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Heme oxygenase 1 mediated neurogenesis is enhanced by Ginkgo biloba (EGb 761®) after permanent ischemic stroke in mice

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Abstract

Stroke is the fourth leading cause of death and a major cause of disability in stroke survivors. Studies have underlined the importance of repair mechanisms in the recovery phase of stroke. Neurogenesis in response to brain injury is one of the regeneration processes that, if enhanced, may offer better stroke treatment alternatives. Previously, we have demonstrated antioxidant, neuritogenic, and angiogenic properties of Ginkgo biloba/EGb 761® (EGb 761) in different mouse models of stroke. In the present study, we were interested to study whether EGb 761 could protect mice from permanent middle cerebral artery occlusion (pMCAO) and enhance neurogenesis. EGb 761 pre- and post-treated mice had lower infarct volume and improved motor skills with enhanced proliferation of neuronal stem/progenitor cells (NSPCs) at 24 h and 7 days post-treatment. Netrin-1 and its receptors (DCC and UNC5B) that mediate axonal attraction and repulsion were observed to be over-expressed in NSPCs only, implying that netrin-1 and its receptors might have partly played a role in enhanced neurogenesis. Interestingly, in heme oxygenase 1 knockout mice (HO1^{-/-}), neurogenesis was significantly lower than in vehicle-treated mice at day 7. Furthermore, EGb 761 post-treated mice also demonstrated heme oxygenase 1 (HO1)-activated pathway of phosphorylated glycogen synthase kinase 3- α/β (p-GSK-3 α/β), collapsin response mediator protein 2 (CRMP-2), semaphorin3A (SEMA3A), and Wnt, suggesting probable signaling pathways involved in proliferation, differentiation and migration of NSPCs. Together, these results propose that EGb 761 not only has antioxidant, neuritogenic and angiogenic properties, but can also augment the repair and regeneration mechanisms following stroke.

INTRODUCTION

There is considerable evidence available in the literature highlighting the active repair and recovery mechanisms following stroke, and it is believed that neurogenesis is one of them. Studies on rodent experimental stroke have shown that adult neurogenesis occurs in the forebrain, and these newly born neuroblasts migrate toward the sites of injury in an attempt to fix the damaged area of the brain [1,2]. Neurogenesis is a complex developmental process involving proliferation, differentiation, migration, survival, maturation and functional integration of neural stem/progenitor cells (NSPCs) into the neuronal circuit [3,4]. In the adult mammalian brain, there are different regions where neurons are generated throughout life: the subventricular zone (SVZ), subgranular zone of the dentate gyrus, and the posterior periventricular area [5,6]. Cerebral ischemia can stimulate proliferation of NSPCs that

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migrate to the damaged brain areas [7,8] to repair the damaged neurons, but only few develop into mature neurons, most of which die due to the lack of a suitable environment for neuronal differentiation in the ischemic lesion. It is still unclear whether these progenitor cells replace necrotic cells and to what extent [9].

Experimental approaches have been undertaken to enhance regeneration or restore lost functions after stroke with exogenously injected or transplanted human-induced pluripotent stem cells [10], umbilical cord blood-derived aldehyde dehydrogenase-expressing progenitor cells [11], and endothelial progenitor cells from umbilical cord [12]. Recent studies predicted that intensifying neuronal differentiation and survival around the ischemic area can improve the clinical outcome. Nevertheless, controversies on ethics, dosing modalities (number of cells to be injected), site of injection, possible immune reactions, and incidence of tumor formations associated with stem cell therapy has stimulated the idea of enhancement of endogenous neurogenesis with exogenous molecules or drugs—specifically natural products—which appears to be an innovative tool for post-stroke neuroregeneration and repair therapy.

Ginkgo biloba/EGb 761® (EGb 761) is a popular and standardized natural extract purportedly used worldwide for the treatment of many diseases, and it has been studied extensively in different disease models. Previously, our group has demonstrated neuroprotective properties of EGb 761 in different models of stroke, and the mechanism of action was reported to be mediated by up-regulation of the antioxidant [Heme oxygenase 1/ (HO1/Nrf2)] pathway and neurotogenic [collapsing response mediator protein- 2 (CRMP-2)], angiogenic [endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF)] [13,14] properties. With all these divergent molecular mechanisms already in place and being evaluated in different stroke models, in the present study, we embarked on finding whether EGb 761 can also augment endogenous neurogenesis and contribute to the repair and regeneration phase of stroke.

In the present study, we investigated the role of EGb 761 on neurogenesis enhancing properties and the possible involvement of HO1, phosphorylated glycogen synthase kinase 3 α and β (p-GSK-3 α/β), CRMP2, semaphorin 3A (SEMA3A), Wnt and the brain-derived neurotrophic factor (BDNF) signaling pathway. Furthermore, the role of HO1 on neurogenesis was studied by using HO1 knockout (HO1^{-/-}) mice.

MATERIALS AND METHODS

Animals

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. Male C57BL/6 mice, 8–10 weeks old and 20–25 grams were procured from Charles River Laboratories and were housed at 22 $\pm$ 1 °C with a 12 h: 12 h light/dark cycle.

HO1 knockout mice

Heterozygous HO1 (HO^{+/-}) mice, originally from the colony of Dr. Susumu Tonegawa, MIT, were obtained from Dr. Anupam Agarwal, University of Alabama. HO1^{-/-} mice were obtained by mating male chimeras with C57BL/6 females. HO^{+/-} animals were intercrossed to produce HO1^{-/-} mice. Genotypes of mice were determined by PCR analysis of progeny tail DNA as previously described [15]. Wild type forward primer: 5'GGTGACAGAAGAGGCTAAG 3' and reverse primer: 5'CTGTAACCTCCACCTCCAAC 3'; mutant forward primer: 5'TCTTGACGAGTTCTTCTGAG 3' and reverse primer: 5'

ACGAAGTGACGCCATCTGT 3', generated 450 and 350 BP, respectively for the wild type and mutant mice.

Permanent distal middle cerebral artery occlusion (pMCAO)

We used our previously optimized method to occlude the distal portion of the middle cerebral artery (MCA) [13,16]. Mice anesthetized with isoflurane, initially with 2 % and then maintained at 1 %, were operated on under a surgical microscope. Aseptic conditions were maintained by using sterile drapes, hand gloves and autoclaved surgical equipment. A thin 1.0-cm vertical skin incision was given between the right eye and ear, the temporal muscle was moved aside by forceps, and the underlying temporal bone was exposed. A 2.0-mm hole was drilled with a dental drill exactly over the region of the distal MCA visible through the temporal bone, and the dura mater was separated carefully. Using a bipolar coagulator, the distal part of the MCA was occluded directly and severed to interrupt the blood flow to the area supplied by the artery. A rectal probe was used to monitor core body temperature, which was maintained at 37.0 ± 0.5 °C during the procedure with a heating blanket. After the surgery, mice were moved to a temperature-regulated incubator and kept for 2 h to recover from surgery; they were later shifted to home cages.

Drug treatment

Egb 761 (100 mg/kg), kindly provided by Willmar-Schwabe Pharmaceutical, Germany, was administered to mice for 7 days before surgery for pre-treatment paradigms. In another cohort of mice, Egb 761 was immediately administered 4 h after pMCAO and continued for 7 days. For neurogenesis experiments, sham, vehicle, Egb 761 post-treated, and HO1^{-/-} mice were injected with 100 μ L of bromodeoxyuridine (BrdU) (10 mg/mL of concentration) at 4 h following surgery and then continually for 7 days.

Motor function

Motor function was measured by a person blinded to the treatment groups. Mice were placed on a moving rod of a rotarod, which was preprogrammed to rotate at 1 rpm with an acceleration rate of 1 rpm every 10 seconds until the animal fell from the rod. All animals were trained to run on a rotarod assembly prior to the surgery. The time at which the animal fell from the accelerating rod was recorded and registered as the latency to fall. The readings obtained prior to surgery were noted as baseline for each mouse.

Infarction volume analysis

Animals from all the treated groups were euthanized on day 7 after the pMCAO procedure. Brains from mice were dissected out, sliced into five 2-mm thick sections and incubated in 1 % triphenyltetrazolium chloride (TTC, Sigma Co) in a normal saline solution for 20 min at 37 °C. Brain sections fixed overnight in 4 % formaldehyde solution were used for infarct volume analysis using Image J software provided by NIH and calculated as the percentage of the contralateral cortex, with correction for swelling.

Brain sectioning and Immunohistochemistry

Mice were anesthetized with sodium pentobarbital and transcardially perfused with saline followed by 4 % paraformaldehyde. Mice brains were removed and stored for 24 h in 4 % paraformaldehyde at 4 °C before sectioning into 6 μ m sections, and four to five brain sections were placed on each slide and stored at -80 °C until use. Brain sections were 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 were allowed to cool for 10 min at room temperature (RT). Permeability was performed by incubating the sections with 1 % Triton- X100 in 1X PBS buffer for 30 min then washing

again with 1X PBS buffer. Denaturation was accomplished with 2N HCl for 30 min at RT, and the slides were neutralized with 0.1M sodium borate for 20 min at RT and washed again. The slides were 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-BDNF (Abcam) using following dilutions: 1:300, 1:300, 1:250, 1:250, and 1:100, respectively. The slides were 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 were mounted with DAPI (Santa Cruz, Dallas, TX) and then sealed with nail polish.

For BrdU analysis, 12-15 captures were taken from the side of the injury for each section/ four to five brain sections/mice/3mice each group. The sections were cut approximately at the coordinates between -0.94 to -2.92 from bregma. The Netrin-BrdU positive cells were counted and averaged, and the ratio was determined by dividing Netrin-BrdU positive cells by the total DAPI cell counts.

Isolation of cytosolic fraction and Western blot analysis

Brain cortices of mice were dissected out, weighed, and homogenized as described before [13,14]. Protein concentration was determined by Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and samples were analyzed by loading equivalent amounts of total cytoplasmic proteins (25 µg) onto 10 % or 12 % SDS-polyacrylamide gels. Proteins were transferred from the gel to PVDF membrane (Millipore, Billerica, MA) and blocked with 5 % dry nonfat milk and /or 3 % BSA for 1 h at RT followed by overnight incubation at 4 °C with the following antibodies: rabbit anti HO1 (Assay Designs, Ann Arbor, MI), mouse anti-actin, (Millipore), rabbit anti-CRMP-2 (Millipore), rabbit anti-SEMA3A (Abgent, San Diego, CA), rabbit anti-BDNF (abcam), rabbit anti-Wnt (abcam), rabbit anti-p-GSK-3 α/β (Thermo Scientific), mouse anti- GSK-3 α/β (Thermo Scientific) and rabbit anti-GAPDH (Thermo Scientific) using the following dilutions: 1:1000, 1:2000, 1:60000, 1:400, 1:1000, 1:1000, 1:1000, and 1:2000, respectively. After washing with 1X PBS-T buffer three times (15 min/wash), membranes were incubated with the secondary antibody, goat anti-rabbit and/ or goat anti-mouse, at a dilution of 1:4000 (Jackson Immuno-Research laboratories). Images were analyzed using Bio-Rad chemi Doc™ XRS+ image analyzer. The densitometric values were normalized with respect to the values of actin immunoreactivity to correct for any loading and transfer differences between samples.

Statistical analysis

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

RESULTS

Pre and post-treatment with EGb 761 reduces infarct size and improves locomotor activity

Previously, we have demonstrated that a single dose of EGb 761 4 h after pMCAO protected mice from brain stroke injury and improved neurologic deficits [13]. Here, we wanted to test the protective effects of multiple doses of EGb 761 using pre-treatment and post-treatment paradigms and evaluate its neurogenesis-enhancing properties. Mice pre- and post-treated with EGb 761 recovered significantly faster than the vehicle-treated group 7 days after

pMCAO (Fig. 1). In the pre-treatment study, mice were orally administered EGb 761 for 7 days before pMCAO and survived for an additional 7 days without any drug treatment. Locomotor activity was significantly increased at 24 h ($p=0.03$) and at day 7 ($p=0.04$) after pMCAO, and infarct volume showed a significant decrease (30.9 ± 3.4 ; $p=0.01$) at day 7 (Fig. 1B, C and D) compared to vehicle (43.5 ± 2.7). In the post-treatment paradigm, mice treated with EGb 761 immediately, at 4 h following pMCAO, and then daily for 7 days showed significantly increased locomotor activity at 7 days ($p=0.01$), which was also corroborated with decreased infarct volume (30.4 ± 3.4 ; $p=0.01$) (Figure 1B, C and D). This data suggests that EGb 761 treatment enhanced stroke recovery, as evidenced by improved neurobehavioral parameters and lower stroke volume.

EGb 761 post-treatment enhances neurogenesis

Several reports, including ours, have documented the neuroprotective properties of EGb 761, but its neurogenesis-enhancing properties are yet to be investigated. To address this question, we selected 24 h and day 7 time points for the quantification of BrdU positive cells. As shown in Fig. 2A and B, neurogenesis was detected in sham mice, and there was no specific distribution; rather, it was scattered throughout the brain. We were able to distinguish between NSPCs and non-stem cells by netrin-1 protein expression. In this study, we showed that netrin-1 protein expression was specifically expressed at the highest level in NSPCs. After 24 h of stroke, vehicle-treated mice and $HO1^{-/-}$ mice showed significantly increased neurogenesis compared to sham mice ($p=0.038$ and $p=0.01$, respectively), whereas neurogenesis in EGb 761 post-treated mice was significantly increased compared to sham mice ($p=0.025$), $HO1^{-/-}$ mice ($p=0.015$), and vehicle-treated mice ($p=0.024$) (Fig. 2 A-C and E). On the other hand, we didn't detect any significant differences between vehicle-treated and $HO1^{-/-}$ mice 24 h after pMCAO. Seven days after stroke, neurogenesis was observed to be significantly increased in EGb 761 post-treated mice as compared to both vehicle-treated ($p=0.015$) and $HO1^{-/-}$ mice ($p=0.008$). Interestingly, $HO1^{-/-}$ mice had significantly decreased neurogenesis as compared to vehicle mice ($p=0.028$). Neurogenesis in EGb 761 post-treated mice at 24 h and 7 days was not statistically significant. The distribution of NSPCs in the brain at 24 h showed a differential pattern from vehicle-treated to EGb 761-treated mice. In vehicle-treated mice, the distribution of NSPCs was concentrated in the striatum and lateral ventricle area, and only few neurons migrated to the injury site, whereas the distribution of NSPCs in the EGb 761 post-treated mice was concentrated at the border of the injury site and mainly in the cortex, and few cells were found in the striatum. Figure 5D is the representative brain section showing infarct area demarcated by red dotted lines and the square represents the area where BrdU–netrin-1 positive cells were counted.

Expression of netrin-1 and its receptors, DCC and UNC5B in NSPCs

In this study, we were able to demonstrate that netrin-1 and its receptors, DCC and UNC5B, were expressed only in NSPCs, and their expression was found 24 h and 7 days after pMCAO (Fig. 3 A-D). These data suggest that netrin-1 and its receptors are required for NSPCs migration from the subventricular zone to the site of injury.

Expression of BDNF in NSPCs

Next, we investigated the possibility that NSPCs may also express different neuronal markers besides netrin-1 and its receptors. BDNF, a neurotrophic factor involved in proliferation, differentiation, and viability of neurons in the mammalian brain, supports the survival of existing neurons and promotes the growth and differentiation of new neurons and synapses [17]. We found that BDNF was expressed in both NSPCs and neuronal cells (Fig. 4 A and B).

Signaling pathway of EGb 761-induced neuroprotection and neurogenesis

In order to determine the molecular mechanism(s) and the signaling pathway involved in EGb 761 neurogenesis, Western blot analysis was performed to measure protein expression levels of HO1, p-GSK-3 α/β and total GSK-3 α/β , CRMP-2, SEMA3A, Wnt and BDNF in mice brain cortices of the following groups: Sham, EGb 761+no pMCAO, EGb 761 pre and post-treatment + pMCAO and vehicle-treatment + pMCAO. Consistent with our previous results [13], HO1 protein expression was not significantly increased in vehicle ($p=0.06$) as compared to sham group. However, a significant increase in HO1 expression as compared to vehicle was observed only in EGb 761 post-treated mice ($p=0.02$) but not in the pre-treatment group (Fig. 5 A and B). CRMP-2 protein expression was significantly decreased in vehicle-treated mice ($p=0.003$) and was observed to be increased in the EGb 761 pre- ($p=0.03$) and post-treated ($p=0.004$) mice (Fig. 5 C and D). SEMA3A protein expression showed an increased trend in the vehicle-treated group, which was further non-significantly increased in the EGb 761 post-treated group (Fig. 5 G and H). Wnt protein expression was significantly down-regulated in vehicle-treated mice compared to the sham group ($p=0.004$), whereas EGb 761 pre- ($p=0.02$) and post-treated ($p=0.01$) group exhibited significantly increased levels of Wnt compared to the vehicle-treated group (Fig. 5 G and I). BDNF protein expression did not exhibit any significant changes across all treatment groups, but an increasing trend was observed from sham to vehicle and vehicle to pre- and post-treated groups (Fig. 5 G and J). p-GSK-3 α/β to total GSK-3 α/β significantly decreased in the vehicle-treated group as compared to sham ($p=0.04$), and expression levels were restored to the levels of sham in EGb 761 pre- ($p=0.02$) and post-treatment ($p=0.02$) groups (Fig. 5 E and F).

DISCUSSION

In this study, we report a novel signaling pathway involving HO1 for EGb 761-mediated neurogenesis-enhancing properties in mice pMCAO model of ischemia. Our data are based on the findings that EGb 761 significantly reduced stroke damage and protected the brain cortex from stroke damage by reducing infarct volume in both pre- and post-treated mice. These protective effects also translated into improved functional recovery. HO1^{-/-} mice demonstrated reduced neurogenesis at day 7 after pMCAO, further reinforcing the role of HO1 signaling in neurogenesis. Moreover, we were also able to show that EGb 761-enhanced neurogenesis was mediated by HO1-activated pathway of p-GSK-3 α/β /CRMP-2/SEMA3A/Wnt signaling.

Stroke is a devastating disease that leads to permanent or long-term disability in most of the survivors. In a normal brain, neuroblasts from SVZ migrate towards the olfactory bulb via the rostral migratory stream (RMS), whereas during stroke, most of them migrate toward the location of ischemic damage. Factors responsible for the migration of these NSPCs to the peri-infarct area include chemokine/chemokine receptors and matrix metalloproteases [18]. Recent studies have linked the level of adult neurogenesis to brain function in both normal and diseased brain, and these findings make possible the use of NSPCs in the treatment of brain injury-related diseases [19-21]. Previously, we demonstrated that EGb 761 has neuroprotective, antioxidant and neuritogenic properties [13,14], and in this report we extended our investigation to explore the role of EGb 761 as a neurogenesis-enhancing nutraceutical by using the standard procedure of BrdU to detect NSPCs in different groups subjected to pMCAO and EGb 761 treatment.

The process of neurogenesis involves large number of secreted extracellular molecules that have been shown to regulate the migration of NSPCs including netrin-1 and semaphorins [22,23]. Netrin-1 and its receptors, DCC and UNC5B mediate axon attraction and repulsion and are highly expressed in the central nervous system during embryonic development as

well as in the adulthood, but at relatively low levels [24,25]. Previous studies have demonstrated that using small molecules such as lithium chloride, sodium-valproate, kenpaullone, or indirubin-3 monoxime increases neurogenesis by inhibiting GSK-3 activity [26]. Curcumin and metformin have also been shown to increase neurogenesis and cognition [27,28]. Netrin-1 is a chemotrophic factor expressed on the floor plate cells in the central nervous system and is involved in either promotion or inhibition of axonal growth, depending on which receptor it binds to [29,30]. Also, there is documented evidence that netrin-1 can act as guidance molecule for neuronal migration [31,32], and its overexpression can induce angiogenesis in the mouse brain, thus it participates in neovascularization and vessel remodeling after ischemic stroke [33]. In the present study, we were able to demonstrate that netrin-1 and its receptor, DCC and UNC5B, were only expressed in NSPCs in all treatment groups, suggesting that netrin-1 plays an important role as a guidance molecule for NSPCs migration. The numbers of netrin-1/NSPCs were significantly increased in EGb 761 post-treated mice brains after pMCAO at 24 h and 7 days post-treatment, which suggests that EGb 761 plays a role in the enhancement of neurogenesis. It is important to mention that we observed a differential distribution of the NSPCs in sham, vehicle, and EGb761 post-treated groups. In the sham group, the NSPCs were distributed in the whole brain section, whereas in EGb 761 post-treated mice, large numbers of NSPCs were observed to have migrated towards the cortex and close to the border of the injury site (penumbral region). On the other hand, vehicle-treated mice showed NSPCs mainly in the striatum, and few cells migrated towards the border of injury site. These results suggest that EGb 761 enhanced the migration of NSPCs towards the penumbral region and consequently enhanced the recovery from stroke. These results also illustrate that EGb 761 targets NSPCs outside the ischemia area and directs their migration towards the penumbra area. This might also explain the global effect of the EGb 761 on the brain of the mice since pMCAO is a permanent occlusion and blood flow to the distal part of the MCA is totally obscured. In agreement with our study, many studies have demonstrated that protein expression was elevated for netrin-1 and its receptors, DCC and UNC5, after brain injury or brain ischemia [34,24], signifying their role in regulating the growth of axons and preserving cell survival. However, there is a disagreement between our results showing slight decline but not a significant one in the number of netrin-1/NSPCs 7 days after pMCAO and those of Tsuchiya et al. (2007) [35], which showed that netrin-1/NSPCs peak at 14 days after stroke. This controversy may be attributed to the differences in the stroke model or species and/or the end-point of 7 days used in our study. Also, we were able to show that NSPCs expressed BDNF protein, which is a specific marker for neuronal cells and enhances NSPCs proliferation and differentiation [36,37]. Recently stroke-induced migration of neuroblasts to the ischemic site was shown to be controlled by BDNF signaling [38], and its expression post-stroke, is associated with stroke recovery and improved learning and memory [39]. Previous results from our lab revealed that EGb 761 treatment up-regulated the *in vitro* expression of both HO1 and CRMP-2 proteins. Consistent with our previous study, HO1 was up-regulated only by EGb 761 post-treatment, and CRMP-2 was up regulated in both EGb 761 pre- and post-treatment paradigms. The link between the up-regulation of HO1 and CRMP-2 has already been demonstrated in our previous study using HO1 inhibitor in *in vitro* neuronal cultures [14]. CRMP-2 overexpression stimulates nerve regeneration, suggesting the involvement of CRMP-2 in neuronal survival [40]. In addition, we have also reported that HO1^{-/-} mice suffer from higher stroke damage at 7 days after pMCAO than wild type mice, and EGb 761 treatment did not attenuate stroke damage in HO1^{-/-} mice [13]. Pertinent to the previous evidence, we only planned to study the level of neurogenesis in HO1^{-/-} mice at 24 h and 7 days after pMCAO. Concomitant with the findings of higher infarct volume at day 7 after pMCAO, we observed significantly reduced levels of NSPCs in HO1^{-/-} mice, suggesting that HO1 partly plays a role in neurogenesis after ischemic injury.

CRMP-2 acts as a component of the SEMA3A signaling pathway [41,42]. SEMA3A is a secreted protein and a potent neuronal chemo repellent for sensory, sympathetic, and motor axons [43]. SEMA3A is a repulsive axon guidance cue that causes dramatic collapse of the growth cone [41,44] due to the redistribution of F-actin cytoskeleton [45]. Other studies demonstrated that SEMA3A acts as a chemoattractant and chemotropic molecule [22,46] that guides the migrated cells towards their destination [47]. The switch of SEMA3A from chemorepulsion to chemoattraction requires activation of the cGMP/NO pathway [46,48], which is activated by HO1 mediated upregulation of VEGF and eNOS [49]. We reported an increasing trend in SEMA3A expression levels from sham to vehicle-treated groups and from vehicle-treated to EGb 761 (significant increase) post-treated groups, which suggest its role as a chemoattractant in our studies. GSK-3, a serine-threonine kinase family protein also involved in the neurogenesis pathway, plays a key role in regulating neuronal morphogenesis, including the determination and maintenance of axonal identity. Studies have also shown that inhibiting GSK-3 β expression induces angiogenesis in myocardial ischemia and promotes neurorestoration [50]. It is also required for neurotrophin-mediated axon elongation and can antagonize the canonical Wnt pathway, thus playing a central role in neural development and adult neurogenesis [51]. In the present report, we demonstrated that EGb 761 inhibited GSK-3 activity by increasing its phosphorylation on Ser 21/9 of both GSK-3 α and β subunits.

Many studies have indicated that the canonical Wnt signaling pathway is crucial for mammalian central nervous system development and regulates diverse processes including cell proliferation, differentiation, and migration [52,53]. Moreover, the Wnt signaling pathway is an obligate component of NSPCs differentiation into neurons [54]. A recent study demonstrated that Wnt signaling is able to increase the survival of newly produced neurons [9]. A recent study from Vanella et al. (2013) showed that HO1 acts upstream of the canonical Wnt signaling cascade [55]. In our study, we observed increased Wnt protein expression in EGb 761 post- and pre-treatment groups as compared to the vehicle-treated group. These results suggest that EGb 761 enhances Wnt expression through activation and elevation of HO1 protein expression, which in turn activates the neurogenesis signaling pathway.

EGb 761 post-treatment versus pre-treatment

In this report, we demonstrated that most of the signaling molecules required for neurogenesis were up-regulated in post-treatment paradigms at day 7. Although both treatments were able to protect mice brain from ischemic stroke, the difference in expression levels may be attributed to repeated EGb 761 doses after pMCAO instead of no EGb 761 treatment after pMCAO in pre-treatment paradigms. Although we did not look beyond 7 day post-stroke, it would be worthwhile to conduct future longitudinal studies with 14 to 29 days of survival time-points.

CONCLUSIONS

In conclusion, we propose that EGb 761 pre- and post-treatment protects mice from pMCAO-induced damage and post-treatment helps the recovery and regeneration process of stroke by enhancing neurogenesis. In this study, we were able to dissect out the EGb 761 neuroprotective signaling pathway. Based on our previous studies [13,14,56], HO1 has turned out to be a key candidate that activates several signaling cascades. HO1 is a rate-limiting enzyme required for the conversion of deleterious heme into neuroprotective products like bilirubin, biliverdin and carbon monoxide (CO). CO is required for the activation of soluble guanylyl cyclase and increases the intracellular cyclic guanosine monophosphate (cGMP) production [57], consequently inhibiting the GSK-3 [58]. HO1 also increases Wnt protein expression, which is required for neurogenesis [55]. Furthermore,

HO1 activation of VEGF and eNOS leads to an increase in nitric oxide (NO) production, which can act as a second messenger and enhance learning ability and neurogenesis [49]. The novel signaling pathway (illustrated in Figure 6) is suggested to involve HO1 in activating the signaling pathway mechanisms of angiogenesis, cell survival and/or neuroplasticity and neurogenesis. Discovering natural products that can enhance post stroke regeneration process provides an innovative tool for improving treatments for stroke recovery.

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Abbreviations

BrdU	5-Bromo-2'-deoxyuridine
CRMP-2	collapsin response mediator protein 2
EGb 761	Ginkgo biloba/EGb 761 [®]
HO1	heme oxygenase 1
pMCAO	permanent middle cerebral artery occlusion
TTC	triphenyltetrazolium chloride

NSPCs	neuronal stem/progenitor cells
SEMA3A	semaphorin3A
DCC	deleted in colorectal cancer
UNC5B	uncoordinated gene 5B
BDNF	Brain-derived neurotrophic factor
GSK-3 α/β	Glycogen synthase kinase 3 α and β
p-GSK-3 α/β	phosphorylated form of glycogen synthase kinase-3 α/β

Fig. 1.

Pre- or post-treatment with EGb 761 reduces stroke damage. **(A)** Schematic diagram of the experimental protocol. Mice were orally administered with vehicle (n=12), EGb 761 (100 mg/kg; n=16), daily for 7 days before pMCAO in the pre-treatment group, and at 4 h after pMCAO and then daily for 7 days (n=5) in the post-treatment group. Neurobehavioral parameters were performed 24 h before and after pMCAO and then at day 7. Mice were sacrificed 7 days after pMCAO in both pre-treatment and post-treatment, and brains were dissected out and cut into 2mm coronal sections for TTC staining. **(B)** Rotarod activity was improved at 24 h and on day 7 in the EGb 761 pre-treated group, whereas the post-treated group showed improvement at day 7. **(C)** Representative coronal brain section stained with TTC showing reduced infarct volume in pre- and post-treated groups. **(D)** Infarct volume was significantly reduced with both pre- and post-treated groups. Data are expressed as mean±SEM; * $p < 0.05$ vs. vehicle.

Fig. 2.

EGb 761 post-treatment enhances neurogenesis. Mice post-treated with EGb 761 at 4 h after pMCAO and then daily for 7 days were sacrificed at 24 h and at 7 days. BrdU was injected 4 h after pMCAO and then daily for 7 days (schematic diagram of Fig. 1A) (A) Triple immunofluorescence staining of DAPI (blue), BrdU (green), and netrin-1 (Red) and overlay of brain sections (6 μ m) of mice sacrificed at 24 h after pMCAO. (B) Brain sections of mice sacrificed at day 7. (C) Brain sections of HO1^{-/-} mice subjected to pMCAO and sacrificed at 24 h and at day 7. (D) Representative brain section showing infarct area demarcated by red dotted lines and the square represents the area where BrdU–netrin-1 positive cells were counted. (E) Analysis of BrdU–netrin-1 showed increased neurogenesis at 24 h, which dwindled by day 7, but EGb 761 post-treated mice showed sustained increase in the number of BrdU–netrin-1 positive neurons at 24 h and at day 7. HO1^{-/-} mice showed reduced neurogenesis at day 7 after pMCAO as compared to vehicle. Data are expressed as mean $\pm$ SEM; ^a p < 0.05 vs. Sham; * p < 0.05 vs. vehicle; # p < 0.05 vs. HO1^{-/-}; ns = non-significant (n = 2–3 each group). The pictures were taken by Nikon Eclipse Ti microscope using 100X objective.

Fig. 3.

Expression of netrin-1 and its receptors, UNC5B and DCC in NSPCs. Mice post-treated with EGb 761 at 4 h after pMCAO and then daily for 7 days were sacrificed at 24 h and at 7 days. BrdU was injected 4 h after pMCAO and then daily for 7 days. **(A-B)** Triple immunofluorescence staining of DAPI (blue), BrdU (green), UNC5B (Red) and overlay of brain sections (6 μ m) of mice treated with EGb 761 and sacrificed at 24 h and at 7 days after pMCAO. **(C-D)** Triple immunofluorescence staining of DAPI (blue), BrdU (green), DCC (Red) and overlay of brain sections (6 μ m) of mice treated with EGb 761 and sacrificed at 24 h and at 7 days after pMCAO. Netrin-1 receptors, DCC and UNC5B were only observed to be expressed in the NSPCs. The pictures were taken by Nikon Eclipse Ti microscope using 100X objective.

Fig. 4.

BDNF protein expression in NSPCs. Mice post-treated with EGb 761 at 4 h after pMCAO and then daily for 7 days were sacrificed at 24 h and at 7 days. BrdU was injected 4 h after pMCAO and then daily for 7 days. **(A-B)** Triple immunofluorescence staining of DAPI (blue), BrdU (green), BDNF protein (Red) and overlay of brain sections (6 μ m) of mice sacrificed at 24 h and at 7 days of pMCAO. Immunofluorescence shows that BDNF was expressed in NSPCs. The pictures were taken by Nikon Eclipse Ti microscope using 100X objective.

Fig. 5. Cortical protein expression levels after EGb 761 treatment. Mice were sacrificed after 7 days of pMCAO in both pre-treatment and post-treatment; cortical tissue, along with infarct area were dissected out for protein expression by Western blot analysis. (A, C, E and G) Protein expression levels of HO1, p-GSK-3 α/β, CRMP-2, GAPDH, β-actin, SEMA3A, Wnt and BDNF. (B, D, F, H, I and J) Values shown are percent of ratio of each protein expression to β-actin and are expressed as mean±SEM. (n=4-5/group); **p* < 0.05 vs. vehicle; #*p* < 0.05 vs. sham.

Fig. 6.

Proposed signaling pathway of EGb 761-induced neuroprotection and neurogenesis. We postulate that putative neuroprotective mechanism of EGb 761 is mediated through; 1). The first sub-mechanism is EGb 761 activates HO1 pathway and HO-1 cleaves heme to form biliverdin and CO. Biliverdin is subsequently converted to bilirubin by biliverdin reductase, an antioxidant CO has the ability to bind to the iron atom of the heme moiety associated with soluble guanylyl cyclase, thereby activating the enzyme and increasing intracellular cyclic guanosine monophosphate (cGMP) production. cGMP can regulate axon branching by converting SEMA3A from chemorepulsion to the chemoattraction and also by inhibiting glycogen synthase kinase 3 (GSK-3). GSK-3 inhibition increases the activity of CRMP-2, which leads to cell survival and cell neuroplasticity. 2). The second sub-mechanism is EGb 761 activates VEGF which activates eNOS leading to elevated NO levels that mediates the conversion of SEMA3A from chemorepulsion to chemoattraction and ultimately to angiogenesis. 3). The third sub-mechanism is through the elevation of Wnt protein which leads to the activation of Wnt/ β -Catenin pathway subsequently displacing GSK-3 from the complex which leads to the neurogenesis.

Activation ($\rightarrow$), inhibition ($\neg$), mutual activation ($\leftrightarrow$).