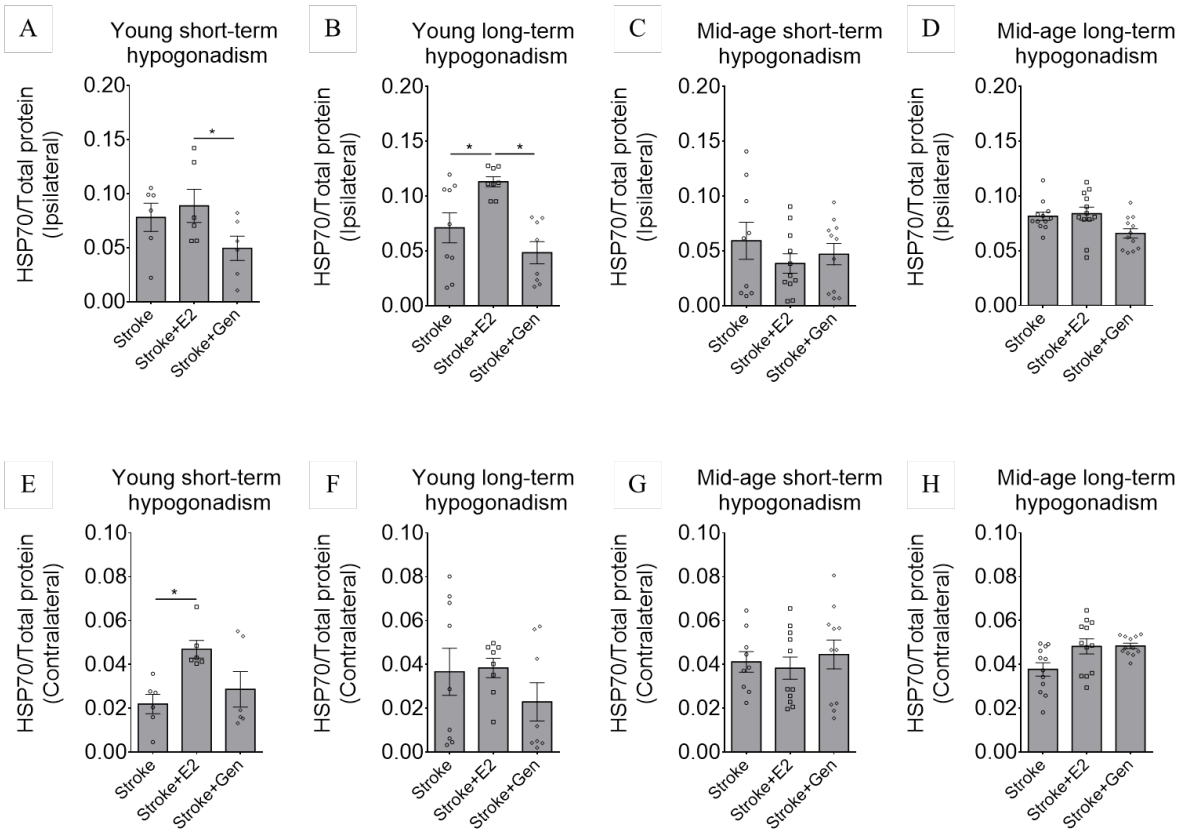

A
Young short-term hypogonadism
B
Young long-term hypogonadism
C
Mid-age short-term hypogonadism
D
Mid-age long-term hypogonadism
E
Young short-term hypogonadism
F
Young long-term hypogonadism
G
Mid-age short-term hypogonadism
H
Mid-age long-term hypogonadism
HSP70/Total protein (Ipsilateral)
HSP70/Total protein (Contralateral)
0.20
0.15
0.10
0.05
0.00
0.10
0.08
0.06
0.04
0.02
0.00
*
Stroke
Stroke+E2
Stroke+Gen

Figure 3

527

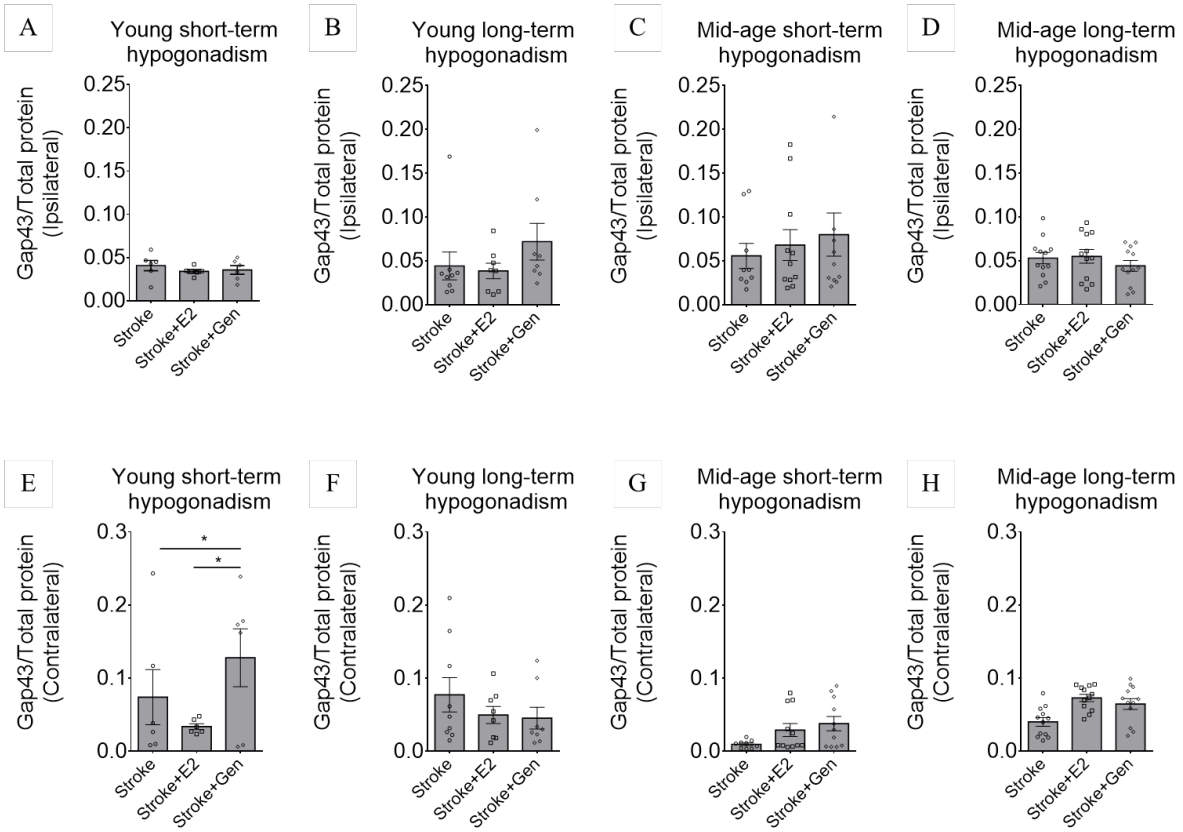

A
Young short-term hypogonadism
B
Young long-term hypogonadism
C
Mid-age short-term hypogonadism
D
Mid-age long-term hypogonadism
Gap43/Total protein (Ipsilateral)
0.25
0.20
0.15
0.10
0.05
0.00
Stroke
Stroke+E2
Stroke+Gen
E
Young short-term hypogonadism
F
Young long-term hypogonadism
G
Mid-age short-term hypogonadism
H
Mid-age long-term hypogonadism
Gap43/Total protein (Contralateral)
0.3
0.2
0.1
0.0
*
*

Figure 4

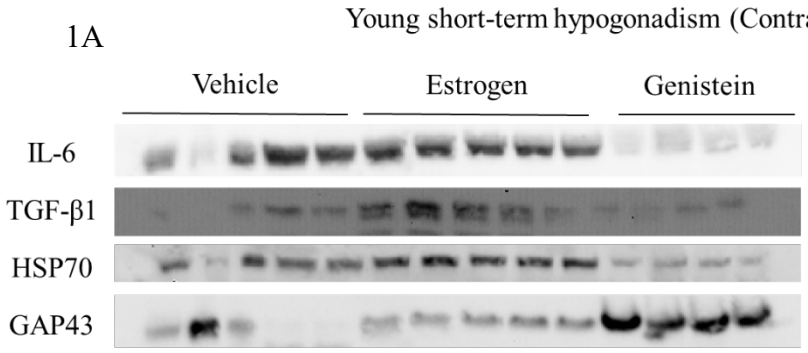
**Table 1.** Summary of contralesional effects of E2 and Gen on probed proteins compared to the vehicle

| Treatment | Estrogen | | | | Genistein | | | |
|---|---|---|---|---|---|---|---|---|
| Age | Young | | Middle-aged | | Young | | Middle-aged | |
| Hypogonadism length | STD | LTD | STD | LTD | STD | LTD | STD | LTD |
| Interleukin-6 | ↔ | ↔ | ↔ | ↔ | ↓ | ↓ | ↔ | ↔ |
| Transforming growth factor | ↑ | ↓ | ↔ | ↔ | ↔ | ↓ | ↑ | ↔ |
| Growth associated protein | ↔ | ↔ | ↔ | ↔ | ↑ | ↑ | ↔ | ↔ |
| Heat shock protein 70 | ↑ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |

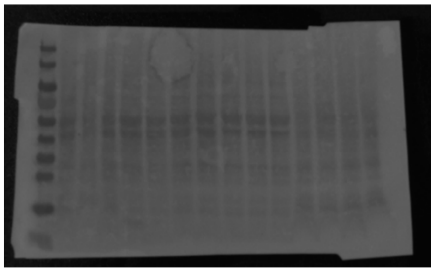
**Table 2.** Details of antibodies used for Immunohistochemistry (IHC) and Immunoblotting (WB)

| Antibody | Catalog number | Source | Concentration |
|---|---|---|---|
| Arginase 1 | PAS-32267 | ThermoFisher Scientific | IHC- 1:200 |
| Growth-Associated Protein 43 (GAP43) | 5307 | Cell Signaling Technology | WB- 1:1000<br>IHC- 1:500 |
| Interleukin-6 (IL-6) | ab9324 | Abcam | WB- 1:1000 |
| Transforming Growth Factor-β1 (TGF-β1) | ab92486 | Abcam | WB- 1:500<br>IHC- 1:500 |
| Heat Shock Protein 70 (HSP70) | MAS-35578 | ThermoFisher Scientific | WB- 1:500 |
| Cluster of Differentiation-16 (CD16) | bs-6028r | BiossUSA | IHC- 1:200 |
| Inducible Nitric Oxide Synthase (iNOS) | SAB4502011 | Sigma Life Science | IHC- 1:200 |

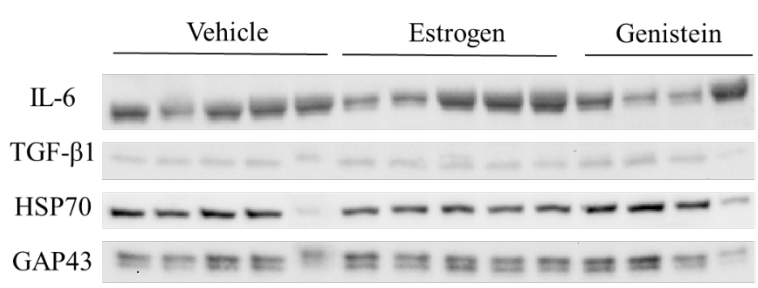
1A


Young short-term hypogonadism (Contralateral)
Vehicle
Estrogen
Genistein
IL-6
TGF-β1
HSP70
GAP43

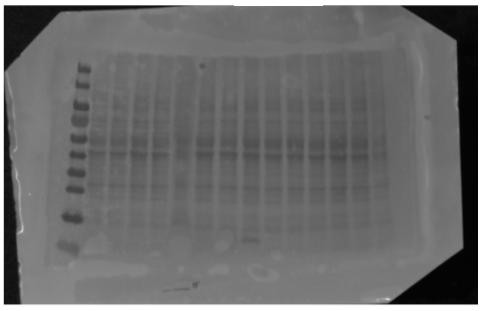
1B



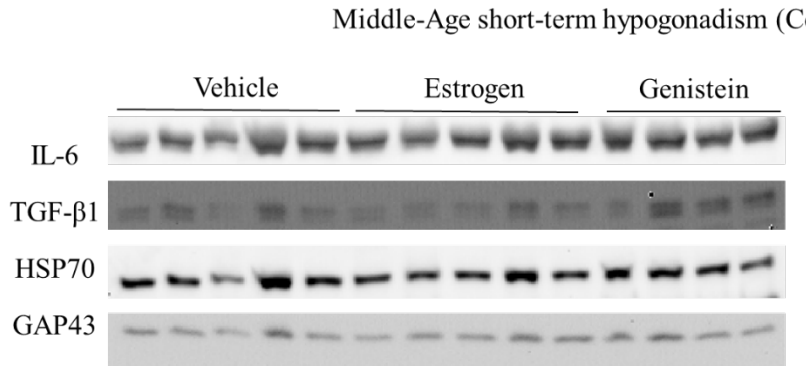
2A


Young long-term hypogonadism (Contralateral)
Vehicle
Estrogen
Genistein
IL-6
TGF-β1
HSP70
GAP43

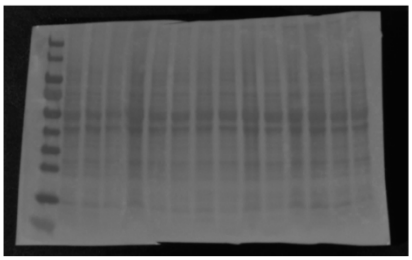
2B



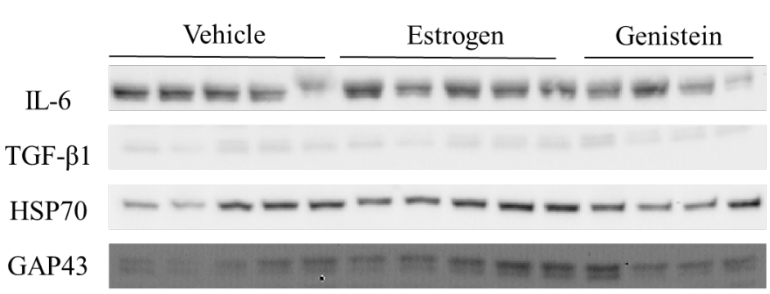
3A




3B



4A




4B

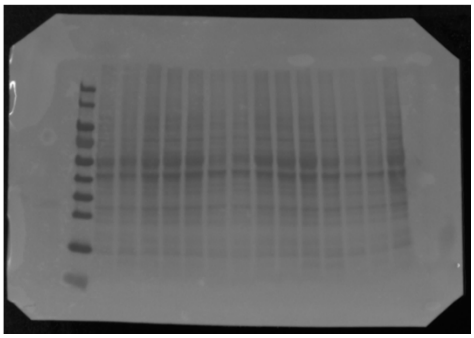


Figure A: Representative immunoblots of contralesional expression of IL-6, TGF-β1, HSP70 and GAP43 after 21-day reperfusion poststroke. 1A) after short-term hypogonadism in the young cohort, 2A) After long-term hypogonadism in the young, 3A) after short-term hypogonadism in the middle-aged, 4A) after long-term hypogonadism in the middle-aged. 1B-4B) Ponceau stains of blots.

# CHAPTER 5

## 1 SUMMARY AND DISCUSSION

Stroke risk increases with age in both sexes but more disproportionately in the female population [10]. A major contributor to this discrepancy is the sudden and significant drop of female sex hormones beyond the menopause transition compared to men of similar age. Because of the challenges with estrogen therapy for menopausal symptom management and possible prevention of chronic cardiovascular diseases, other agents are investigated as alternatives for estrogen. This dissertation aimed to identify the effect of varying lengths of hypogonadism on the neuroprotective properties of dietary genistein, a well-studied and commonly used plant-based estrogenic compound. We used young and middle-aged female Sprague-Dawley rats in the overall study design under experimental conditions of hypogonadism and focal cerebral ischemia. In *Chapter 2*, I investigated the effect of hypogonadism on dietary genistein's neuroprotection under ischemic conditions in young adult ovariectomized rats. For *Chapter 3*, I repeated similar experimental conditions of *Chapter 2* in middle-aged rats as a function of age. *Chapter 4* sought to investigate the influence of dietary genistein on poststroke inflammatory markers and the possible intra-conversion of microglia phenotypes following 21-day reperfusion after stroke. Collectively, the data suggest that dietary genistein differentially influences motor and cognitive functions in age and length of hypogonadism-dependent manner. This was evidenced by the improvement in cognitive flexibility after short-term hypogonadism in both young and middle-aged Sprague-Dawley rats and improved locomotor deficits in the middle-aged subjects after long-term hypogonadism.

Genistein is a selective estrogen modulator, possessing dose-dependent estrogenic and anti-estrogenic actions [477, 478]. In the current study, it was observed that dietary genistein but not 17β-estradiol (E2) improves cognitive flexibility and spatial reversal learning after short-term hypogonadism. In both human and rodent studies, dietary genistein has been shown to improve cognitive performance in both male and female subjects [392, 479, 480]. A possible reason for the difference in effect on cognition between genistein and E2 could stem from their effects on ER-mediated activities in different regions of the brain including the hippocampus and the prefrontal cortex. Whishaw et al showed that rats with compromised or damaged hippocampus may have no difficulty in initial learning based on visual cues but may exhibit gross impairment on reversal learning [481, 482]. Here, all treatment groups showed reduced path lengths to the hidden platform, depicting an intact capacity to learn initially based on their environmental cues, accounting for the absence of a statistically significance within the learning phase (acquisition) among the treatment groups. However, the benefit of genistein on the reversal task, as opposed to the lack of improvement by E2, means that genistein can affect extra-hippocampal regions that mediate cognitive function, one that may not be apparent with E2 under the conditions of the studies where there may be a compromised hippocampus [393]. Additionally, in the prefrontal cortex, that in part influences spatial flexibility, there is disproportionately high expression of estrogen receptor-beta (ERβ) compared to estrogen receptor-alpha (ERα). Considering a preferential avidity of Genistein for ERβ [394-396], this may also serve as the molecular basis for which improved reversal learning was seen with Genistein treatment but not E2, with the backdrop E2 have higher binding affinity and potency on ERα [397-399]. Previous long-term hypogonadism studies in rodents have shown that extended reduction of circulating E2 results in downregulation of ER receptors accompanied by a reduced estrogenic activity [401]. For this, the absence of effect

after long-term hypogonadism in young animals means that the long absence of circulating E2 majorly contributes to a blunted cognitive function due in part to the diminished ER expression [321, 322, 357]. Genistein did not affect motor function. Even though genistein reduced infarct size, the effect was not apparent on the motor tasks including the cylinder and rotorod tests. One reason for this observation is a link with the length of the occlusion period. In focal ischemic studies with genistein, the beneficial effect of genistein had been seen on neuroinflammatory markers within the motor cortex [300, 301, 309, 382]. Under the experimental stroke conditions, there was a possible residual cortical activity across the treatment groups as evidence by a minimal cortical impact from the immunostaining. Hence the treatment with genistein or E2 did not cause appreciable cortical improvement to result in change between groups. Following stroke in the young cohort, both dietary genistein and E2 reduced Iba1 expression most notably at the contralateral side of stroke injury but not at the ipsilateral side. What this means is that genistein maintains its constitutive anti-inflammatory properties in line with previous accounts [272, 402-405]. However, at the ipsilateral side, the effect of genistein might not be overt to reverse the inflammatory events accompanying the stroke. These differences in response to genistein in the young cohort, thus suggest that aspects of cognition are differentially sensitive to early administration of dietary genistein which may diminish with extended delay under conditions of surgical menopause [483].

Estrogen's use is limited by its peripheral detrimental effects and its propensity to increase the risk of stroke with age and after menopause [484]. In Chapter 3, we investigated dietary genistein for its neuroprotection under ischemic conditions following hypogonadism in middle-aged ovariectomized rats. This compared to previously observed effects of dietary genistein in young ovariectomized rats under similar short-term hypogonadal conditions (Chapter 2). Here we

observed improved cognitive flexibility on spatial performance tasks by Genistein-treated rats compared to both control and E2-treated rats after stroke. This finding is in line with published data on cognitive tasks where four-week and ten-week treatments with dietary soy isoflavones showed improved performance on the Morris water maze task [485, 486]. Further to that, the improvement of reversal learning by genistein underscores its cognitive benefits under conditions of short-term absence of circulating estrogen as previously observed in the young cohorts (Chapter 2). While dietary genistein improved cognitive flexibility, E2-treatment failed to do so. Although estrogen therapy over several decades has been linked to improved cognitive performance [487], findings from the Women's Health Initiative study among other human and preclinical studies have shown conflicting results where benefits were observed in some cohorts [488-490] while impairments were seen in others [491, 492]. This observation further adds to the complexity of the cognitive effects of estrogen considering that in the current study, there was no improvement regardless of the age (as in the case of the young also) and length of hypogonadism. There is evidence to suggest estrogen's impairment of cognitive tasks may involve brain regions other than the hippocampus including the striatum and prefrontal cortex [493-495]. Prominent among the reasons for the differential cognitive responses to estrogen is the diverse distribution of estrogen receptors alpha ERα and ERβ. While there are similar proportions of both ERs within the hippocampus, there is a disproportionate expression of the two in the prefrontal cortex, favoring ERβ [394-396]. Hence, the absence of E2's benefit on the reversal phase of the Morris water maze test suggests a minimal ERβ activity on extra-hippocampal regions involved in cognition [494, 495]. On the other hand, a higher affinity of genistein for ERβ, a mediator of ER-dependent memory function suggests that the beneficial effect of genistein on cognitive flexibility that is partly an executive function may involve ERβ activity within the prefrontal cortex where it mostly

concentrates [496, 497]. Both E2 and dietary genistein, like in the young cohort, failed to improve spatial learning and memory after chronic hypogonadism. Several factors could account for the disparities between dietary genistein and E2 treatment. First, the widely reported impaired ER activity following long periods of hypogonadism could similarly be at play in the middle-aged subjects as was seen in the young ones knowing that up to 4 weeks after ovariectomy is enough to see overt cognitive impairments in both young and old rats. [321, 322, 357]. This finding is corroborated by human studies where late recipients of estrogen therapy after menopause show impaired cognitive performance with an increased risk of dementia [323-325]. The abovementioned has further been recapitulated in preclinical studies where up to 10 weeks delay in estrogen therapy was associated with impaired cognitive performance. Given the beneficial roles of both ERα and ERβ in learning and memory [498, 499], the absence of the expected benefit by E2 and Genistein in the middle-aged also may stem from a reduced ER expression and loss of sensitivity to ER ligands within the brain. Additionally, aging contributes to the reduced activity of estrogen on cognition. Jezierski et al showed that administering estrogen to reproductively senescent rats was associated with impaired BDNF release and a loss of estrogen responsiveness [500]. Hence, a prolonged absence of estrogen or genistein and aging may have led to a decreased sensitivity by the neurons. Even though genistein has been used among some postmenopausal populations for the management of vasomotor symptoms, there is little preclinical evidence to support its improvement of motor function [285, 286, 294, 360, 501]. Thus far, Rajput et al have reported the improvement of motor performance in combination with metformin after transient global ischemia in diabetic mice with no effect in normal mice [502]. In non-stroke studies, Kyuhou et al and Pierzynowska et al have also reported that genistein treatment improves forelimb deficits in 6-hydroxydopamine (6-OHDA) Parkinson's disease model and locomotor activity in

streptozotocin-induced sporadic Alzheimer's disease model respectively [383, 384]. On the contrary, several works have reported the absence of effect of genistein on motor performance. No effect of genistein was observed by Lu et al and Arbabi et al on the motor performance following scopolamine-induced amnesia and 6-OHDA Parkinson's models respectively [385, 386]. This paucity of evidence on stroke together with the absence of motor benefit by genistein for most of the current study suggests that genistein may improve inflammatory markers and the extent of injury that may not translate into motor performance. E2 improved forelimb deficits after long-term hypogonadism across both ages. Previous studies have shown worse effects of E2 or loss of efficacy after long-term hypogonadism [355-357] which would suggest an expected detrimental effect. However, further assessment of the available information on E2 under extended absence was mainly related to cognitive impairment in addition to an exacerbated inflammatory response with little effect on motor tasks. This suggests that the detrimental effect of E2 may be mostly confined to the cognitive regions as were observed with the cognitive tests in the current study, but not on motor function. Additionally, E2 can promote neurogenesis within the subventricular zone and other brain regions [387, 388]. Hence, there is a possible facilitated tissue repair after the injury, translating into the E2 motor benefit here. Suffice it to say that, regardless of the minimal effect of genistein, there remained an age-dependent deficit in motor function where the young cohort showed better performance than the middle-aged on the motor tasks. Findings from the current study, therefore, support the notion that there is a time window of opportunity for estrogen, and for genistein within which their cognitive and motor benefits may be realized, as have been widely reported [503, 504].

As part of efforts to identify molecular changes that could explain the disparate outcomes from genistein and estrogen treatment, we sought to investigate the impact of both genistein and

E2 on poststroke inflammation 21 days after stroke. Overall, there was increased expression of IL-6 and HSP70 at the ipsilateral side of stroke compared to the contralateral side. Transforming Growth Factor-β1 levels were elevated at the contralateral side compared to the ipsilateral side. Previous studies have shown several-fold increases in the expression of proinflammatory cytokines including IL-6, TNF-alpha and IL-1β within the ipsilateral hemisphere compared to the contralateral side [74, 76, 77]. IL-6 serves as one of the earliest recruited cytokines following microglial and astrocyte activation remains elevated and integrally contributes to the extent of ischemic brain damage [94, 95]. In line with distinct expressions of IL-6, 60-min middle cerebral artery occlusion disproportionately increased the IL-6 levels in the current study at the ipsilateral side compared to the contralateral side, confirming the previously observed significant increase in GFAP and Iba1 activation.

What was intriguing about the study was that, neither estrogen nor genistein affected TGF-β1 and IL-6 induction at the ipsilateral side. This runs contrary to what has been reported with other studies where both genistein or E2, through their constitutive antioxidant properties or ER-mediated actions have adequately reduced IL-6 or increased TGF-β1. Since little information is available to support this finding, a possible reason could be because of a temporal waning of the inflammatory response to both agents in the perilesional area over the period studied. Additionally, the ipsilateral side was associated with a significant increase in HSP70 compared to the contralateral side. This demonstrates the increased brain response to salvage the ischemic neurons within and around the lesion as has been reported by other studies [125, 127-129]. The subtle contralateral effects of genistein and E2 as were observed underscored the anti-inflammatory properties of both Genistein and E2 following ischemia [447, 505]. Genistein in the young, adequately reduced IL-6, an effect that was not apparent in the middle-aged cohort. Elevated IL-6

plasma levels are associated with increased cognitive deficits independent of the presence of stroke [506-509]. Consistent with previous reports, a moderate positive correlation between spatial learning and the expression of IL-6 was observed. This depicts that the stroked rats which expressed high levels of IL-6 learned slower on the cognitive task as evident in their longer path lengths. Again, Genistein increased GAP43 in the young cohort and TGF-β1 in the middle-aged after short hypogonadal length. Axonal regeneration is generally induced following ischemic stroke as a restorative process [115-118, 510, 511]. However, axonal sprouting is impaired following large strokes [512]. With a suppressed regeneration as evident in low GAP43 expression across ages, it is possible that the extent of degeneration induced by our experimental protocol in the ipsilateral hemisphere could not be rescued by either Genistein or E2. However, at the contralateral side, Genistein could still induce axonal regeneration, hence the observed increase. Overall, the different anti-inflammatory effects per cohort suggest that both age and length of hypogonadism can limit the anti-inflammatory effects of Genistein and E2 when assessed several days after the initial injury.

## 2 Future directions

### 2.1 Establishing a therapeutic window for GEN

Tissue-Plasminogen Activator (tPA) remains the gold standard pharmacologic treatment for ischemic stroke [513]. Strategies to increase the number of tPA recipients within its therapeutic window have intensified over the years but there remains a large ineligible candidate population (~96%) for its use [514]. For this, there is a pressing need to identify diverse strategies especially suited for the postmenopausal population in whom the risk of stroke increases exponentially and less likely to receive tPA [515-517]. In the current study, we identified mixed effects of genistein on behavioral functions in different age groups at different times of hypogonadism. It is, therefore,

necessary to advance the study to identify the temporal molecular changes at which the onset of a dichotomy of genistein's benefit and lack of it may be observed. This will provide clarity on the time-dependent beneficial effect of genistein in attenuating stroke risk with potential for clinical translation.

### 2.2 Potential synergy between E2 and Genistein for neuroprotection

Loss of or reduced circulating endogenous estrogens is associated with an increased risk of stroke, cognitive decline, and metabolic syndrome [323-325, 518]. However, hormone therapy with estrogen has not proven substantial in reducing cardiovascular risk. In combination treatment with both Genistein and estrogen, Genistein acting as a selective estrogen receptor modulator reduced the negative effects of estrogen on cells from mammary glands, ovaries and the prostate [406-408]. There is, however, limited information on the impact of combined therapy of Genistein and estrogen on brain function and possibly on cardiovascular risk [519]. In the current study, we observed that both genistein and estrogen confer beneficial effects at different time points of hypogonadism and age with genistein favoring cognitive function while estrogen favored motor benefits. Hence, a possible concurrent administration of genistein and estrogen within safe limits in further studies could render a dual-purpose beneficial effect for both locomotor and cognitive functions. The off-target detrimental effects of E2 including hyperplasia of the endometrium were beyond the scope of this study. However, that can equally be tested in future studies with genistein as an adjuvant to estrogen under similar conditions of brain ischemia. Altogether, it will provide the guidance that will inch closer to identifying strategies that provide the needed protection to the brain against ischemic insults while keeping a high safety profile in the target population.

## 2.3 Possible involvement of gut biome in phytoestrogen neuroprotection

About half of stroke patients experience different degrees of gastrointestinal complications including impaired gut motility, increased gut permeability and gut-induced sepsis [520-522]. Dysfunctional intestinal microbiota and accompanying bacterial translocation have recently been shown to influence the inflammatory milieu of the brain during stroke, contributing to poor outcomes, especially in the aging population [523-527]. This observation is further compounded by the bidirectional interaction between the brain and gut to sustain the exacerbating immune responses [523, 528-532]. A recent study by Benakis et al shows that modification of the gut microbiota by interleukin-10 (IL-10) and interleukin-17 (IL-17) play integral roles in neuroprotection, impacting poststroke neuroinflammatory responses and functional recovery [533]. Considering poorer behavioral outcomes with aging, peripheral influences such as poststroke infection and immune dysfunction could be contributing factors that could equally serve as a therapeutic target [534, 535]. Soy isoflavones, including genistein, have demonstrated the ability to reduce intestinal injury by reducing inflammatory responses from Toll-Like Receptor-4 (TLR-4) signaling and increase the expression of B-defensins and mucins [536]. Genistein has been shown to maintain gut integrity through the reduction in NF-kβ/ TLR-4 signaling in dextran sulfate sodium-induced intestinal injury study [537]. Previous beneficial effects of genistein during stroke especially in the middle-aged rats hence suggest that there could be a positive peripheral involvement which could stem from the gut-brain-axis [300, 305, 306]. Therefore, further studies should investigate how changes in the gut microbiota and immune responses poststroke are directly influenced by genistein administration.

## 3 Conclusion

Vasomotor symptoms, cerebrovascular disease and cognitive decline are associated with increasing age especially beyond menopause. There are, however, limited therapeutic options in meeting the clinical needs of individuals in such a target population. This dissertation project has shown that the neuroprotective benefits of dietary genistein as a potential alternative for estrogen therapy is sensitive to age and the length of hypogonadism under ischemic condition. Findings from this dissertation, therefore, proffers newer thoughts for consideration on the use of the soy isoflavone genistein under conditions of low circulating estrogens. By genistein's benefits seen in this dissertation, it still holds a therapeutic potential and could serve as the substantive agent or adjuvant for maintenance of cognitive function after menopause with further studies needed to validate the benefits.

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