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To investigate neuroprotective mechanism in female brain

Jatin Tulsulkar
University of Toledo

A Dissertation

entitled

To investigate neuroprotective mechanism in female brain

by

Jatin Tulsulkar

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Medicinal Chemistry

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An Abstract of

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It is well known that gender differences exist in the experimental or clinical stroke with respect to the tissue damage and the loss of functional outcome. We have previously reported neuroprotective properties of Ginkgo biloba/EGb 761® (EGb 761) in transient and permanent mouse models of brain ischemia in male mice and the mechanism of neuroprotection was attributed to the upregulation of the HO1/Wnt pathway. Here, we sought to investigate the novel hypothesis, whether neuroprotection by EGb 761 in ovariectomized (OVX) female mice is mediated by HO1/Wnt upregulation in a model of permanent cerebral ischemia. The OVX mice pretreated with EGb 761 for 7 days were subjected to permanent ischemia at day 8 and sacrificed on day 14. Infarct volume analysis showed that EGb 761 pretreated OVX mice had lower infarct volume, lower neurological deficits and improved motor skills as compared to OVX female vehicle mice. Protein analysis studies demonstrated that neuroprotection in EGb 761 pretreatment OVX group is not mediated by HO1/Wnt pathway. Furthermore EGb 761 pretreatment group demonstrated overexpression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide (eNOS). In addition, increased expression of TNF-α in the

vehicle group was observed to be comparatively lower in EGb 761 pretreated OVX group. These results suggest that the neuroprotective effect of EGb 761 in females is not associated with the activation and upregulation of HO1/Wnt pathway. To further understand the cell death mechanism involved, we studied expression levels of apoptotic protein cleaved caspase-3 and caspase-8 which were found to be significantly elevated in the Veh/OVX group as compared to the EGb 761/OVX group. We previously showed that EGb 761 post and pretreatment enhances neurogenesis by increasing the number of neural stem progenitor cells (NSPC's) in male mice 7 days following permanent ischemia, similarly in this study we demonstrated that in absence of ovarian hormone estrogen, EGb 761 increased NSPC's 7 days after ischemia. To test whether EGb 761 in absence of estrogen activates another gonadal steroid androgen, luciferase assay and immunofluorescent studies suggested that EGb 761 significantly binds and results in activation of androgen receptor (AR) in female brain. Taken together these results suggest that the possible mechanism of EGb 761 mediated neuroprotection in females following permanent ischemia is independent of HO1/Wnt signaling and via caspase dependent pathway and neurogenesis.

Acknowledgements

I consider this as a proud moment to express my venerable gratitude to all the people who have been involved directly and indirectly with the completion of this dissertation. Since it is required more than my solitary efforts, I therefor take this opportunity to thank all who in one way or other have helped me.

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My family has overwhelmed me with their care, blessings, support and love during testing times.

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Introduction

1.1 What is brain stroke?

Stroke is the fifth leading cause of death and permanent disability affecting nearly 700,000 Americans each year with either new or recurrent stroke. On an average someone is having a stroke every 40 seconds (Kochanek, Murphy et al. 2014). The direct and indirect financial burden for the treatment of stroke is approximately $58 billion each year. It is expected that the cases of stroke will increase by 20.5% in 2030 as compared to what was observed in 2012 (Mozaffarian, Benjamin et al. 2015). Apart from the expensive stroke treatment, there is an enormous burden on the patient and the care givers due to permanent disability associated with the disorder. Public awareness on the warning signs is absolutely necessary regarding the risk factors of stroke. The common risk factors of stroke include high blood pressure, diabetes, smoking, heart disorder, high cholesterol, obesity and kidney disease (Mozaffarian, Benjamin et al. 2015).

Brain stroke is a neurological disorder characterized by interruption of blood supply to a particular region of the brain, resulting into insufficient supply of nutrients such as glucose and oxygen to neurons leading to neuronal death. Stroke is caused either by

choking of blood vessels due to a clot (ischemic) or severing of arteries leading to blood leaking out into brain parenchyma (hemorrhagic) (Figure 1-1). Brain sensitivity towards low levels of oxygen and glucose results in enormous cell damage leading to permanent loss of function in the blood compromised areas of the brain. Stroke damage leads to a functional deficit which commonly includes loss of motor function, vision problem and cognitive loss (American Heart Association, About Stroke, 2014).

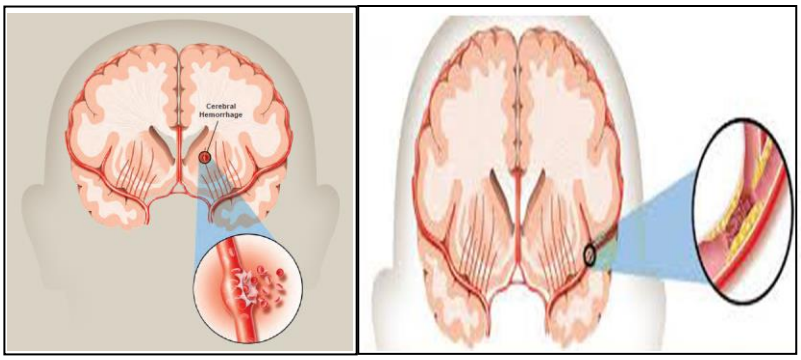


Figure 1-1: Schematic description of hemorrhagic stroke and ischemic stroke. The left panel shows hemorrhagic stroke which occurs due to rupturing of the blood vessel and the right panel shows ischemic stroke that occurs due to choking of the artery by a clot. Both result in blood loss followed by extensive cell death in the affected areas of the brain. (Picture source: National Stroke Association).

The occurrence of ischemic stroke accounts for approximately 85% of all the stroke cases, whereas 15% are the combination of intracerebral hemorrhage and subarachnoid hemorrhage. Therefore, ischemic stroke is the major focus of our study. Ischemic stroke can further be divided into embolic and thrombotic strokes (American Heart Association, About Stroke, 2014). Embolic stroke results from the embolus developed in the heart and the large arteries in the neck and upper chest area which enters the brain via the

circulation resulting in the obstruction of the blood supply to the brain, whereas thrombotic stroke occurs when a clot is developed due to accumulation of fatty acids in the lumen of the artery that supplies blood to the brain. The feeble outcome, astounding mortality and extortionate economic burden associated with this disease have impelled extensive drug development and increased efforts to restore good health. Focus is also on the preventative strategies that reduce the probability of stroke in susceptible individuals. The following section highlights the current stroke therapy.

1.2 Current Scenario of Ischemic Stroke Therapy

The only FDA- approved therapy for stroke is recombinant tissue plasminogen activator (rtPA) (Zivin 2009). Tissue plasminogen activator is a serine protease enzyme present in endothelial cells and is responsible for thrombolysis by catalyzing plasminogen to plasmin thus dissolving the clot. Recombinant technology has been used to produce rtPA, and used on patients in the critical stages of stroke. Due to its narrow time window of 3 hours after the start of the symptoms, this drug is rendered impractical or ineffective as many patients do not qualify for its beneficial effects (Zivin 2009). However, recent studies showed that the 3 hour time window is desirable to control stroke damage. A recent study showed significant improvement in functional outcome after treatment with the drug as late as 6 hours after the onset of stroke (group, Sandercock et al. 2012). Nevertheless, tPA still struggles with time constraint, cost, hemorrhagic effect and other exclusion criteria's like age, sex and hypertension (Zivin 2009), thus preventing large numbers of patients from utilizing the beneficial effects of this stroke therapy.

Due to the limitations in stroke therapy listed above, researchers are investing resources in finding a lead for an effective stroke treatment. Unfortunately, most of the promising neuroprotective agents in pre-clinical studies show poor compliance in clinical trials and ultimately fail to qualify as an effective neuroprotective agent. This bleak performance by these drug candidates has led to a reassessment of the clinical testing parameters for stroke. The below par activity of the drugs at the clinical level could be attributed to the following reasons:

(a) Narrow time window of 3 hours after initial symptoms of stroke for drug administration: Treatment with the combination of prophylactic agent and several doses of drug following stroke will provide a better outcome (Gladstone, Black et al. 2002).

(b) Relying mostly on histological outcomes to conclude the success at the pre-clinical level, whereas improvement in the functional recovery determines the clinical success (Gladstone, Black et al. 2002).

(c) Use of single animal model of stroke to estimate the effectiveness of the drug. It has been exaggerated that the reperfusion model of stroke is the conventional and clinically relevant model (Hossmann 2012) . Recent studies suggested that the permanent model of focal ischemia (discussed in materials and methods) is also a clinically relevant model. It has also been suggested by the Stroke Therapy Academic Industry Roundtable (STAIR) that drugs should be evaluated in both permanent as well as transient model of ischemia in rodents and then replicated in primates before advancing to clinical trials (Stroke Therapy Academic Industry 1999).

(d) Drugs developed so far target only an individual component of the ischemic cascade: Ischemia activates a cascade of cell damaging as well as cell survival pathways,

thus developing multiple drugs or even a single drug that can act at the multiple sites such as to inhibit the cell death and promote cell survival are the preferred candidates (Gladstone, Black et al. 2002).

(e) One of the major reasons for the failure of neuroprotective agents in the clinic is the use of only male populations in the studies; brain injury during stroke largely depends on sex and age. Use of both males and females in studies will improve the perspective and might result in better alternatives for stroke drug therapy.

Collection of data from numerous failed clinical trials has changed the point of view towards stroke research. Natural products being continuously tested for potential leads (Newman and Cragg 2007) furnish distinct chemical properties that could be exploited to design novel structures or used directly as drugs. Sophisticated analytical techniques and screening procedures has allowed researchers to simplify the natural product drug discovery process (Rishton 2008). The ingrained complexity of natural compounds provides a novel idea in drug discovery. It was initially believed that one drug can target one component but recent drug discoveries are more based on one drug targeting many components in the cascade of disease progression. (Reddy and Zhang 2013). Due to the highly complicated polypharmacology exhibited by different constituents of plant extracts, they are considered as potential leads in pre-clinical research for stroke advancement.

1.3 *Ginkgo biloba*: Introduction

Traditional Chinese herbal products have an established history for the treatment of

common ailments to maintain proper balance within the body and ward off illness. *Ginkgo biloba* is one of the oldest living tree species in China. For over 5000 years, fruits and the seeds of this tree have been used in the treatment of asthma, cough and enuresis (Zimmermann, Colciaghi et al. 2002). However the first use of Ginkgo leaves for medicinal purpose dates back to 1505 A.D by Liu Wen-Tai, Ben Cao Pin Hue (DeFeudis and Drieu 2000). Further studies indicated that *Ginkgo* leaves can also be used for the treatment of skin infections. Standardized extracts of *Ginkgo biloba,* EGb 761, is becoming popular as a memory enhancer in the United States.

The percentage of various constituents present in Ginkgo biloba differs considerably with time of harvest, country of origin etc., so it is essential to develop a standardized extract with a known constituent concentration for consumption, clinical research, reproducibility and drug regulation. The standardized form of Ginkgo biloba leaf extract EGb 761 was developed by a French company called Ipsen Pharma (Paris, France) and Dr. Wilmar and Schwabe pharmaceuticals (Karlsruhe, Germany). The extract contains 24% flavonoid glycosides, 6% terpene lactones and less than 5 ppm ginkgolic acid, the component with allergenic properties (Jacobs and Browner 2000). The method of obtaining EGb 761 from Ginkgo leaves is archived and is as follows: Ginkgo biloba leaves are harvested while they are green between July and September, dried and their chemical and microbiological properties are analyzed for trace heavy metals and pollutants (Bilia 2002, Zimmermann, Colciaghi et al. 2002). Furthermore, the active components are extracted from the dried leaves by using an acetone-water mixture. Flavonoid fractions are identified by HPLC/UV, whereas terpenes are quantified by HPLC coupled with retention index detector (Bilia 2002, Zimmermann, Colciaghi et al.

2002). *Ginkgo biloba* itself was one of 2011's top 10 selling dietary supplements across the United States with sales around $15mn.

1.4 Components of EGb 761 and Neuroprotection

The unique polypharmacological activity of the extract depends on two major fractions, the flavonoids and the terpenes. The terpene lactones are classified by ginkgolides A, B, C, J and M and bilobalide. Extensive research on ginkgolides demonstrated their use resulted in reduced platelet activation and aggregation which helped to improve blood circulation. Improvement in the blood circulation allows more oxygen and glucose to be transported to the brain following ischemic stroke (Defeudis 2002). Bilobalide is a sesquiterpene trilactone and is known to reduce infarct volume after brain stroke (Chandrasekaran, Mehrabian et al. 2003). The flavonoid fraction of the extract has antioxidant properties and appears to be more effective on hydroxyl radicals than the terpene fraction (Bastianetto, Ramassamy et al. 2000).

The chemical structure of flavonoids is composed of an aromatic ring and double bond which favors direct scavenging of the hydroxyl radicals. Additionally, the phenolic hydroxyl group chelates heavy metal ions (e.g: Fe^{+2}) thus preventing the new hydroxyl radical formation (Ni, Zhao et al. 1996). One of the EGb 761 components, bilobalide has shown neuroprotective properties by preventing the uncoupling of oxidative phosphorylation in mitochondria and increasing ATP levels (Defeudis 2002), enhances neuronal plasticity (DeFeudis and Drieu 2000), attenuates apoptosis in hippocampus

(Tulsulkar and Shah 2013) and neuronal cultures (Bastianetto, Ramassamy et al. 2000). Clinical applications include improvement of peripheral arterial insufficiency (DeFeudis and Drieu 2000), treatment of tinnitus, acute cochlear deafness, vertigo and disturbance of equilibrium (DeFeudis and Drieu 2000) and improvement in cognitive deficits from stress and traumatic brain injury (DeFeudis and Drieu 2000).

1.5 Neuroprotection studies

Both in vitro and in vivo studies have identified the therapeutic properties of Ginkgo biloba extract EGb 761 and have recommended its use in clinical studies (Luo 2001). In 2000, Defeudis proposed several mechanisms of action that point towards the beneficial effects of EGb 761 in Alzheimer's disease (AD) (DeFeudis and Drieu 2000). Our lab has showed the mechanistic approach of EGb 761 and its components, bilobalide, ginkgolide A, B and TFM (terpene free material) in a model of transient global ischemia induced by cardiac arrest (Tulsulkar and Shah 2013). Here we demonstrated that none of the constituents except EGb 761 extract as a whole showed protection against neuronal death in CA-1 and CA-2 region of the hippocampus against ischemic cell death suggesting that EGb 761 has a synergistic effect. The mechanism was mainly attributed to the robust expression of the HO1/Nrf2 pathway resulting in the activation of the antioxidant pathway. Furthermore, EGb 761 extract successfully enhanced anti-inflammatory effects by reducing expression of microglial cells and astrocytes and enhancing angiogenic effect

by increasing expression of vascular endothelial cell growth factor (VEGF). In our recent study, we further demonstrated the repair and regenerating properties of EGb 761 in a model of permanent ischemia. After 7 days of EGb 761 pretreatment followed by permanent ischemia on day 8, mice showed lower infarct volume, improved neurological deficits and functional recovery and enhanced newly born cells or neural stem precursor cells (NSPC's) on day 14. The Fig 1-2 depicts the neuroprotective mechanism of EGb 761 after ischemia (Shah, Nada et al. 2011, Nada, Tulsulkar et al. 2014, Raghavan and Shah 2014).

1.6 EGb 761 provides neuroprotection by upregulating the HO1/Wnt pathway

Hemeoxygenase-1 (HO1) is an inducible enzyme that catabolizes heme to biliverdin (BVD) which is later converted to bilirubin (BR) (Dore, Takahashi et al. 1999), carbon monoxide (CO) and iron, and its activity is increased in the brain during stroke. A plethora of studies have shown that HO1 is partially involved in the protective mechanism in stroke, cardioprotection in diabetic rats, lipopolysaccharide preconditioning in intestine and lungs and protection against apoptosis in myocardial cells (Tamion, Richard et al. 2001, Jancso, Cserepes et al. 2007, Li, Li et al. 2007, Thirunavukkarasu, Penumathsa et al. 2007, Zhu, Zhang et al. 2007). It is well established that brain stroke causes generation of free radicals and blood brain barrier disruption thus releasing free heme from heme containing proteins such as hemoglobin, myoglobin, neuroglobin and others which enter the brain. Over expression of HO1 after injury cleaves heme and releases by-product such as CO, BVD/BR and iron which together

rescue neurons from apoptotic death and inflammatory responses (Balla, Jacob et al. 1993, Wang, Wang et al. 2007) by attenuating NFκB and TNF-α expression (Zeynalov and Dore 2009). Studies have shown higher cellular and ischemic damage in HO1 knockout ($HO1^{-/-}$) mice following permanent and transient ischemia. This can be attributed to un-metabolized heme due to the absence of HO1 (Zeynalov, Shah et al. 2009) and its beneficial by-products such as CO, biliverdin and bilirubin.

Many studies have shown that central nervous system development is dependent on the Wnt signaling pathway which is involved in cell proliferation, cell differentiation and migration (Zheng, Ying et al. 2010). Moreover, Wnt signaling is necessary for the self-renewal and differentiation of neural stem cells to neurons in the hippocampal region of dentate gyrus (Kuwabara, Hsieh et al. 2009). The role of Wnt signaling in extending the survival of newly produced neurons in a non-niche environment has also been extensively studied (Shruster, Ben-Zur et al. 2012). The activation of Wnt has been found to be neuroprotective in models of AD (Toledo, Colombres et al. 2008). It has also been shown that Wnt signaling is likely to be involved in the pathogenesis of neuronal death after ischemia due to activation of the Wnt inhibitor Dickkopf-1 (DKK-1) secreted in the ischemic area resulting in neuronal death (Cappuccio, Calderone et al. 2005). We previously showed that EGb 761 enhances Wnt expression in male mice after 7 days of permanent ischemia thus; resulting into neurogenesis and neuroprotection mediated through the HO1/Wnt pathway (Nada, Tulsulkar et al. 2014).

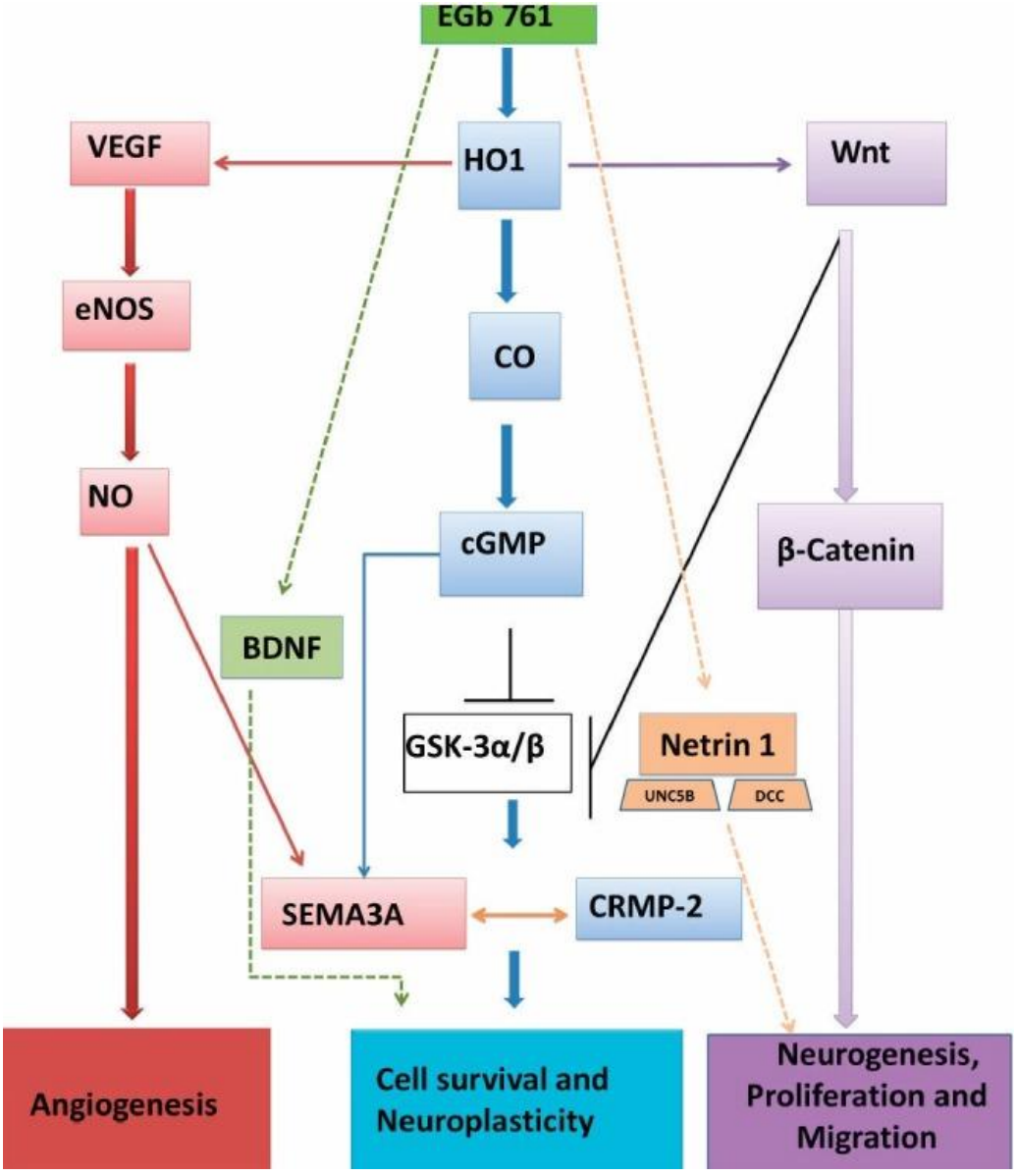


Fig. 1-2. The Signalling pathway of EGB 761 induced neuroprotection and neurogenesis

In Fig 1-2 The possible neuroprotective mechanism of EGb 761: 1) The initial

activation of the HO1 pathway by EGb 761, in which the HO1 cleaves free heme to biliverdin and CO. Biliverdin is further metabolized to bilirubin by biliverdin reductase, and an antioxidant CO, which directly binds to the iron of the heme moiety associated with soluble guanylyl cyclase (sGC), thereby activating the enzyme and increasing intracellular cyclic guanosine monophosphate (cGMP) production. Axonal branching is regulated by cGMP production which converts SEMA3A from chemoreplusion to chemoattraction and also inhibits glycogen synthase kinase-3 α/β. Inhibition of GSK-3 α/β increases the activity of CRMP-2 ultimately leading to neuronal survival and cell plasticity. 2) In the second sub mechanism EGb 761 increases VEGF expression which further activates eNOS leading to elevated NO levels that mediates the conversion of SEMA3A from chemorepulsion to chemoattraction and ultimately angiogenesis. 3) In the third sub mechanism the elevation of Wnt leads to activation of the Wnt/β-catenin canonical pathway by phosphorylation of GSK-3 α/β from the complex which leads to neurogenesis (Nada, Tulsulkar et al. 2014, Raghavan and Shah 2014) (Copyright permission from NRR, Raghavan and Shah).

Chapter 1

Ginkgo biloba extract (EGb 761) attenuates brain damage independent of the HO1/Wnt pathway in ovariectomized mice following permanent ischemia.

1.0 Introduction

Stroke affects approximately 795,000 people across the U.S., killing more than 137,000 people, with the majority of patients dying within first month after the onset of stroke; a third of stroke survivors experience permanent disability(Turtzo, Li et al. 2013). Men experience higher incidence of stroke than the women throughout their lifespan; however, at older ages, more women than men experience stroke (Roger, Go et al. 2012). The mode of neuroprotection is attributed to estrogen, due to the beneficial effect of the hormone in preventing coronary heart disease (Alkayed, Harukuni et al. 1998). A deficiency of estrogen after menopause is associated with a greater risk of memory decline, cognitive impairment, Alzheimer's disease (AD) and stroke (McCullough,

Alkayed et al. 2001, McCullough and Hurn 2003). Recent studies suggest that ovariectomy exacerbates brain damage in MCA occlusion, and the infarct size is comparable to that of male rodents. Estrogen pretreatment reduced the ischemic lesion in ovariectomized (OVX) female rats and showed similar reduction in male rats, an effect that was due to changes in the regional cerebral blood flow (Hurn, Littleton-Kearney et al. 1995). However, Dubal et al. found no changes in regional cerebral blood flow between estrogen-pretreated and oil-treated animals. Post-stroke estrogen administration has been shown to play a beneficial role in reducing ischemic lesion in postmenopausal women (Dubal, Kashon et al. 1998, Sampei, Goto et al. 2000, Murphy, McCullough et al. 2003). However, long term exposure to estrogen replacement therapy in menopausal women showed poor compliance and side effects. In addition, a women's health initiative study demonstrated that estrogen or hormone replacement therapy increases stroke and heart attack in healthy post-menopausal females as compared to placebo (Billeci, Paciaroni et al. 2008). In spite of advances in understanding the pathophysiology of underlying cerebral ischemia, current treatments are limited in terms of efficacy (e.g., recombinant tissue plasminogen activator) and utility (Gibson, Coomber et al. 2011). Thus, there is a need for basic research to investigate potential neuroprotective candidates in order to determine whether their clinical investigation should be warranted in future.

There is a wealth of experimental evidence suggesting neuroprotective properties of EGb 761 in various neurological conditions such as AD, Parkinson's and Stroke (Janssen, Sturtz et al. 2010, Tanaka, Galduroz et al. 2013). Over a decade of research on EGb 761 and stroke provides convincing data explaining its neuroprotective role in male mice

(Shah, Nada et al. 2011, Tulsulkar and Shah 2013). Recently, our lab showed neurogenesis-enhancing properties of EGb 761 following stroke, and the neuroprotective mechanism has been attributed to the HO1/Wnt canonical pathway.(Nada, Tulsulkar et al. 2014) However, all the studies were conducted in male animals, so EGb 761's protective effects and mechanism of action in females is yet to be determined.

In the current study, we investigated whether pretreatment with EGb 761 in OVX mice is neuroprotective after permanent ischemia. In order to determine whether EGb 761 is neuroprotective, we combined the measures of infarct volume, grip strength, neurological outcome, protein expression, neurogenesis, and androgen receptor (AR) activation-enhancing properties. This study allowed us to understand the molecular mechanism of EGb 761 neuroprotection in females, which might lead to safer and alternative treatments for stroke.

2 Materials and Methods

2.1 Animals and treatment

All animal protocols were approved by The University of Toledo Health Science Campus Institutional Animal Care and Utilization Committee and the guidelines of the National Institutes of Health were followed throughout the study. Female C57BL/6 mice, 8–10 weeks old and 20-25 grams in weight, were procured from Charles River Laboratories, and were housed at 22 ± 1°C with a 12 h: 12 h light/dark cycles.

2.2 Ovariectomy and Drug treatment

Sham, vehicle, EGb 761 pretreated group underwent bilateral ovariectomy and sham intact and WT intact groups were used as a positive control. All the female mice were anesthetized under 5% isoflurane and then stabilized at 1.5% throughout the surgical procedure (Idris 2012). A single incision of 5.5-10 mm long is made into the muscle wall on both the right and left sides approximately 1/3 of the distance between the spinal cord and the ventral midline. The ovary and the oviduct are exteriorized through the muscle wall. A hemostat is clamped around the uterine vasculature between the oviduct and uterus. Each ovary and part of the oviduct are removed with single cuts through the oviducts near the ovary. The hemostat is removed and the remaining tissue is replaced

into the peritoneal cavity. The ovary on the other side is removed in a similar manner. All the animals are allowed to recover for 7 days following OVX prior to pMCAO. In the drug treatment group EGb 761 was orally administered 24 hr after OVX and continued for 7 days. For neurogenesis study BrdU was administered intraperitonially to all the animals prior to surgery and continued until the day of sacrifice.

2.3 Permanent middle cerebral artery occlusion (pMCAO)

Our previously optimized protocol for middle cerebral artery occlusion was used (Nada, Tulsulkar et al. 2012, Nada, Tulsulkar et al. 2014). An intracranial approach was used to occlude the middle cerebral artery permanently. Sterile conditions were maintained throughout the surgical procedure by using sterile drapes, surgical gloves and autoclaved equipment. Animals were briefly anesthetized using 5% isoflurane and later stabilized at 1-2% under a surgical microscope. A rectal thermometer was used to monitor the body temperature, and a heating blanket was used to maintain the body temperature at 37^{o} C ± 0.5. An approximately 1 cm vertical skin incision was made between the right eye and ear; using forceps, the temporal muscle was separated, and the underlying skull was exposed. A dental drill was used to drill a 2.0mm hole above the visible MCA region, and the dura was carefully separated. The distal part of the MCA was carefully occluded and severed using a bipolar coagulator. Animals were moved to a temperature-controlled incubator to recover from surgery and later moved to home cages.

2.4 Infarct Volume analyses

Animals from both the treatment group were euthanized seven days after pMCAO using carbon dioxide overdose. Brains tissues were harvested and sliced into five 2mm-thick coronal section and immediately immersed into 1% triphenyltetrazolium chloride (TTC) (SigmaAldrich) dissolved in 1X phosphate buffered saline (PBS) solution for 20 min at 37° C. Oxidoreductase activity in live tissues allows cells to turn reddish pink on incubating with TTC, while leaving the dead tissue unstained or pale in color. This unstained region is considered as the ischemic area and analyzed for quantification purpose. After staining these brain sections they are incubated in 4% formyldehyde for 12h which allows the tissue to get fixed and easier to handle for analysis. Both rostral and caudal surface of the brain sections are photographed and analyzed for infarct areas using image J software. The infarct area was estimated from five slices of each brain, measuring rostral and caudal surfaces of each individual piece in conjunction with the thickness of each slice to obtain the volume and express the percentage of the volume of the contralateral hemisphere. These readings were corrected for the swelling in the ipsilateral hemisphere by using the differences between the volumes of normal tissue in the two hemispheres to calculate the actual infarct.

$$\%I = 100x\left(\frac{Vc-V_i}{V_C}\right)$$

Where %*I*= Percent of the cortex in the ipsilateral hemisphere that is infarcted

V_c= Volume of the normal matter in the cortex of the contralateral (control) hemisphere.

V_i= Volume of the normal matter in the cortex of the ipsilateral (lesion) hemisphere.

This original equation by Swanson et al was meant for infarct calculation within any structure in the brain, and was adapted to measure cortical infarct in our case as the pMCAO produces infarct only in cortex. The percentage infarcts so obtained for the EGb 761/OVX treated group and intact group were expressed as a percentage of the Veh/OVX treated group (Swanson, Morton et al. 1990).

2.5 Grip Strength

Grip strength is evaluated by holding the mice by the tail and placing their forelimbs on a specially designed pull bar assembly (Grip strength meter, Columbus Instruments, OH). The peak amount of force an animal exert is shown on the digital display and is noted. Each animal is tested three times per trial 4h before and 24h and 7 days after pMCAO surgery.

2.6 Neurological deficit scoring (NDS)

NDS is evaluated by a 28-point score pattern optimized by our group.(Nada, Tulsulkar et al. 2012) A person blinded to the treatment groups but expert in the NDS evaluation performed NDS 7 days after pMCAO; the evaluation includes a battery of both sensory

and motor deficits, such as body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each of the seven tests included in the 28-point NDS is graded from 0 to 4, with higher scores indicating severe deficits.

2.7 Western blotting

For western blot analysis, mice were sacrificed 7 days post ischemia using carbon dioxide overdose and brain cortices of ischemic and non-ischemic mice are dissected, weighed, and homogenized. The brain cortex is homogenized to extract cytoplasmic protein using a buffer containing 10mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl and 1mM EDTA. Prior to extraction, freshly prepared 0.5 mM DTT and 0.2mM PMSF along with phosphatase and protease inhibitors are added to the buffer. The cytoplasmic protein was isolated by homogenizing the brain in buffer A and centrifuging at 5000 rpm for 10 mins. The supernatant was collected as the cytoplasmic fraction and the pellet was treated with buffer B. The nuclear fraction was isolated by centrifuging the suspension at 10,000 rpm for 10 mins. Both the fractions were stored at -20 °C until further use. Bradford reagent (Bio-Rad laboratories) is used to determine protein concentration and samples are analyzed by loading equivalent amounts of cytoplasmic proteins (20 μg) on 12% SDS polyacrylamide-gel. Proteins are transferred from the gel to PVDF membrane and blocked by 5% dry nonfat milk for 1 h at room temperature followed by overnight incubation at 4 °C with the following antibodies: mouse anti-actin (1:2000; Sigma); mouse anti-HO1 (1:1000); rabbit anti VEGF(1:1000, Santa Cruz, TX) rabbit anti TNF-α(1:1000, Santa Cruz, TX) anti rabbit Wnt(1:1000, Abcam, MA) , rabbit anti caspase-8

(1:1000, Bioss, MA)and rabbit anti Caspase-3 (1:1000, cell signaling, MA). After washing, membranes are incubated with the secondary antibody, goat anti-mouse or anti-rabbit (1:2000; Jackson ImmunoResearch Laboratories).

The bands were photographed and analyzed using the ChemiDoc molecular imager (Bio-Rad). The densitometric values were normalized with respect to the values of actin immunoreactivity to correct for any loading and transfer differences between samples.

2.8 Immunohistochemistry Procedure

The role of every protein varies depending on their expression or translocation to the cellular sub compartments. We used immunohistochemistry to detect the cellular localization, expression and thereby infer the action of proteins. A series of procedures were used to detect these proteins. A detailed account of each procedure is given below:

2.8.1Perfusion of Mice Brains and their Isolation

A separate group of mice received pre-treatment with EGb 761 (120mg/kg, orally) or vehicle (Polyethylene glycol) was used for this experiment. At the end of the survival period of 7 days, mice were anesthetized using sodium pentobarbital (50-65mg/kg, i.p) and transcardially perfused with physiological saline (0.9% NaCl) followed by 4% buffered paraformaldehyde using a peristaltic pump. The perfusion with saline insured draining of entire blood from the body otherwise interferes with the histochemical staining. Paraformaldehyde fixes the soft brain tissue, preparing it for further processing.

The perfusion was discontinued upon the first sign of rigidity in the tail of the mouse. Brains were then dissected from the skulls and post fixed for 24 h in 4% paraformaldehyde. The isolated brains were stored at -80°C.

2.8.2 Tissue Sectioning

The frozen brains were sectioned using a cryostat machine. About 6-7 µ sections were obtained and positioned onto positively charged slides (Superfrost plus, VWR, Radnor, PA,USA) that absorb thin tissue sections by means of electrostatic attraction. Slides were immediately stored at -20°C to prevent dehydration. These sections were then used for the immunostaining methods.

2.8.3 Immunohistochemistry

Brain sections are incubated in 4% paraformaldehyde for 20 min, washed with 1XPBS buffer, and antigen-retrieved with 10 mM citrate buffer (pH=6.0) using a pressure cooker for 4 min; sections are allowed to cool for 10 min at room temperature (RT). Permeability is performed by incubating the sections with 1% Triton- X100 in 1X PBS buffer for 30 min then washed again with 1X PBS buffer. Denaturation is accomplished with 2N HCl for 30 min at RT, and the slides are neutralized with 0.1M sodium borate for 20 min at RT and washed again. For neurogenesis study the slides are blocked with 3% bovine serum albumin fraction IV (RPI Corp., Mount Prospect, IL) for 1 h at RT, followed by incubation with primary antibodies for: anti-BrdU mouse (Thermo Scientific, West Palm

Beach, FL), chicken anti-Netrin (Thermo Scientific), rabbit-anti UNC5B (Abcam, Cambridge, MA), rabbit anti-DCC (Abcam), and rabbit anti-AR (Cell signaling) using following dilutions: 1:300, 1:300, 1:250, 1:250, and 1:100, respectively. The slides are incubated overnight at 4°C, washed with 1X PBS for 10 min and incubated with fluorescent anti-mouse, anti-rabbit and/or anti-chicken secondary antibody (Jackson Immuno Research, West Grove, PA) at the dilution of 1:500 for 2 h at RT. After another set of washings, the slides are mounted with DAPI (Santa Curz, Dallas, TX) and then sealed with nail polish. For BrdU analysis, 12-15 captures are taken from the side of the injury for each section/four to five brain sections/mice/3mice each group. The sections are cut approximately at the coordinates between -0.94 to -2.92 from bregma. The Netrin-BrdU positive cells are counted and averaged, and the ratio is determined by dividing Netrin-BrdU positive cells by the total DAPI cell counts.

2.9 Assay for caspase-3 activity

Aliquots of cytosolic extracts were mixed with equal volumes of 40 µm Ac-DEVd-AMC in the buffer provided by the company. Free AMC accumulation, which resulted from cleavage of the aspartate-AMC bond, was monitored continuously in each sample over 30 mins in 96-well microtiter plates using ELISA plate reader at 360 nm excitation and 460 emission wavelengths. The emission from each well was plotted against time.

2.10 Transfection and Reporter Assays.

Expression constructs containing AR or empty vector were transiently transfected into COS-7 cells (African green monkey kidney cells lacking an endogenous AR). AR activity was measured by luciferase assay using the AR-responsive minimal reporter $pGRE_2EIB$-Luc (Allgood, Oakley et al. 1993) and pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was achieved using GeneFect (Alkali Scientific, Inc.), according to the manufacturer's protocol. Twenty-four hour post-transfected cells were treated with vehicle or 10 nM R1881 or 0.1, 1.0, 10, or 100 mg/ml EGb 761 for an additional 24 h until harvest. Cell lysates and assay were performed using the Promega dual luciferase assay system. Statistical analyses employed the Student's t-test using GraphPad Prism v6.0f for Mac (GraphPad Software).

2.11 Statistical Analysis

All behavioral parameters and infarct volume analyses among different groups were analyzed by using one-way ANOVA and Newman-Keuls post hoc test. For the WB protein expression analysis and reporter assay, the differences between groups were determined by student's t test. A value of $p< 0.05$ was considered to be significant for all parameters.

3.0 Results

3.1 Effect of EGb 761 pretreatment on OVX female mice following permanent ischemia

Previously, we demonstrated that EGb 761 pretreatment protected male mice from brain injury. Here, we wanted to investigate whether EGb 761 pretreatment also protects OVX female mice from ischemic brain injury. Randomly cycling female mice underwent ovariectomy and were allowed to recover for 7 days. EGb 761 was orally administered 24 h after ovariectomy and daily for 7 days followed by pMCAO at day 8, and mice were sacrificed after 8 days. OVX female mice pretreated with EGb 761 showed lower NDS and improved grip strength on day 8 after pMCAO (Fig.1 A and B). Similarly, infarct volume was significantly reduced in the EGb 761/OVX pretreated group as compared to the Veh/OVX group following pMCAO (Fig. 1C). Together, these results suggest that EGb 761 pretreatment improved stroke recovery in OVX female mice by improving neurobehavioral parameters and reducing stroke lesions. To further understand the molecular mechanism involved in neuroprotection we performed western blot analysis.

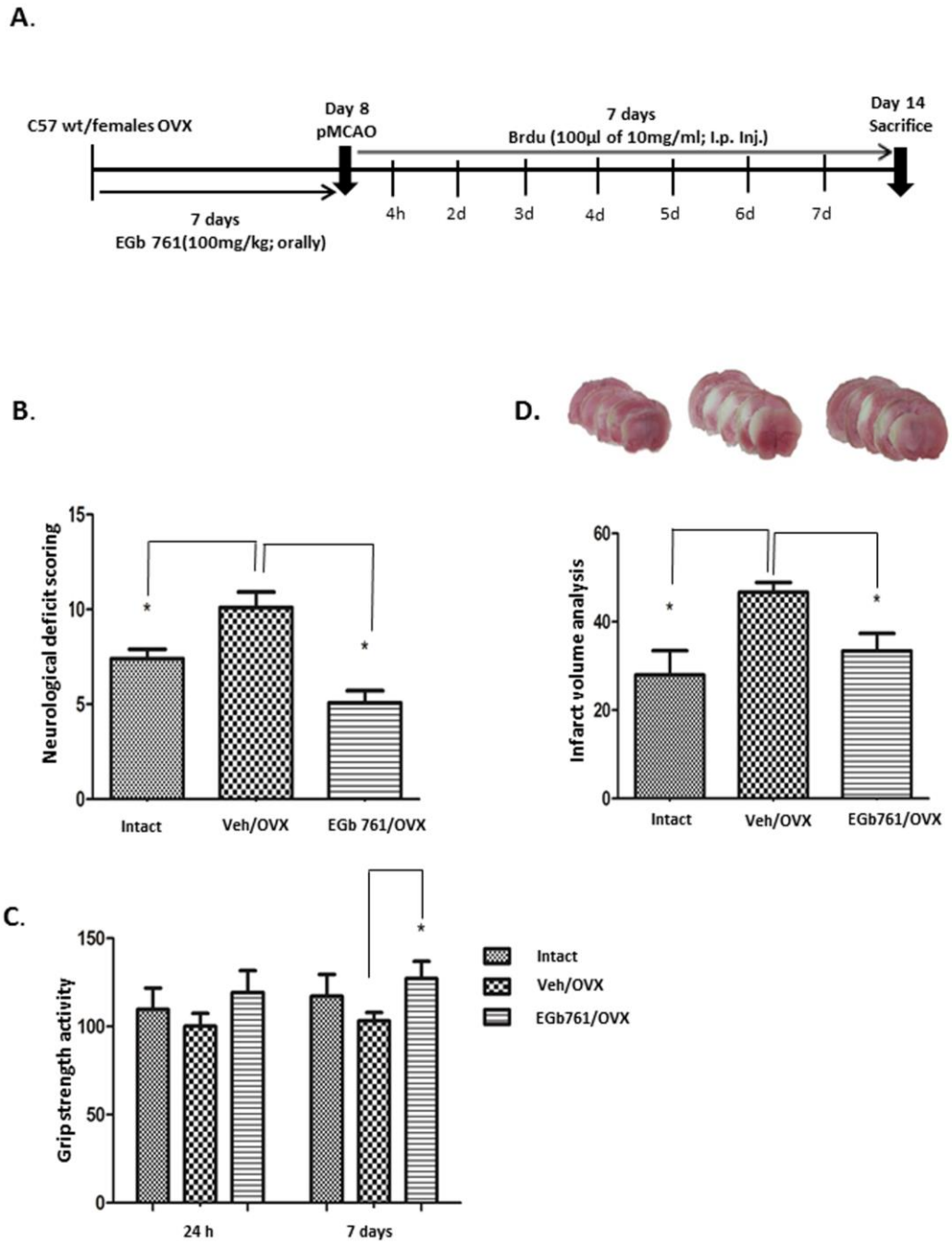


Figure 1: Pretreatment with EGb 761 attenuates brain damage following pMCAO.

A) Schematic diagram showing experimental protocol. Mice were randomly selected and ovariectomized and allowed to recover for 7 days. Female mice were divided into vehicle and treatment groups and administered either vehicle or (EGb 761 for 7 days prior to pMCAO and survived additional 7 days followed by sacrifice on day 8. B) NDS in EGb 761 pretreated OVX mice were significantly lower as compared to Veh/OVX. C) Grip strength showed significant improvement on day 7 in EGb 761/OVX pretreated group as compared to Veh/OVX. D) Representative coronal brain sections show higher infarction in Veh/OVX female mice which was significantly reduced in EGb 761 treated OVX mice. E). Infarct volume was significantly lower in EGb 761 pretreated group as compared to Veh/OVX. Data are expressed as mean ± SEM; * $p<0.05$, vs. vehicle-treated control or Intact n=7; Veh/OVX n=8 and EGb 761/OVX n=8

3.2 Neuroprotection of EGb 761 is independent of the HO1/Wnt pathway in OVX female mice

Several studies on male mice, including ours, showed that EGb 761 mediated neuroprotection is dependent on the HO1/Wnt canonical pathway (Vanella, Sodhi et al. 2013, Nada, Tulsulkar et al. 2014). Here, we examined whether neuroprotective properties of EGb 761 in female mice were attributed to over expression of HO1 and Wnt following permanent ischemia. HO1 and Wnt expression levels were observed to be unchanged in the EGb 761/OVX (n=5) pretreated group when compared to the Veh/OVX group (n=5) after ischemia (Fig.2 A-B). Intact female mice were used as positive control. Together, these results suggest that neuroprotection mediated by HO1/Wnt expression

after EGb 761 treatment is beneficial only in males(Nada, Tulsulkar et al. 2014), and this neuroprotective pathway is ineffective in OVX female mouse brain.

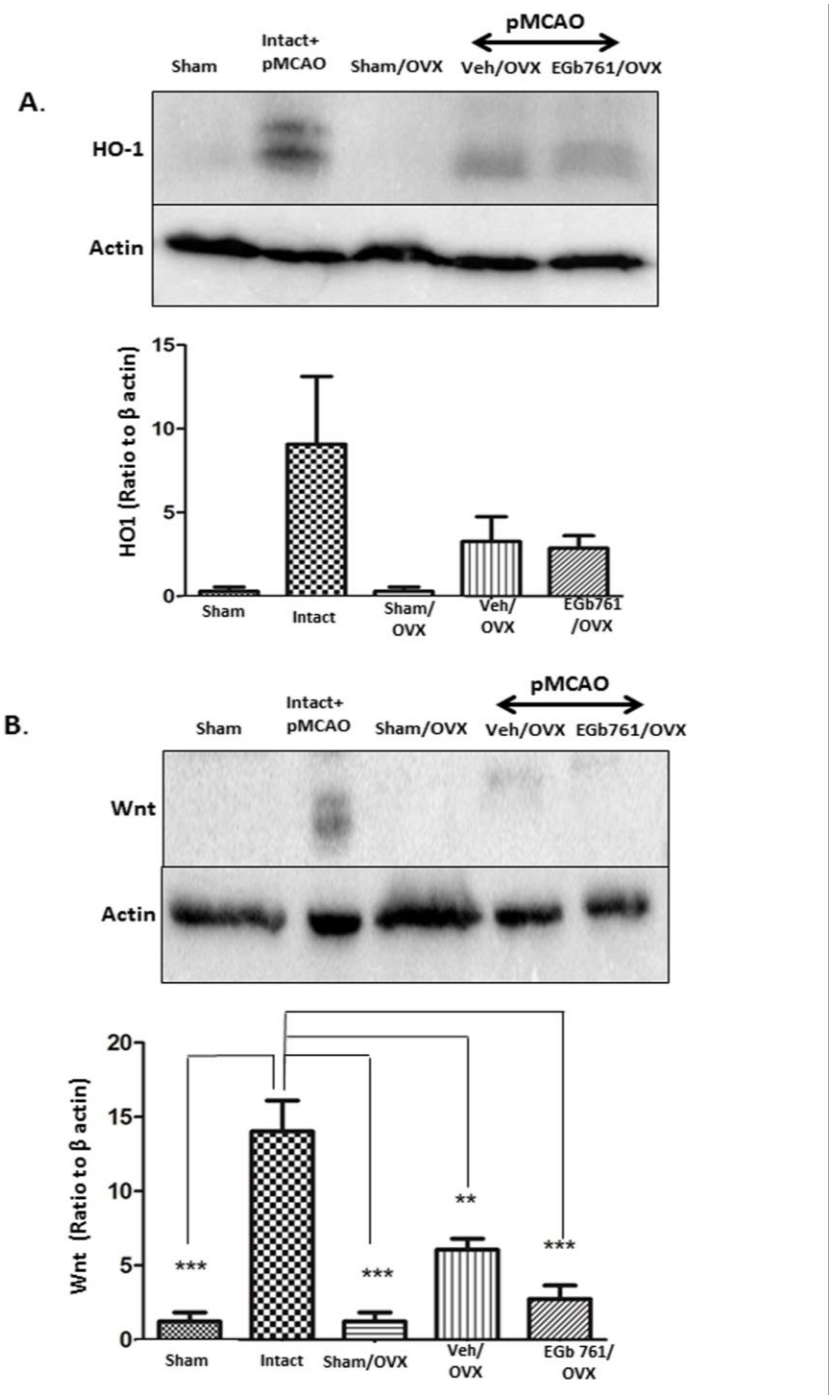


Figure 2: Non-involvement of HO1/Wnt pathway in EGb 761 neuroprotection.

A) Protein expression levels showed that EGb 761 pretreatment did not upregulate HO1 expression after pMCAO as a protective mechanism. Intact female mice were used as positive control. C) Wnt expression also showed similar pattern as that of HO1. (B and D) corresponding graph shows the densitometric analysis normalized to actin. Data are expressed as mean ± SEM; * $p<0.05$, vs. vehicle-treated control; Intact n=5, Veh/OVX n=5 and EGb 761/OVX 761 n=5

3.3 EGb 761 inhibits the extrinsic apoptotic pathway by targeting cleaved caspase-3 and caspase-8 expression, inhibits inflammation and induces angiogenesis in OVX female mice

To assess the apoptotic mechanism in OVX female mice subjected to permanent ischemia, we measured the caspase-3 activity in both Veh/OVX and EGb 761/OVX treated groups and further confirmed the results by western blot analysis. Caspase-3 activity assay showed significantly lower caspase-3 activity in the EGb 761/OVX group (n=3) as compared to the Veh/OVX group (n=3) (Fig. 3A). To confirm our results we performed western blot analysis and expression of cleaved caspase-3 was significantly down-regulated in the EGb 761/OVX treated group as compared to the Veh/OVX group (Fig 3B and C). Our results are in conjunction with the previous studies suggesting a major role of caspase-3 activation in apoptotic cell death in female mice (Liu, Li et al. 2009, Siegel, Li et al. 2011). The expression levels of cytochrome C in the EGb 761/OVX treated group subjected to pMCAO showed no changes (data not shown); however, caspase-8 expression in the EGb 761/OVX group (n=5) was significantly

reduced when compared to the Veh/OVX group (n=5) (Fig 3D and E). To further illustrate the neuroprotective properties of EGb 761 in female mice, we investigated the role of the inflammatory marker, TNF-α, which was observed to have significantly reduced expression in the EGb 761/OVX treated group (n=5) as compared to Veh/OVX group (n=5) (Fig. 4A). In addition, angiogenic markers, VEGF and eNOS were significantly elevated in the EGb 761/OVX treated group (n=5) when compared to the Veh/OVX group (n=5) (Fig 4A, B, C and D). Our results from this study are in parallel to our previous data suggesting angiogenic properties of the EGb 761 in male mice (Tulsulkar and Shah 2013). Together, these results suggest that in female mice, EGb 761 mediates neuroprotection by inhibiting extrinsic apoptotic pathway mediated cell death, reducing TNF-α mediated inflammation and upregulating the angiogenic proteins, VEGF and eNOS.

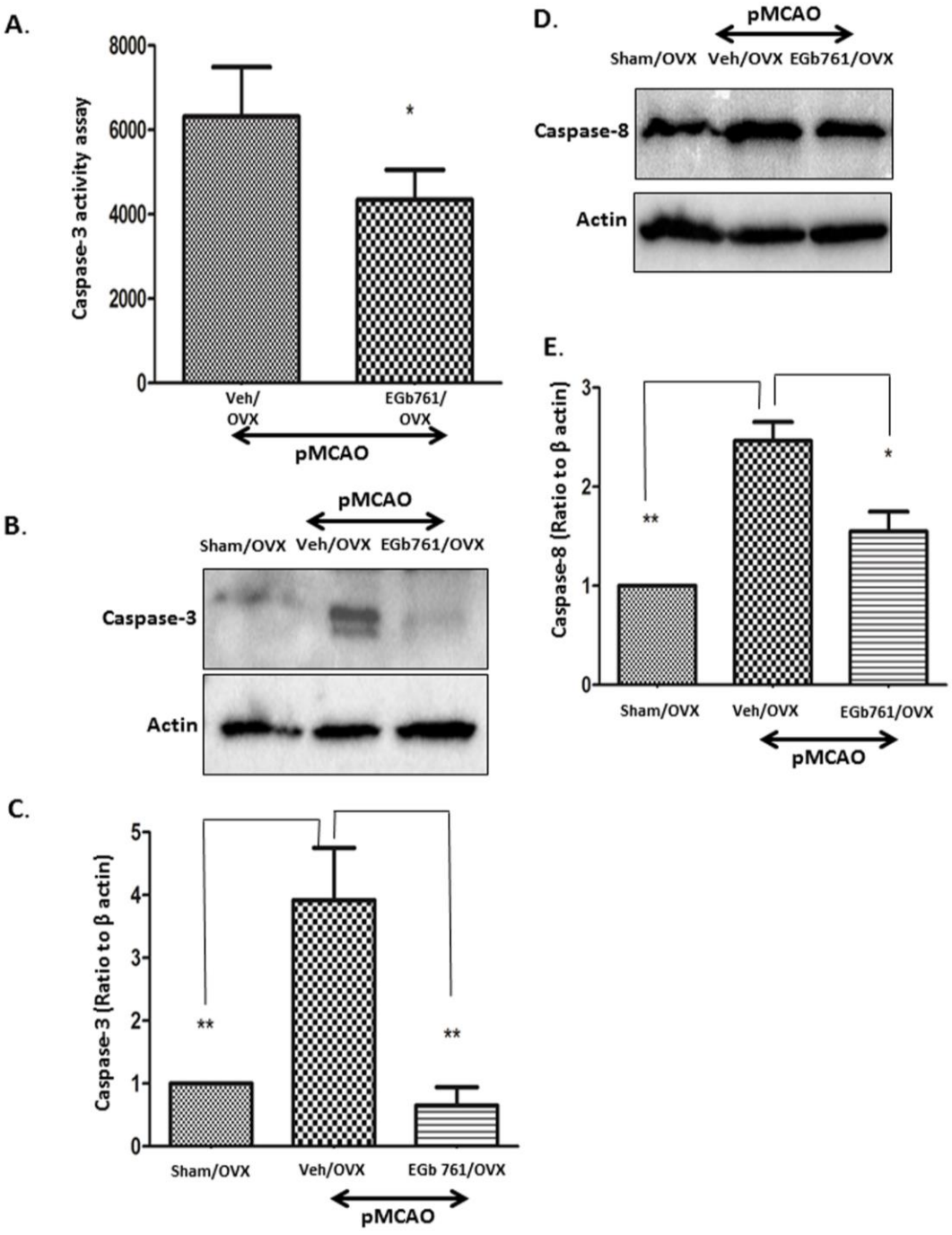


Figure 3: EGb 761 blocks caspase-3 activity assay and expression.

A) Caspase-3 activity assay showed that EGb 761 significantly inhibits caspase-3 activity as compared to OVX vehicles. B) Protein expression analysis showed that OVX animals have higher expression of cleaved caspase-3 and EGb 761 treated OVX group showed significantly reduced levels of caspase-3 as compared to Veh/OVX. D). Significant reduction in expression levels of caspase-8 in EGB 761 treated OVX group as compared to OVX vehicle group following pMCAO C) Corresponding graph shows the densitometric analysis normalized to actin. Data are expressed as mean ± SEM; * $p<0.05$, vs. vehicle-treated control; Veh/OVX n=5 and EGb 761/OVX n=5.

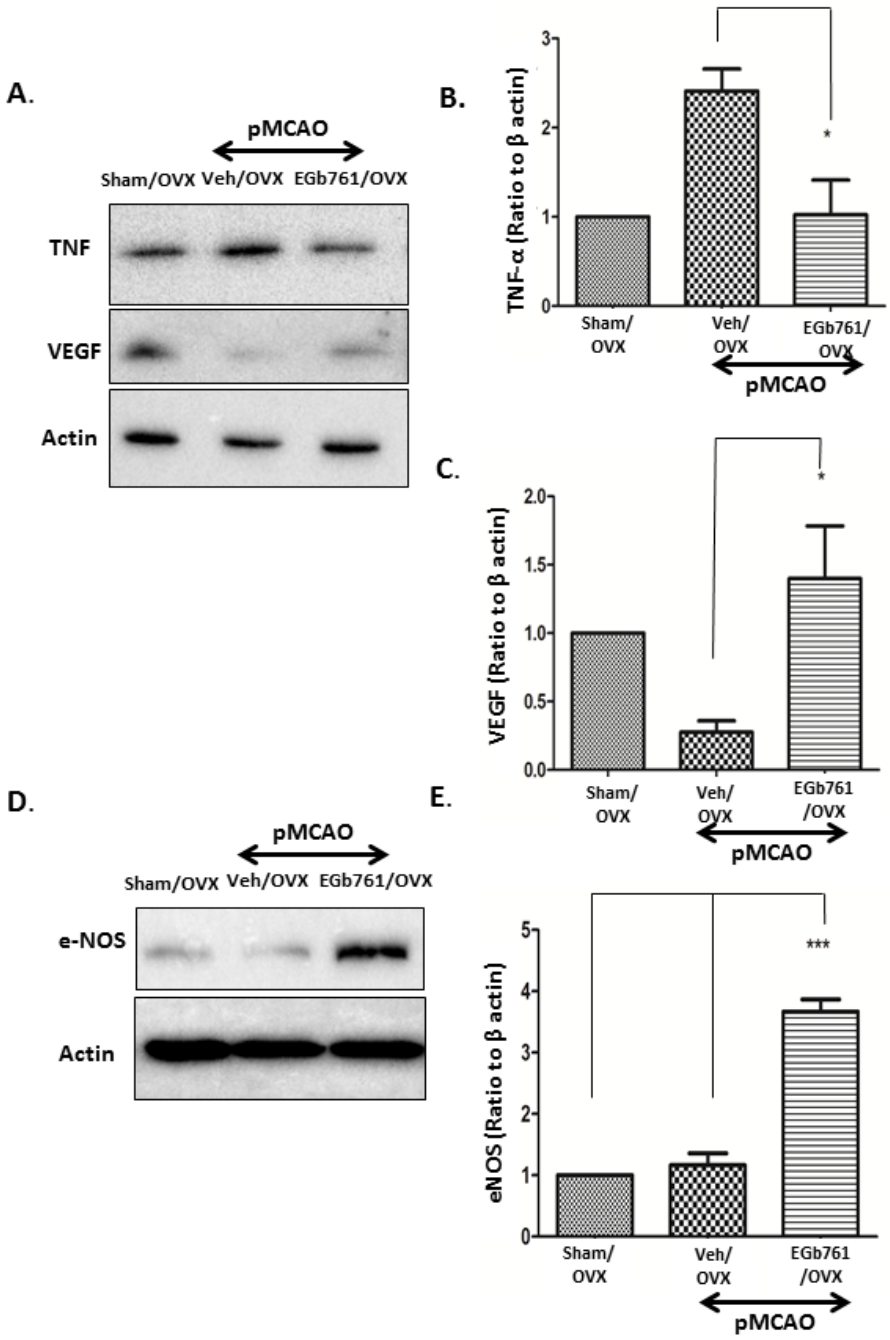


Figure 4: EGb 761 reduces inflammation and induces angiogenesis.

A) EGb 761 pretreatment significantly reduced TNF-α expression as compared to Veh/OVX. On the other hand, expression of VEGF was significantly higher in EGb 761 treated OVX group as compared to that of OVX vehicles. D) eNOS expression was significantly higher in EGb 761 treated OVX group as compared to that of OVX/vehicle group following pMCAO. (B, C and E) Corresponding graph shows the densitometric analysis normalized to actin. Data are expressed as mean ± SEM; * $p<0.05$, vs. vehicle-treated control; Veh/OVX n=5 and EGb 761/OVX n=5.

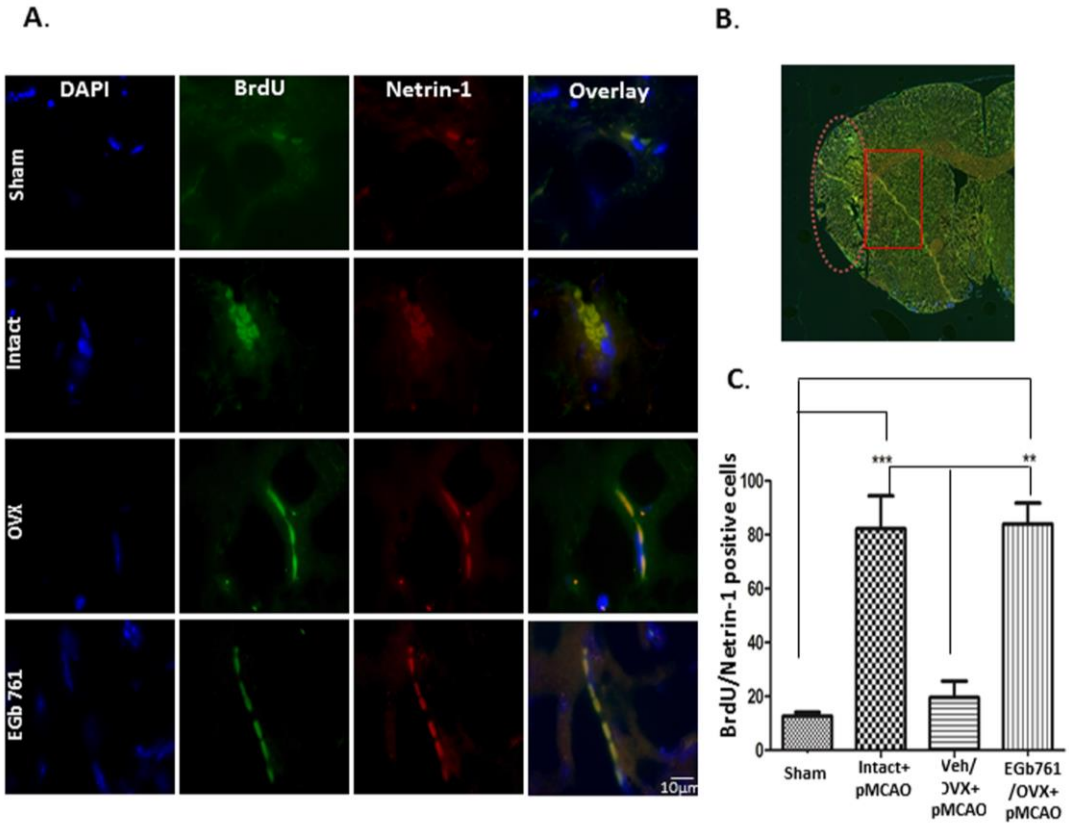
3.4 EGb 761 pretreatment enhances neurogenesis independent of the HO1/Wnt pathway in female mice

Our group previously reported the neurogenesis-enhancing properties of EGb 761 after ischemia in male mice, but this phenomenon has not been elucidated in female mice. The neurogenesis-enhancing properties of estrogen have been widely studied, so female mice with intact ovaries were used as a positive control. Immunohistochemical studies on OVX female mice treated with EGb 761 were subjected to permanent ischemia and sacrificed after 7 days showed similar levels of BrdU/Netrin-1 positive cells to that of intact females. Surgical removal of ovaries showed a markedly reduced number of BrdU/Netrin-1 positive cells Fig. 5A and B). As shown in Fig. 5A, stem cells were distinguished from non-stem cells by using a stem cell marker called netrin-1. Studies from our lab and various others have already shown that ischemia itself upregulates neurogenesis after 24h; however, in this study, we showed higher levels of netrin-1 expression in NSPCs after 7 days of ischemia. NSPCs were counted manually in the

region near the peri infarct area, only cells expressing BrdU and Netrin-1 were counted, and the graph was plotted (Fig. 5B). We also demonstrated that netrin-1 and its receptors, deleted colorectal cancer (DCC) and uncoordinated gene 5B (UNC5B), were expressed only in NSPCs. Expression of DCC and UNC5B was elevated after 7 days of ischemia in the EGb 761/OVX treated group (Fig.6A-B). For all the immunohistochemistry experiments control slide was stained with only secondary antibody was used as a negative control (data not shown). Together, these results suggest that netrin-1 and its receptors, DCC and UNC5B, are required for migration of NSPCs from SVZ to the injury site.

Figure 5: EGb 761 pretreatment enhances neurogenesis.

Mice pretreated with EGb 761 after OVX for 7 days prior to inducing pMCAO and allowed to survive an additional 7 days without drug treatment were sacrificed at day 8. BrdU was injected 4 h after pMCAO and then daily for 7 days (schematic diagram of Fig. 1a). A) Triple immunofluorescence staining with DAPI (blue), BrdU (green), and netrin-1 (red) and overlay of brain sections (6 μm) from female mice.. B) The image represents the area where the netrin+BrdU cells were counted; the image was taken with Cytation 5 immunofluorescence microscope from Biotek, the ischemic region is in the oval shaped (dotted) area whereas as the NSPC's are counted from the rectangular area. C) Number of netrin+BrdU positive cells where counted near the peri infarct area and the graph was plotted. Data are expressed as mean±SEM; $p < 0.05$ vs. sham; ***$p < 0.05$ vs. Intact, $p < 0.05$ vs. Veh/OVX; **$p < 0.05$ vs. EGb 761/OVX (n =2–3 each group). The pictures were taken with a Nikon Eclipse Ti microscope using × 100 objectives


A.
DAPI
BrdU
Netrin-1
Overlay
Sham
Intact
OVX
EGb 761
10μm
B.
C.
BrdU/Netrin-1 positive cells
***
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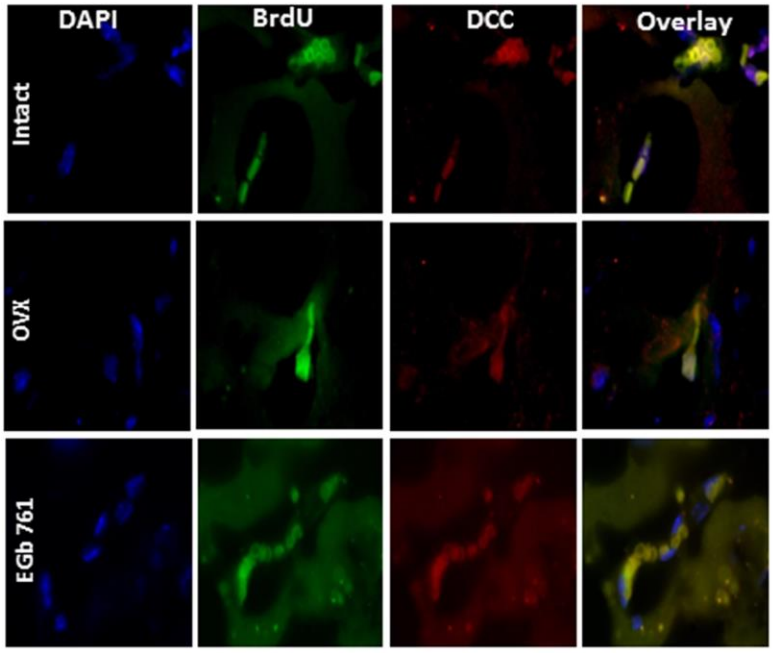
100
80
60
40
20
0
Sham
Intact+ pMCAO
Veh/ OVX+ pMCAO
EGb761 /OVX+ pMCAO

A.



B.

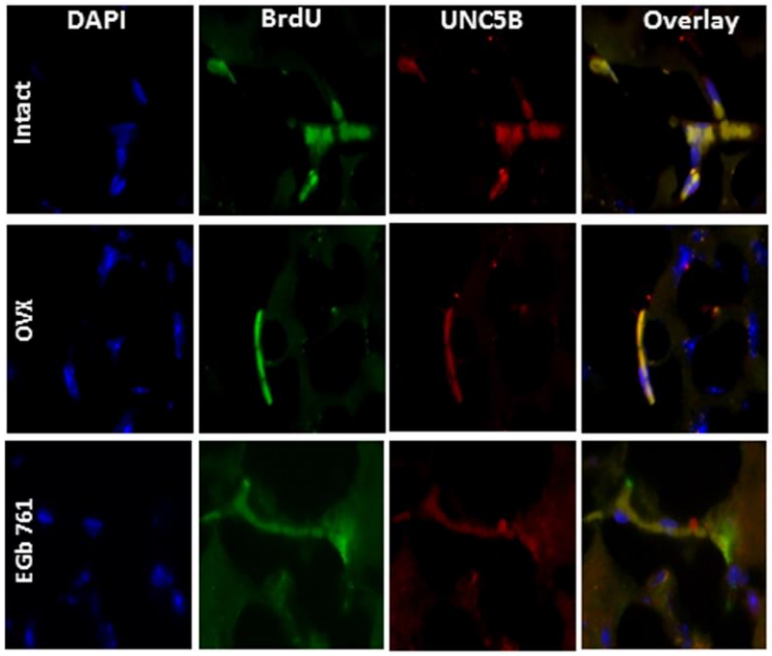


Figure 6: Expression of netrin-1 and its receptors, UNC5B and DCC, in NSPCs.

A) Triple immunofluorescence staining with DAPI (blue), BrdU (green), and DCC (red) (B) staining of UNC5B (red) and overlay of brain sections (6 μm) of intact, Veh/OVX and EGb 761/OVX mice sacrificed at day 8 after pMCAO. Netrin-1 receptors, DCC and UNC5B, were only observed to be expressed in the NSPCs. The pictures were taken with Nikon Eclipse Ti microscope using × 100 objectives.

3.5 EGb 761 induces neuroprotection via androgen receptors in ovariectomized female mice

Androgens function by binding and activating the AR, and are typically considered male hormones (Dart, Waxman et al. 2013). Females, however, secrete androgens from adrenal, ovarian and adipose tissues, with circulating levels much lower than those of males. On the other hand, brain androgen activity is essential for the initiation of sexual activity, libido and mating behavior in both male and females (Ogawa, Chan et al. 1999, Dart, Waxman et al. 2013). Since the removal of the female hormone, estrogen by excising the ovaries combined with pretreatment with EGb 761 protected mice from ischemic brain injury, here we investigated whether the neuroprotective mechanism of EGb 761 is through activation of AR in the brains of these mice. Cos7 cells, which do not express AR were transfected) with an AR vector plasmid that constitutively expresses the receptor and were further co-transfected with a minimal luciferase response element, ARE-luc, and a pRL-CMV plasmid for renilla luciferase as a control for transfection efficiency (Figure 7A). As a positive control, we treated Cos7 cells with androgen activator, R1881, which did not activate the ARE-luc in the vector with no AR present.

With AR expressed, the R1881 significantly ($p<0.0001$) increased the ARE-luc, 4.75-fold. AR activity increased 3.17-fold on treatment with 1 mg/ml ($p<0.001$) of EGb 761 and further showed a dose-dependent increase. Immunohistochemical studies also showed activation of AR in EGb 761 treated OVX female mice after ischemia (Fig 7B).

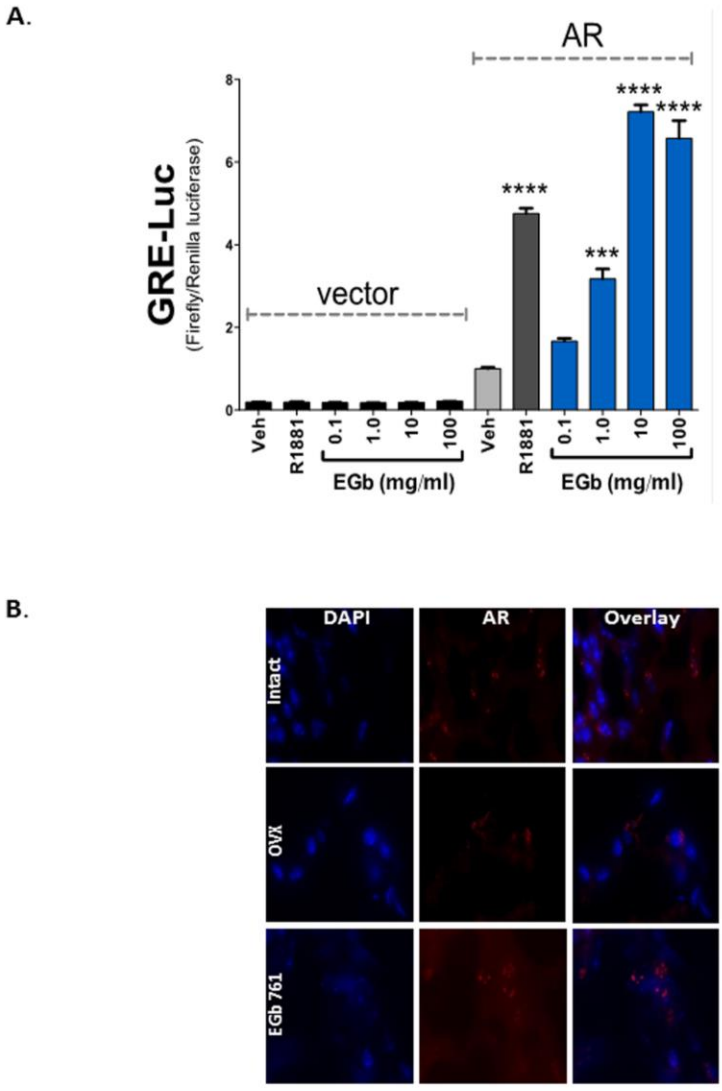


Figure 7: EGb 761 treatment increased AR expression level in OVX female brain after ischemia.

A) A dose dependent increase of EGb 761 showed that at 1.0 mg/ml significantly ($p<0.001$) increased AR activity (3.17-fold). The highest increase in AR activity with EGb 761 treatment was with 10 mg/ml (7.21-fold) and 100 mg/ml (6.57-fold). B) Immunohistochemical studies showed that DAPI (blue) and AR receptors (red) were expressed highly in the EGB 761 treated group as compared to the Veh/OVX group.

4.0 Discussion

In this study, we report important and novel findings: EGb 761 pretreatment reduced infarct volume in OVX female mice and improved functional recovery by increasing grip strength, and reduced neurological deficits after permanent ischemia. The surgical removal of ovaries reduced the expression of HO1 and Wnt, and the EGb 761/OVX treated mice did not show any upregulation of this pathway, this suggests that the neuroprotective mechanism is independent of the HO1/Wnt pathway. Furthermore, EGb 761 treatment inhibited apoptotic cell death pathway by preventing the cleavage and activation of caspase-3 and downregulating caspase-8 and TNFα expression and increased VEGF and endothelial NOS production. The pretreatment with EGb 761 enhanced proliferation, differentiation of neural stem precursor cells (NSPCs).It was also observed that EGb 761 treatment was able to bind to and activate the AR. Together these results suggest that EGb 761 neuroprotection is independent of HO1/Wnt expression, and sexual differences may exist in HO1/Wnt mediated neuroprotection and the mechanism of neuroprotection is attributed to inhibition of caspase dependent apoptotic cell death pathway and over expression of angiogenic proteins possibly resulting in new blood vessel formation and activation of AR.

Stroke is considered to be one of the most devastating diseases in the United States, leading to death or permanent disability. Epidemiological studies in females showed that estrogen is considered to be protective against cardiovascular disease, and incidence of coronary heart disease is lower among women as compared to men prior to the menopause; however, these differences are abolished after menopause (Posa, Kupai et al.

2013). A number of studies have reported beneficial effects of estrogen in various neurological disorders including stroke, but recent clinical studies have shown that estrogen replacement after menopause does not enhance cognitive behavior and has serious side-effects, including risks of breast and uterine cancer (McCullough and Hurn 2003, McCullough, Zeng et al. 2005, Genazzani, Pluchino et al. 2007, Takuma, Hoshina et al. 2007). Moreover, studies also report the negative indication of hormone replacement therapy in prevention of stroke due to increased occurrence of death in the estrogen-treated group as compared to placebo (Billeci, Paciaroni et al. 2008). The role of EGb 761 has been widely studied in male rodents and to our knowledge, only one study has investigated OVX female animals and its protective mechanism in chronic stress. EGb 761 treatment attenuated cognitive impairments and prevented CA3 hippocampal neuronal loss in OVX/stress subjected female rats, as was observed with estrogen replacement (Walesiuk, Trofimiuk et al. 2005, Walesiuk, Trofimiuk et al. 2006). EGb 761 is an all-purpose antioxidant that has been shown to provide protection against cardiovascular and neurological disorders (Chandrasekaran, Mehrabian et al. 2003, Nada and Shah 2012). A number of groups, including ours, have previously shown that EGb 761 attenuates brain damage in male rodents after permanent, transient and global models of ischemia (Sung, Shah et al. 2012, Tulsulkar and Shah 2013, Nada, Tulsulkar et al. 2014). The neuroprotective properties of EGb 761 are mainly attributed to HO1 induction and we have shown the synergistic effect of EGb 761 in attenuating brain damage by upregulating the HO1/Wnt pathway and inducing neurogenesis in male mice (Tulsulkar and Shah 2013, Nada, Tulsulkar et al. 2014). The mechanism of ischemic protection against ischemic injury by EGb 761 in females has not yet been determined. Therefore,

we tested whether pretreatment with EGb 761 attenuates brain injury in OVX mice, and whether the mechanism of action is mediated through the HO1/Wnt pathway and enhanced neurogenesis.

Our previous studies have shown the importance of HO1 in neuroprotection during ischemia and that deleting the HO1 exacerbated ischemic damage. Ischemic preconditioning (IPC) induces neuroprotection in transient and permanent ischemia, and this mechanism is attributed to HO1 upregulation, whereas the IPC-induced neuroprotection was completely abolished in $HO1^{-/-}$ mice subjected to either of the ischemic models. (Zeynalov, Shah et al. 2009) Previous studies on male mice and estrogen replacement studies in females as well as in males have shown that the Wnt canonical pathway plays a crucial role in central nervous system development. It was shown that the estrogen replacement therapy regulates diverse processes including proliferation, differentiation and migration (Zheng, Ying et al. 2010, Valvezan and Klein 2012); and plays a role in differentiation of NSPCs to neurons (Kuwabara, Hsieh et al. 2009). Recent studies from our lab and by Vanella et al. showed that HO1 acts upstream of the canonical Wnt signaling cascade (Vanella, Sodhi et al. 2013). We also observed that HO1 and Wnt expression are down-regulated after ovariectomy. Surprisingly, EGb 761 treatment showed no upregulation in HO1 expression in OVX females, as was observed previously in males. These results suggest that the neuroprotective mechanism of EGb 761 is sexually dimorphic and that the HO1/Wnt canonical pathway is beneficial in males but not in female mice. While a great part of the observed gender differences in vascular reactivity has been attributed to genomic modulation by sex hormones, nongenomic effects of those hormones also exist (Posa, Kupai et al. 2013). Choudhary et

al. (Choudhry, Bland et al. 2007) showed that estrogen administration upregulates HO activity during traumatic brain injury and hemorrhage. However, Wang et al. (Wang and Dore 2007) showed that upregulating HO1 contributes to increased hemorrhagic damage. Posa et al. showed that inhibiting HO1 increases contraction in the aorta in females and males, but at the basal level, female rats with intact ovaries had lower blood pressure and lower aortic contraction as compared to male rats. Several possible reasons can be postulated for the difference between Posa et al.'s study and our current study, such as type of disease, organ system and non-hormonal treatment (Posa, Kupai et al. 2013). In fact, very little is known about the HO activity in the female brain after ischemia.

The involvement of inflammation has been widely documented in the exaggeration of brain injury (Arvin, Neville et al. 1996, Jin, Yang et al. 2010). One of the known inflammatory cytokines expressed during cerebral ischemia is TNF-α, however the function of TNF-α is elusive in brain ischemia. At the pathophysiological level, TNF-α is shown to be involved in not only necrosis but also in the regulation of caspases and other apoptotic factors followed by disruption of the blood brain barrier. Our data suggest that EGb 761 treatment in OVX females significantly attenuates TNF-α expression as compared to OVX female mice, and the caspase-3 activity showed significant reduction in EGb 761 treated OVX females as compared to Veh/OVX treated females. To further determine caspase activation, we began by assessing cytochrome C expression for the intrinsic apoptotic pathway and caspase-8 expression for the extrinsic apoptotic pathway. We did not observe any changes in cytochrome c expression (data not shown); however, caspase-8 expression was significantly lower in the EGb 761/OVX treated group as compared to the Veh/OVX group. In addition, EGb 761 treatment in the OVX female

mice significantly upregulated VEGF and eNOS expression, signifying an increase in angiogenesis. Together, these results suggest that EGb 761 promotes cell survival after ischemic injury by preventing activation of the extrinsic apoptotic pathway of cell death, reducing inflammation via TNF-α inhibition, and augmenting new blood vessel formation via expression of VEGF and eNOS.

To address the recovery and repair properties of EGb 761, we further investigated its neurogenesis-enhancing properties that were previously observed in male mice. It is well established that during ischemic injury, neuroblasts migrate towards the damaged site. Axon attraction and repulsion are mediated by netrin-1 and its receptors, DCC and UNC5B, which are expressed highly during embryonic development as well as in adulthood, albeit at lower levels (Manitt, Thompson et al. 2004, Bradford, Cole et al. 2009). Studies suggest that netrin-1 (Masuda, Watanabe et al. 2008) and its overexpression result in angiogenesis, thus inducing neovascularization and vessel remodeling after stroke (Lu, Wang et al. 2012). Our previous study illustrated that ischemia itself acts as an inducer of neurogenesis (Nada, Tulsulkar et al. 2014). In the present study, we demonstrate that netrin-1 and its receptors, DCC and UNC5B, are only expressed in NSPCs in all treatment groups, which suggests the role of netrin-1 in the migration of NSPCs towards the injury site. The number of netrin-1/NSPCs positive cells was higher in animals with intact ovaries, suggesting the role of estrogen in neurogenesis, whereas Veh/OVX showed lower numbers. However, EGb 761 treatment enhanced netrin-1/NSPCs positive cells, signifying enhanced endogenous neurogenesis after permanent ischemia. Taken together, our findings suggest that EGb 761 enhances

neurogenesis during stroke in female mice, independent of the ovarian hormone, estrogen.

At the cellular and molecular levels, gonadal steroids have a profound effect on target neurons, including the sexual development in neural structure and organization (Phoenix, Goy et al. 1959). Gonadal steroids have been shown to exert a neuroprotective effect by rescuing neurons from cell death in cases of various neurological insults (Bialek, Zaremba et al. 2004, Pike, Nguyen et al. 2008). Until the late 1990s, estrogen was thought to be neuroprotective in the central nervous system; (Garcia-Segura, Azcoitia et al. 2001) however, recent studies have shown that cerebellar granule neurons can be protected from oxidative stress by androgens (Ahlbom, Grandison et al. 1999, Ahlbom, Prins et al. 2001). Several laboratories have described nerve regeneration activity of androgens in motor neuron injury, where androgen treatment showed enhanced axon regeneration in both male and female rats (Yu 1982, Yu 1982, Yu and Yu 1983). In cerebral ischemia, androgen receptor over-expression in AR-Tg male mice showed neuroprotection following MCAO and reperfusion (Ayala, Uchida et al. 2011). The role of androgen receptor activation is still poorly understood in OVX female mice. Our in vitro studies using a vector plasmid expressing the androgen receptor showed that EGb 761 dose-dependently increased AR activation, and immunohistochemical studies on brain sections also suggest increased expression of the AR in OVX females mice treated with EGb 761.

Conclusion

In summary, our results suggest that EGb 761 pretreatment prevents cell death and protects the brain from further damage, specifically in the cortex region, which further

aids in improvement of neurological and grip strength deficits induced by the combination of estrogen loss and permanent brain ischemia. The molecular studies on OVX female mice treated with EGb 761 show its beneficial neuroprotective actions are independent of HO1/Wnt expression. Furthermore, EGb 761 prevented cellular apoptosis by inhibiting the extrinsic pathway of apoptosis and augmenting the angiogenic response. EGb 761 pretreatment in OVX females showed increased neurogenesis and AR activation in the brain, in the absence of ovarian estrogen. We propose that EGb 761 enhances the neuroprotective actions in an ER-independent manner.

Chapter 2

HO1 and Wnt expression is independently regulated in female mice brains following permanent ischemic brain injury

5.0 Introduction

Heme oxygenase (HO) is a microsomal enzyme that includes inducible heme oxygenase-1 (HO1), and constitutive HO2 and HO3 (Li, Song et al. 2014). The function of HO1 or HSP32, is controlled by number of stimuli such as the presence of heme, heavy metals, hormones, oxidative stress (Platt and Nath 1998) and traumatic brain injury (Okubo, Xi et al. 2013). First reports on HO enzymes were established by Tenhunen and co-workers in liver microsomes (Tenhunen, Marver et al. 1968). Heme oxygenase catalyzes the first and rate-limiting step in the oxidative degradation of heme (Fe-protoporphyrin- IX) to carbon monoxide (CO), ferrous ion (Fe^{+2}), and biliverdin-IX (Stocker and Perrella 2006). HO is not a heme protein but uses heme as both its active center and substrate. CO

activates cGMP to promote vasodilation and also acts as a potent anti-inflammatory agent whereas biliverdin is converted to bilirubin by bilirubin reductase (Platt and Nath 1998, Li, Song et al. 2014), which serves as an antioxidant and contributes to protective role of HO1 in cerebral ischemia (Sharp, Zhan et al. 2013). Apart from its role in heme catabolism, HO1 plays important role in various pathological states associated with cellular stress, and possess anti-atherogenic properties (Platt and Nath 1998).

Stroke is one of the leading causes of death and permanent disability across the United States. Neurological dysfunction caused by transient ischemic attack (TIA) is from a focal temporary ischemia which is not associated with the cerebral infarction (Ren, Fan et al. 2013). The modality of stroke is highly dependent on age and sex, thus woman experience a lower stroke risk as compared to men pre menopause (Sudlow and Warlow 1997), but post menopause, the incidence and severity of stroke is higher in woman, predominantly after the age of 80. Therefore it is the aging female population that bears the brunt of reduced post stroke related recovery and institutionalization (Lai, Duncan et al. 2005). Early, epidemiological findings suggested that the ovarian hormone estrogen is responsible for early neuroprotection in females. However, the comparative study of male versus female tissue injury and neurologic deficits at the time of stroke is understudied. A decade of research has shown that stroke is a sexually dimorphic disease and worldwide databases have consistently showed significantly lower incidence of stroke in females. However, stroke kills 16% of post-menopausal females as compared to 8% of males (Bousser 1999) (Sacco, Benjamin et al. 1997). The differences in males and females are largely dependent on reproductive hormones.

Experimental stroke studies in rodents highlight the loss of neuroprotection in females on removal of ovaries which was restored upon exogenous estrogen treatment, indicating the importance of estrogen in neuroprotection (Dubal, Kashon et al. 1998). Tolerance against ischemic brain injury by estrogen is attributed to maintaining cerebral blood flow, anti-inflammatory effect and protecting cells from apoptosis (Alkayed, Goto et al. 2001, Yang, Liu et al. 2005, Park, Cho et al. 2006). Experimental data pertaining to stroke gender differences suggests that cell death in the brain may follow different routes depending on sex steroid exposure. Evidence of sexual dimorphism has also been authenticated by studying the iNOS expression in male and female mice. The reduction in ischemic injury was observed in iNOS null male mice but not in iNOS null female mice. Ovariectomy (OVX) resulted in higher infarct volume with increased iNOS expression; furthermore treatment with iNOS inhibitor, aminoguanidine (AG) reduced the injury in males but not in females suggesting that the reduction and deletion of iNOS contributes to protection only in females (Park, Cho et al. 2006). Cumulative data on mixed neuronal cultures and male animal studies suggests that targeted deletion of nNOS or PARP-1 render male mice resistant to brain injury from focal or global ischemia. When studied in females, ischemic NOS and PARP-1 is observed to be highly sex specific. Targeted gene deletion or pharmacological inhibition of NOS and PARP-1 increases ischemic damage in female mice. Which is in contrast to male mice, where PARP-1 expression post ischemia activates bioenergetic failure, proapoptotic signaling, mitochondrial dysfunction and cell death (McCullough, Zeng et al. 2005). The protective role of HO1 has been previously identified and reported in; mouse stroke models (Dore, Sampei et al. 1999, Dore, Goto et al. 2000, Zhuang, Pin et al. 2002), a rat model of age-related memory

deficits (Dore, Goto et al. 2000), and Alzheimer's Disease (AD) mouse models (Takahashi, Dore et al. 2000).

Recent study has shown that HO1 is a key player in neuroprotection following the transient middle cerebral artery occlusion (t-MCAO) model of ischemia and reperfusion injury (Saleem, Zhuang et al. 2008). It has also been shown that a neuroprotective agent such as Ginkgo biloba binds to the antioxidant responsive element (ARE) within the promotor region of HO1 and activates the unique endogenous pathway of neuroprotection (Saleem, Zhuang et al. 2008). Doré et al showed no differences in the infarct volume between the WT and $HO1^{-/-}$ animals in *t*-MCAO model (1h occlusion, 23 h reperfusion) (Dore, Sampei et al. 1999). However, a studies conducted by Shah et al showed the delayed ischemic effect (7 days post ischemia) of HO1 in the permanent distal middle cerebral artery occlusion (pMCAO) ischemia model through a higher survival rate of animals post ischemia as compared to survival in the transient model (Shah, Nada et al. 2011). Interestingly, the study also highlighted that the protective effect of Ginkgo biloba was abrogated in $HO1^{-/-}$ animals.

To our knowledge, the role of HO1 in the female brain is yet to be elucidated. In this study we hypothesize that the role of HO1 neuroprotection is not beneficial in the female brain after stroke, and the HO1/ Wnt pathways are independently regulated in the female brain after pMCAO.

6.0 Materials and Methods

6.1 Animals and treatment

All animal protocols were approved by the University of Toledo Health Science Campus Institutional Animal Care and Utilization Committee and the guidelines of the National Institutes of Health were followed throughout the study. Female C57BL/6 mice, 8–10 weeks old and 20-25 grams were procured from the in-house facility, and were housed at 22 ± 1°C with a 12 h: 12 h light/dark cycles. All animals were randomized and distributed in different groups.

6.2 HO1 Knockout Mice

Heterozygous HO1 ($HO^{+/-}$) mice, from the colony of Dr. Susumu Tonegawa, MIT, were obtained from Dr. Anupam Agarwal, University of Alabama. $HO1^{-/-}$ mice were obtained by breeding male chimeras with C57BL/6 females. Afterwards, $HO1^{+/-}$ animals were intercrossed to produce $HO1^{-/-}$ mice. Genotypes of mice were determined by PCR analysis of progeny tail DNA as previously described (Chen, Sega et al. 2004). Primers for wild type (forward primer: 5′ GGTGACAGAAGAGGCTAAG 3′ and reverse primer: 5′ CTGTAACTCCACCTCCAAC 3′) and mutant (forward primer: 5′TCTTGACGA GTTCTTCTGAG 3′ and reverse primer: 5′ ACGAAGTGA CGCCATCTGT 3′) generated 450 and 350 BP, respectively, for the wild-type and mutant mice.

6.3 Tin mesoporphyrin (SnMP) treatment

All animals were divided into two groups; control and SnMP treatment. Controls were treated with 0.4M Na_3PO_4 (pH 7.6) alone while SnMP was dissolved in 0.4M Na_3PO_4

(pH 7.6) and 5 mg/Kg of SnMP was injected intraperitoneally, 24 h before surgery and continued for 7 days.

6.4 Ovariectomy

Only one group of mice underwent bilateral ovariectomy (OVX). The animals were anesthetized under 5% isoflurane and then stabilized at 1.5% throughout the surgical procedure (Idris 2012). A single incision of 5.5-10 mm long is made into the muscle wall on both the right and left sides approximately 1/3 of the distance between the spinal cord and the ventral midline. The ovary and the oviduct are exteriorized through the muscle wall. A hemostat is clamped around the uterine vasculature between the oviduct and uterus. Each ovary and part of the oviduct is removed with single cuts through the oviducts near the ovary. The hemostat is removed and the remaining tissue is replaced into the peritoneal cavity. The ovary on the other side is removed in a similar manner. All the animals are allowed to recover for 7 days following OVX and prior to pMCAO.

6.5 Permanent middle cerebral artery occlusion (pMCAO)

A previously optimized protocol for permanent distal middle cerebral artery occlusion was used (Nada, Tulsulkar et al. 2012, Nada, Tulsulkar et al. 2014). Craniotomy was used

to occlude the distal part of the middle cerebral artery permanently. Sterile conditions were maintained throughout the surgical procedure. Animals were briefly anesthetized using 5% isoflurane and later stabilized at 1-2%. Body temperature was observed using rectal thermometer and the body temperature around 37^{o} C ± 0.5, and was maintained using heating blanket. A vertical skin incision of approximately 1 cm was made between right eye and ear, and using surgical forceps under a microscope, the temporal muscle was separated and the underlying skull was exposed. A dental drill was used to drill a hole of about 2.0mm which was above the visible MCA region and dura was carefully separated. The distal part of MCA was carefully occluded and severed using a bipolar coagulator. Animals were moved to a temperature controlled incubator to recover from surgery and later shifted to their home cages.

6.6 Neurological deficit scoring (NDS)

NDS are evaluated by a 28-point score pattern optimized by our group (Nada, Tulsulkar et al. 2012). A person blinded to the treatment plan evaluated NDS 7 days after p-MCAO; the evaluation includes both sensory and motor deficits attributes, such as body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each of the seven tests included in the 28-point NDS are graded from 0 to 4, with higher scores indicating more severe deficits and lower scores indicating recovery.

6.7 HO1 activity Assay

Determination of total HO activity (n=4) in brain tissues was performed as described previously (Vera, Kelsen et al. 2007). Briefly, brain samples were homogenized in radio-immunoprecipitation assay (RIPA) buffer, and 0.5mg of protein was combined in 2mM glucose-6-phosphate, 0.2U of glucose-6-phosphatedehydrogenase, 0.8mM of nicotinamide-adenine dinucleotide phosphate, and 20.0 µM hemin in a total reaction volume of 1200 µl. The samples were incubated at 37 ºC for 1 hour. The bilirubin produced in that time was chloroform extracted, and its concentration was determined by change in optical density at 464nm to 530nm with an extinction coefficient of 40mM/cm. Activity was expressed as picomoles of bilirubin per milligram of protein per hour. The totals of 3 animals were tested from each group.

6.8 Western blotting

For western blots, mice were sacrificed 7 days after ischemia using carbon dioxide overdose and brain cortices of ischemic and non-ischemic mice are dissected, weighed, and homogenized. The brain cortex was homogenized to extract cytoplasmic protein using a buffer containing 10mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl and 1mM EDTA. Prior to extraction, freshly prepared 0.5 mM DTT and 0.2mM PMSF along with phosphatase and protease inhibitors was added to the buffer. The cytoplasmic protein was isolated by homogenizing the brain in buffer A and centrifuging at 5000 rpm for 10 mins. The supernatant was collected as the cytoplasmic fraction and the pellet was treated with buffer B. The nuclear fraction was isolated by centrifuging the suspension at 10,000 rpm for 10 mins. Both the fractions were stored at -20 °C for future use. Bradford

reagent (Bio-Rad laboratories) is used to determine protein concentration and samples are analyzed by loading equivalent amounts of cytoplasmic proteins (20 μg) on 12% SDS polyacrylamide-gel. Proteins are transferred from the gel to PVDF membrane and blocked by 5% dry nonfat milk for 1 h at room temperature followed by overnight incubation at 4 °C with following antibodies: mouse anti-GAPDH (1:2000; Santacruz, TX); and antirabbit Wnt (1:1000, Abcam, MA). The bands were photographed and analyzed using the ChemiDoc molecular imager (Bio-Rad). The densitometric values were normalized with the values of actin immunoreactivity to correct for any loading and transfer differences between samples.

6.9 Statistical Analysis

All behavioral parameters and infarct volumes between different groups were analyzed by one-way ANOVA with Newman–Keuls post hoc test. For the protein analysis, the differences between groups were determined by Student's t test. In all statistical analyses performed, a value of $p < 0.05$ was considered to be significant.

7.0 Results

7.1 SnMP administration significantly abrogated HO1 enzyme activity in the female mice brain

To establish the inhibitory effect of SnMP on HO1 activity in the treated group, HO1 activity in the brain was determined *ex vivo* by monitoring the conversion of bilirubin to tissue lysate exposure. As seen in figure 2, brain HO activity was significantly attenuated in SnMP treated female mice (233 ± 57 vs. 55 ± 31 pg/mg/h; $p < 0.05$), demonstrating an approximately 75% reduction. This data suggests that 7 days of continuous administration of SnMP has a significant inhibitory effect on HO activity *in vivo.*

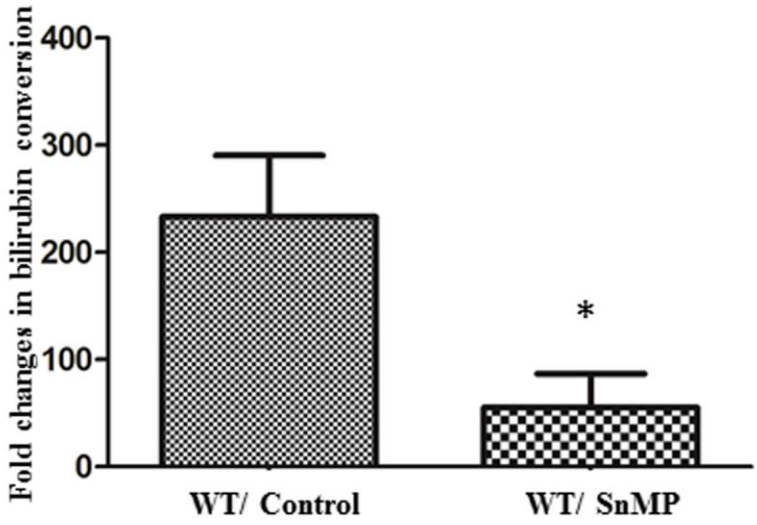


Figure 1: SnMP treatment attenuates HO activity in female mice brain.

Vehicle animals were treated with Na_3PO_4 and the inhibitor group was treated with SnMP (5mg/Kg; i.p) prior to pMCAO and continued for 7 days and sacrificed on day 8. Data are expressed as mean ± SEM; * $p<0.05$, vs. vehicle-treated control n=3; SnMP intact n=3.

7.2 Inhibiting HO1 showed no significant differences in brain damage following pMCAO.

Our lab has previously reported that $HO1^{-/-}$ male mice has increased infarct volume 7 days after pMCAO (Shah, Nada et al. 2011). Nevertheless, the role of HO1 in the female brain after ischemia is yet to be elucidated. Animals were treated with the HO activity inhibitor SnMP 24 hours prior to pMCAO and continued for 7 days and sacrificed on day 8 (Fig 2 A). Intact /$HO1^{-/-}$, WT/intact and WT/OVX animals also underwent pMCAO and were sacrificed on day 8. Neurological deficits were significantly lower in female animals with intact ovaries ($HO1^{-/-}$ and WT) mice as compared to that of WT/OVX group. Our group previously showed that knocking out HO1 in males significantly increases neurological deficits when compared to control animals (Shah, Nada et al. 2011). Surprisingly, $HO1^{-/-}$ female animals with intact ovaries did not show any significant differences in neurological deficits as compared to WT/intact mice. To confirm these results we further used SnMP, an HO activity inhibitor in female WT/intact mice subjected to pMCAO. SnMP treatment for 7 days did not show significant changes in neurological deficits between WT/intact and intact/$HO1^{-/-}$ females, but intact $HO1^{-/-}$ and WT/SnMP treated group showed significant reduction in neurological deficits when compared to WT/OVX animals (Fig 2B). Infarct volume analysis demonstrated that WT/intact, intact/$HO1^{-/-}$ and WT /SnMP group showed no significant differences in infarct volume after 7 days of pMCAO but all groups where significantly lower than the WT/ OVX group (Fig 2C). Together these results suggest that HO1 neuroprotection is beneficial only in males and has no role in neuroprotection in female brain after stroke.

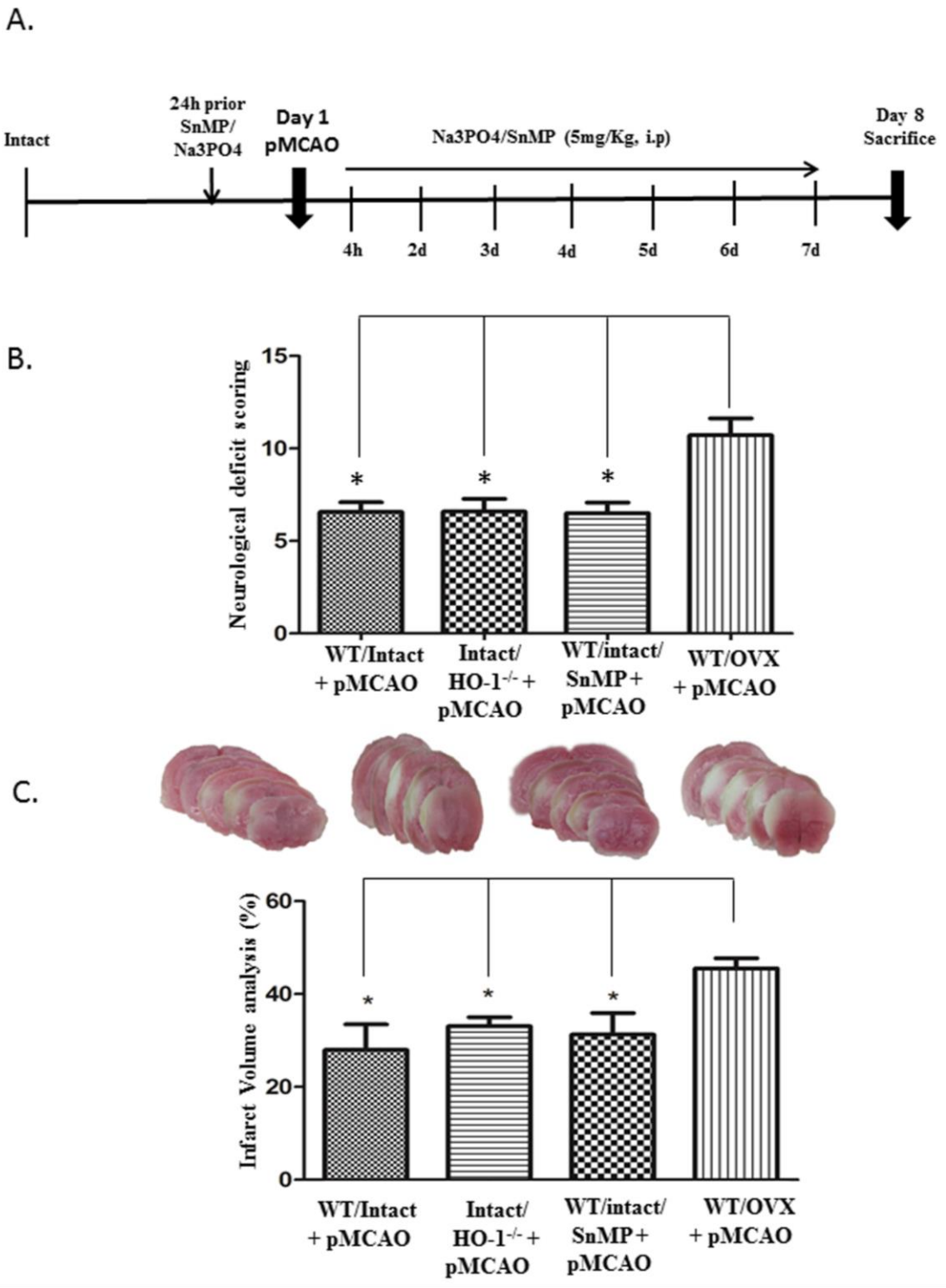


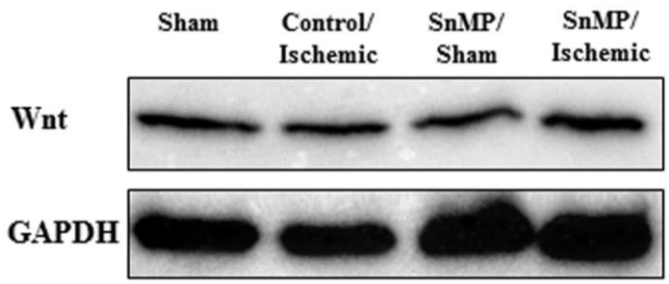
Figure 2: Effect of HO1 inhibition in female brains after 7 days of pMCAO.

A) The schematic representation of the study design for inhibitor treatment. B) Neurological deficit scoring showed no significant differences between the groups except WT/OVX which was significantly higher as compared to other groups after 7 days of ischemia. C) Infarct volume analysis at day 8 showed no differences between intact WT and $HO1^{-/-}$ and WT/SnMP treated group except in WT/OVX group. Data are expressed as mean ± SEM; * $p<0.05$, vs. OVX/pMCAO; Intact n=6; OVX n=7, $HO1^{-/-}$ n= 6 and SnMP n=8.

7.3 HO1/Wnt pathway of neuroprotection is independently regulated in female brains after stroke.

It has been previously shown that HO1 acts upstream of the canonical Wnt signaling cascade and decreases lipogenesis (Vanella, Sodhi et al. 2013). Furthermore, it was showed in the mouse brains that neurogenesis is enhanced after ischemia and it is dependent on the HO1/Wnt canonical pathway (Nada, Tulsulkar et al. 2014). Our protein analysis shows that SnMP treatment does not affect Wnt expression in female brains after 7 days of permanent stroke (Fig 3A). These results suggest that HO1 and Wnt are independently regulated in the female brain.

A.



B.

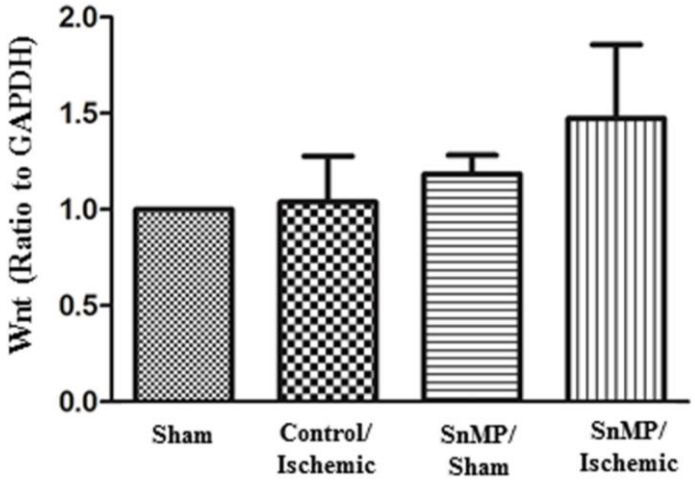


Fig 3: Effect of SnMP treatment on Wnt expression after permanent ischemia.

A) SnMP treatment for 7 days showed no significant differences between vehicle and SnMP treated groups. B) Corresponding graph shows the densitometric analysis normalized to GAPDH. Data are expressed as mean ± SEM; Sham=3, vehicle/ischemic n=3; SnMP sham n=4; and SnMP treated n=4

8.0 Discussion

In the present study we demonstrate the dimorphic role of the HO1/Wnt pathway in the female brain after permanent ischemia, which may correspond to gender related differences during stroke. Treatment with the HO1 inhibitor SnMP significantly attenuated HO1 enzyme activity. Surprisingly, $HO1^{-/-}$ female animals and SnMP treated groups showed no significant differences in neurological deficits when compared to the WT/Intact animals but all of the groups had lower neurological deficits as compared to the OVX group. Furthermore, upon analyzing the brain infarct volume of the intact/$HO1^{-/-}$ and intact/SnMP treatment group there was no significant differences in brain damage as compared to the WT/intact group, but the WT /OVX group showed significantly higher brain damage. Wnt expression also showed no differences between SnMP and WT/intact animals, suggesting the expression of Wnt is independent of HO1 expression in the brain after ischemia. Taken together these results suggest that in female brains the HO1/Wnt pathway is independently regulated during stroke.

Degradation of the pro oxidant free heme by HO result in iron, carbon monoxide (CO) and biliverdin which is further reduced to bilirubin (BR). It has been previously shown that CO possesses a vasodilatory effect, and that biliverdin and BR act as antioxidants. It has also been reported that $HO1^{-/-}$ and WT male mice show no differences in brain infarct volume in the transient MCAO reperfusion model (Dore, Sampei et al. 1999) due to one 1 h occlusion and 23 hr reperfusion. In another study by Shah et al (Shah, Nada et al.

2011) it was demonstrated that after 7 days of permanent ischemia $HO1^{-/-}$ male animals had significantly higher infarct as compared to WT male animals suggesting neuroprotective role of HO1. This HO1 neuroprotection was also evidenced in NMDA induced neurotoxicity model (Saleem, Shah et al. 2010) and by knocking out HO1, which showed higher brain damage as compared to WT mice at 48 hrs of permanent ischemia (Zeynalov, Shah et al. 2009). We recently reported that $HO1^{-/-}$ animals showed lower neurogenesis as compared to WT animals following permanent ischemia (Nada, Tulsulkar et al. 2014). However, HO1 exacerbates stroke damage in hemorrhagic stroke (Wang and Dore 2007). HO1 has been studied in various models and its role in ischemic and remote organ preconditioning (Lai, Chang et al. 2006) has already been established. Cell specificity, sex, origin and nature of damage are possible reasons for the variability in the HO1 function. In a recent study, HO1 has been shown to be protective in a model of myocardial infarction (Posa, Kupai et al. 2013) in the female heart.

Here, in this study we have investigated role of HO1 in the female brain after 7 days of ischemia and to our knowledge this is the first study showing the dimorphic role of HO1 after permanent ischemia. On treatment with SnMP, the conversion of bilirubin was significantly reduced as compared to the vehicle group suggesting inhibition of HO activity. Furthermore, intact/$HO1^{-/-}$ female animals showed no significant differences in brain damage as compared to the WT/intact female after 7 days of permanent ischemia. These results were confirmed by using SnMP, an inhibitor for HO activity, and infarct volume analysis demonstrated no significant differences between WT/Intact and intact/$HO1^{-/-}$ female mice. Taken together our results suggest that HO1 is beneficial only in males and has no role in female neuroprotection following stroke.

Wnt proteins are extrinsic proteins that play an important role in the developed and matured nervous system. The proliferation of neural progenitor cells and differentiation of neurons in the sub ventricular zones (SVZ) is regulated by Wnt (Inestrosa and Arenas 2010). Likewise, the Wnt canonical signaling pathway is an important component of neural progenitor cells to transform into neurons. (Kuwabara, Hsieh et al. 2009). The role of HO1 acting upstream of the Wnt canonical pathway has been previously established by Vanella et al group in adipocytes (Vanella, Sodhi et al. 2013). On the other hand, we demonstrated the connection of the HO1/Wnt canonical pathway in the brain after permanent ischemia (Nada, Tulsulkar et al. 2014), and over expression of the HO1/Wnt pathway enhances neuroprotection. However, of all these studies were performed in either a cell culture environment or in male rodents; the role of the HO1/Wnt canonical pathway in female brain is yet to be elucidated. This is the first study showing role of HO1/Wnt canonical pathway in female brain after permanent occlusion. In this study we have demonstrated that SnMP treatment for 7 days after permanent ischemia showed no significant changes in Wnt expression as compared to the vehicle group. This suggests that HO1 and Wnt are independently regulated in female brains in a neuropathological condition like ischemia. Taken together, the HO1/Wnt canonical pathway is beneficial only in males and has no role in female neuroprotection during ischemia.

Chapter 3

9.0 Discussion

The aim of this study was to investigate the neuroprotective mechanism involved in female brain after ischemia. We focused on neuroprotective properties of EGb 761 modulating the post stroke recovery, as well as neuromorphological and tissue changes in OVX/ischemia subjected female mice. We also demonstrated dimorphic role of the HO1/Wnt canonical pathway in the female brain after 7 days of permanent ischemia. Daily treatment of EGb 761 for 7 days prior to inducing ischemia improved neurological deficits and grip strength in mice after 7 days of stroke. Infarct volume analysis showed significantly reduced brain damage in EGb 761 treated OVX animals as compared to the OVX group. The WT/intact group was used as a positive control to understand the role of endogenous estrogen during ischemia. Our molecular studies showed that EGb 761 neuroprotection is independent of the HO1/Wnt pathway. However, EGb 761 pretreatment enhances neuroprotection by preventing the cleavage of apoptotic protein caspase-3 and caspase-8, thus inhibiting the extrinsic apoptotic pathway, and enhancing neurogenesis and angiogenesis, reducing inflammation and activation of androgen receptor. In chapter 2 we focused on investigating whether HO1 modulates Wnt expression in the female brain. Here we observed that $HO1^{-/-}$ animals showed no

significant changes in neurological deficit as compared to WT/intact group. Similarly infarct volume analysis showed no significant differences between intact/HO1$^{-/-}$ and WT/intact female mice suggesting dimorphic role of HO1 in female brains during stroke. These results were confirmed by using an HO1 inhibitor SnMP inhibitor in which neurological deficits in SnMP treated group showed no significant differences between the intact/ HO1$^{-/-}$ and WT/intact groups. Furthermore, upon analyzing the brain infarct volume, similar results were observed by SnMP treatment which showed no differences in infarct volume when compared to intact/HO1$^{-/-}$ and WT/intact female mice. Together these results suggest that HO1 has beneficial role only in males, and the deletion of HO1 has no deleterious effect in female brains. We then investigated the cross talk between HO1 and Wnt canonical signaling pathway and our western blot data suggested that 7 days of SnMP treatment after permanent ischemia did not affect Wnt expression in the female brain. Together these results suggest that HO1 and Wnt canonical pathways are independently regulated in the female brain and HO1/Wnt pathway has no role in female neuroprotection after ischemia.

Woman typically exhibit protection against vascular disease and atherosclerosis- related ischemic stroke as compared to males due to the presence of the ovarian hormone estrogen in their early life. This advantage is lost during the perimenopausal years emphasizing the role of the female reproductive hormone behind sex differences (Murphy, McCullough et al. 2004). Estrogen replacement therapy has been shown to play a beneficial role in the improvement of cognitive functions in post-menopausal woman (Hogervorst, Williams et al. 2000, Genazzani, Pluchino et al. 2007). However, compliance with the long term estrogen replacement therapy for menopausal women is

poor because of the side effects associated with the treatment (Espeland, Rapp et al. 2004). Thus there is an urgent need to develop alternate, safe and sex specific modalities for the treatment of stroke.

We have previously reported a signaling pathway involving HO1 for EGb 761 neurogenesis enhancing properties in male mice after pMCAO model of ischemia (Nada, Tulsulkar et al. 2014). Our data showed that EGb 761 pre and post treatment significantly reduces stroke damage by reducing cortical infarct volume. This neuroprotection also improved the functional recovery. The $HO1^{-/-}$ male mice demonstrated reduced neurogenesis on day 8 after ischemia, thus suggesting role of HO1signalling in neurogenesis. Furthermore, we also showed that EGb 761 enhanced neurogenesis is mediated by the HO1/Wnt pathway (Nada, Tulsulkar et al. 2014). EGb 761 has been suggested to act directly against the necrosis and apoptosis of neurons, and improve neural plasticity (Maclennan, Darlington et al. 2002). Previously it was suggested that EGb 761 can act as a free radical scavenger and prevent lipid peroxidation at the molecular and cellular levels (Barth, Inselmann et al. 1991). EGb 761 is a platelet activating factor antagonist, and maintains ionic balance in damaged cells (Vogensen, Stromgaard et al. 2003). Several studies have reported the beneficial properties of Ginkgo extract in the models of hypoxia and ischemia in male rodents and gerbils (Lin, Yang et al. 2004), rats (Zhang, Fu et al. 2000), mice (Tulsulkar and Shah 2013) and rabbits (Fan, Wang et al. 2006). Clinical studies conducted across Europe and US have demonstrated that EGb 761 is an effective therapy for cerebral dysfunctions such as dementia, cognitive decline and severe type of senile dementias (Walesiuk and Braszko 2009). Our previous study with a transient global ischemia mouse model demonstrates that EGb 761

predominantly protects the CA-1 hippocampal region neurons from the ischemic insult. However the neuroprotection was evident in EGb 761 as a whole extract but none of its components, BB, GA, GB and TFM, showed neuroprotection. Together, these results suggest that EGb 761 has a synergistic effect. Further immunohistochemical studies suggested that EGb 761 neuroprotection is dependent on activation of the HO1/Nrf2 anti-oxidant pathway (Tulsulkar and Shah 2013). Our studies are in conjunction with other investigators showing that many polyphenolic compounds exert their anti-oxidant properties by binding to ARE's of anti-oxidant proteins, and provide protection against oxidative stress (Shah, Li et al. 2010). All of these studies on EGb 761 and HO1 neuroprotection were investigated on male animals; however role of EGb 761 neuroprotection in the female brain after ischemia had yet to be elucidated. To our knowledge there has been only one study showing the beneficial properties of EGb 761 in the female brain hippocampus in the model of chronic restrain stress (Takuma, Hoshina et al. 2007).

In this current study we focused on neuroprotective properties of EGb 761 in female brains in absence of ovarian hormone estrogen. EGb 761 treatment for 7 days significantly improved the neurological deficit in the OVX group as compared to Veh/OVX and these results were corroborated with the reduction in the infarct volume. These results suggest the beneficial effect of EGb 761 in female brains after ischemia. We previously showed that the beneficial properties of EGb 761, were abrogated in $HO1^{-/-}$ mice suggesting HO1 has a major role in mediating the mechanism of EGb 761 neuroprotection (Saleem, Zhuang et al. 2008). It has also been established that HO1 acts upstream of Wnt signaling during an ischemic insult, and the role of Wnt in

differentiating neural precursor stem cells to matured neurons is well documented (Nada, Tulsulkar et al. 2014). Our protein analysis studies showed that HO1 expression in Veh/OVX group was abolished after ovariectomy and stroke, and surprisingly, EGb 761 pretreatment in OVX group showed no upregulation in HO1 expression. Likewise, a reduced expression of Wnt was observed in the Veh/OVX and EGb 761 treated group after ovariectomy and stroke. Together these results suggest that EGb 761 neuroprotection mediated by HO1/Wnt pathway is not beneficial in female brain after ischemia.

Cerebral ischemia causes extensive cell death mainly due to the activation of caspases, which have been established as events occurring downstream that commit cells to die. Caspase-3 is an individual component involved in activating the cell death pathway independently either by mitochondrial dysfunction or by activating the death receptor ligand (Nagata 1997). The death receptor pathway involves caspase-8, caspase-10 and caspase-2 (Nagata 1997). These apoptotic receptors are the members of tumor necrosis factor (TNF) receptor family which initiates the apoptotic cascade by triggering the adaptor proteins (Chinnaiyan, O'Rourke et al. 1995). Activated and self-cleaved caspase-8 drives the activation of caspase-3 during ischemic insult (Ashkenazi and Dixit 1998). In this study we demonstrated that EGb 761 pretreatment in OVX mice significantly reduced the cleaved caspase-3 activity as compared to the Veh/OVX group, and furthermore, protein analysis showed significant reduction in caspase-3 cleavage and activation in the EGb 761/OVX group as compared to the Veh/OVX group. To further understand the intrinsic and extrinsic pathway of apoptosis we looked into cytochrome C expression, a component involved in intrinsic pathway of apoptosis, which was observed

to be unchanged (data not shown) in EGb761/OVX and Veh/OVX groups. However, the expression of caspase-8, a pro apoptotic protein in extrinsic pathway EGb761/OVX group was observed to be significantly down-regulated in the EGb 761/OVX group as compared to the Veh/OVX group. To confirm that EGb 761 inhibits the extrinsic pathway of apoptosis, we investigated the role of TNF-α and observed that TNF- α expression was significantly abrogated in the EGb761/OVX group as compared to the Veh/OVX group. It has been previously shown that neuroprotection by EGb 761 is attributed to improvement in cerebral blood flow in rats (Lee, Chen et al. 2002) and mice (Saleem, Zhuang et al. 2008) possibly due to angiogenesis. Here we wanted to study the role of angiogenesis in female brain after ischemia. Our protein expression data showed significant upregulation in an angiogenic protein vascular endothelial growth factor (VEGF) and endothelial nitric oxide (eNOS) in the EGb761/OVX group as compared to Veh/OVX animals, suggesting increased angiogenesis which results in increase in cerebral the blood flow.

Many studies have shown that CNS insults such as seizures, oxidative damage, lesions, traumatic injury, and both global and focal ischemia increase progenitor cell proliferation in the adult brain (Parent, Yu et al. 1997, Ernst and Christie 2006, Wiltrout, Lang et al. 2007). Previous studies have also demonstrated that these newly formed cells migrate to the damaged areas of the brain, particularly after cerebral ischemia (Hicks, Hewlett et al. 2007). In the model of transient ischemia progenitor proliferation was significantly enhanced in SVZ and DG of rats and mice following MCAO (Dempsey, Sailor et al. 2003, Hicks, Hewlett et al. 2007). We previously showed that EGb 761 pre and post treatment for 7 days before and after ischemia significantly increased the number of

neural precursor cells and were migrating towards the ischemic lesion (Nada, Tulsulkar et al. 2014). Here we demonstrated that 7 day pretreatment with EGb 761 enhanced neurogenesis in absence of estrogen in the female brain after pMCAO, and the majority of cells were encircling near the ischemic area and the surrounding border. To further understand the role of neuroprotection, we investigated the expression of androgen receptor (AR). It has been previously shown that androgen expression can improve nerve regeneration activity in motor neuron injury by enhancing axonal regeneration in both male and female rats (Yu 1982, Yu 1982, Yu and Yu 1983). In a recent study on cerebral ischemia it was demonstrated that androgen receptor over expression in AR-Tg male mice improves neuroprotection following MCAO and reperfusion (Ayala, Uchida et al. 2011). However, the role of androgen receptor activation is still poorly understood in OVX female mice. Our *in vitro* studies showed that EGb 761 enhances androgen receptor activation and these results were further confirmed using immunohistochemistry on brain sections obtained from EGb 761/OVX and OVX/Veh groups. Histochemical analysis showed androgen receptor activation in EGb761/OVX animals after 7 days of ischemia as compared to OVX/Veh group suggesting that EGb 761 mediates neuroprotection via androgen receptor activation.

Chapter 5

10.0 Conclusion and Future Direction

The results of this study demonstrate the neuroprotective and reparatory potential of EGb 761 in OVX female mice in the model of ischemic stroke. Pretreatment was effective in improving the functional outcomes and infarct volume. We investigated disease relevant markers to identify targets responsible for EGb761 action. Surprisingly we found that EGb 761 protection is independent of HO1/Wnt mediated pathway in OVX female mice. We also showed that neuroprotective function of HO1 is sexually dimorphic in female brain. Furthermore, we found that EGb 761 treatment inhibits extrinsic pathway of

apoptosis by preventing caspase-3, suggesting predominance of caspase dependent apoptosis in female brain following ischemia. We also found that EGb 761 enhances new blood vessel formation by upregulation of VEGF and eNOS resulting in enhanced cerebral blood flow in the obscured area of the brain. Likewise in males, EGb 761 also enhanced neurogenesis in female brains after 7 days of stroke. However, we were not able to associate the Wnt pathway of neurogenesis. The exact mechanism of neurogenesis is still inconclusive and it is hoped that these results will refine future target elucidation effort for this extract. This study revealed many important findings pertaining to sexual differences and EGb 761 therapeutic potential. Nevertheless, it has some limitations. Different constituents of EGb 761 haven't been tested and keeping sexual differences in mind it would make for an interesting future study. Lastly role of neurogenesis in $HO1^{-/-}$ female mice needs to be thoroughly explored.

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