Neuroprotective effects of short-term and long-term administration of genistein on ET-1 induced ischemic stroke rats

Thesis submitted in partial fulfilment of the requirements of the

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By

Niharika Sane

20101087

Biology

Under the guidance of

Dr. Laxmi T. Rao

Associate Professor, Department of Neurophysiology

NIMHANS, Bengaluru

Certificate

This is to certify that this dissertation entitled **"Neuroprotective effects of short-term and long-term administration of genistein on ET-1 induced ischemic stroke rats"** towards the partial fulfilment of the five year BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Ms. Niharika Sane** at Department of Neurophysiology, NIMHANS under the supervision of **Dr. Laxmi T. Rao**, Associate Professor, Department of Neurophysiology, NIMHANS during the academic year 2014-2015.

Dr. Laxmi T. Rao

Associate Professor,

Department of Neurophysiology,

NIMHANS, Bengaluru

Date: 30/4/15

Declaration

I hereby declare that the matter embodied in the report entitled **"Neuroprotective effects of short-term and long-term administration of genistein on ET-1 induced ischemic stroke rats"** are the results of the investigations carried out by me at the Department of Neurophysiology, NIMHANS, Bengaluru under the supervision of **Dr. Laxmi T. Rao** and the same has not been submitted elsewhere for any other degree.

Niharika Sane

Five year BS-MS Dual degree student

IISER Pune

Date: 30/4/15

Abstract

Stroke is classically characterized as a neurological deficit caused by an acute focal injury in the central nervous system and is a major cause of disability and death worldwide. Various treatments for stroke are currently available like tPA, including neuroprotective drugs like estrogen. However, estrogen has its own side effects and hence, naturally occurring phytoestrogens like genistein are being looked into as potential neuroprotectors in ischemic stroke. We looked into the effect of an acute dose of genistein (10mg/kg) and a prolonged dose of genistein (1mg/kg every day for ten days) one hour prior to ET-1 induced ischemic stroke in rats. Our behavioural studies, including the reach to grasp task, cylinder task, gait analysis and the horizontal ladder task, showed that both short-term and long-term genistein administration before stroke were able to lessen the motor deficits caused by stroke. These studies were validated by histological studies (Golgi-Cox staining and TTC staining). Golgi-Cox staining showed that both short-term and long-term administration of genistein helps in the recovery of neuronal atrophy evident in stroke induced rat brains. These results show that genistein has neuroprotective effects in stroke. However, the mechanisms have to be elucidated further. This study has implications in medical research and forms a potential basis of development for further therapeutic strategies.

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Acknowledgements

INTRODUCTION

Introduction

Stroke

In the 1970s, the World Health Organization defined stroke as a "neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours". Stroke affects around 15 million people every year. It is considered to be one of the leading causes of long-term motor disabilities in humans with around 76% of people surviving their stroke (Sacco et al., 2013). According to the WHO, 6.2 million people died of stroke in 2008. It is the second leading cause of death in people above 60 years old after heart attack. Stroke affects on an average 84-262 people in rural India and 334-424 people in urban India per 100000 population (Srivastava et al., 2014). Stroke has many risk factors like lack of exercise, smoking, an unhealthy diet and excessive alcohol. Genetics (Francis et al., 2007) and epigenetics (Qureshi and Mehler, 2010) can play a huge role in the onset of stroke.

There are 2 types of strokes, ischemic and hemorrhagic. An ischemic stroke develops due to the blockage of a blood vessel and a hemorrhagic stroke due to the bleeding of blood vessels in the brain. An ischemic stroke occurs especially when there is an irregularity in the blood supply to the brain induced by obstruction within a blood vessel supplying blood to the brain, e.g. occlusion of Middle Cerebral Artery (MCA). Thrombotic stroke (a blood clot or thrombus forms in one of the arteries that supply blood to the brain) and embolic stroke (an embolus travels from the bloodstream and lodges itself in brain arteries) are the most commonly occurring ischemic strokes. A thrombus or embolus might occlude a cerebral artery which causes ischemia in the affected vascular territory. Ischemic stroke can be focal or global. Focal ischemia occurs when a blood clot occludes a cerebral vessel (end arteries) reducing blood flow to a specific brain region which might cause cell death to that area. A global ischemia occurs if blood flow to the brain is stopped or drastically reduced due to an occlusion in the main branch. Ischemia to the brain may cause cellular lesions which lead to a loss of control of fine motor skills, including independent movements of fingers, along with the lack of refinement of other motor functions like postural activity, locomotion, etc. on the contralateral side of the body to the ischemia.

Ischemia results in a shortage of oxygen and glucose needed for cellular metabolism. Following stroke, there are two main regions of injury: the core and the penumbra. Cell death is maximum in the ischemic core and sometimes extends to the penumbra, causing all cellular elements which includes neurons and supportive cells to be affected. The core's tissues undergo hypoxic cell death while the penumbra's tissues may remain viable for a few hours due to the collateral arteries that supply to the penumbral region and this is when pharmacological interventions are most likely to be effective. Brain tissue that is deprived of required nutrients can survive without permanent injury for a significant period of time, hours and sometimes even days while in most other individuals, irreversible damage (infarction) occurs quickly.

Ischemic strokes are the most common type of stroke representing around 80% of all strokes. The normal cerebral blood flow is around 50-60ml/100g/min. After ischemia, due to the decrease in blood flow, the cerebral auto-regulatory mechanisms open the collaterals leading to increased vasodilation for increased extraction of glucose and oxygen from the blood. If the cerebral blood flow goes below 10ml/100g/min, there is irreversible neuronal damage (Jones et al., 1981). There are various mechanisms for neuronal injury which follows ischemia. Endothelial cells are one of the first cells to respond to hypoxia. These cells form microvilli at the surface of the cell and a thrombus is formed by endothelium, leucocytes and platelets. The thrombus grows bigger and eventually mechanical constriction of the blood vessel occurs by leucocytes, erythrocytes and fibrin (Garcia et al., 1994). Ischemia leads to a depletion of the cellular energy stores which are usually used to clear excitatory neurotransmitters like glutamate from the extracellular spaces. The increased glutamate levels in the extracellular space leads to the opening of calcium channels which causes an increased influx of calcium, sodium and chloride ions and an efflux of potassium ions. Intracellular calcium activates destructive enzyme like proteases, lipases and endonucleases. This causes release of cytokines which results in the loss of cellular integrity (Rothman and Olney, 1995).

Cortex organization:

The cerebral cortex is the largest human brain structure and has different areas. A specific part of the cerebral cortex has a causal role in voluntary movement, called the motor cortex. Electrical stimulation of different parts of the motor cortex evokes movements in the contralateral side of the body. Several cortical motor neurons project via various pathways to sub-cortical regions of the brain. All neocortical areas are divided into six layers starting from the outer surface (pia mater) to the white matter but the number and size of cells in each layer differs. Ascending pathways usually originate in the superficial layers (I, II and III) and end in layer IV. Descending pathways generally originate in the deeper layers (V, VI) and end in layer I and VI. The neocortex receives inputs from the thalamus and other cortical structures on both sides of the brain. It has its outputs in other regions of the neocortex, basal ganglia, thalamus and the spinal cord. Layer I, called the molecular layer, receives the dendrites of cells located in deeper layers and has axons passing through this layer to make connections with other areas of the cortex. Layers II and III contain small pyramidal shaped cells with the axons projecting to other neurons within the same cortical layer thus forming intracortical connections. Layer IV usually contains a large number of small spherical neurons but it is almost absent in the primary motor cortex. Layer V in the primary motor cortex contains large pyramidal neurons called as Betz cells. The pyramidal neurons of this layer projects to other cortical areas. The layer VI is a polymorphic layer which contains different types of neurons. The best studied output pathway is the pyramidal tract which usually originates in cortical layer V.

Circle of Willis:

One of the most common types of stroke is the blockage of the middle cerebral artery (Guyton and Hall, 2006).The middle cerebral artery (MCA) supplies blood to many regions such as the motor cortex and sensory cortex. The brain gets its blood supply from two sets of branches from the dorsal aorta, the internal carotid artery and the vertebral artery. The internal carotid arteries branch into the middle cerebral arteries and the anterior cerebral arteries. The right and left vertebral arteries join to form the

basilar artery. The basilar artery divides into the posterior cerebral arteries which join the middle cerebral artery through the posterior communicating artery. This forms a circulatory ring called the Circle of Willis (Fig. 1). The function of the Circle of Willis is to create redundancies in the cerebral circulation such that if one of the arteries or one part of the circle becomes blocked, blood flow from the other arteries might help to preserve the cerebral flow if not too severe. (Purves, 2004)
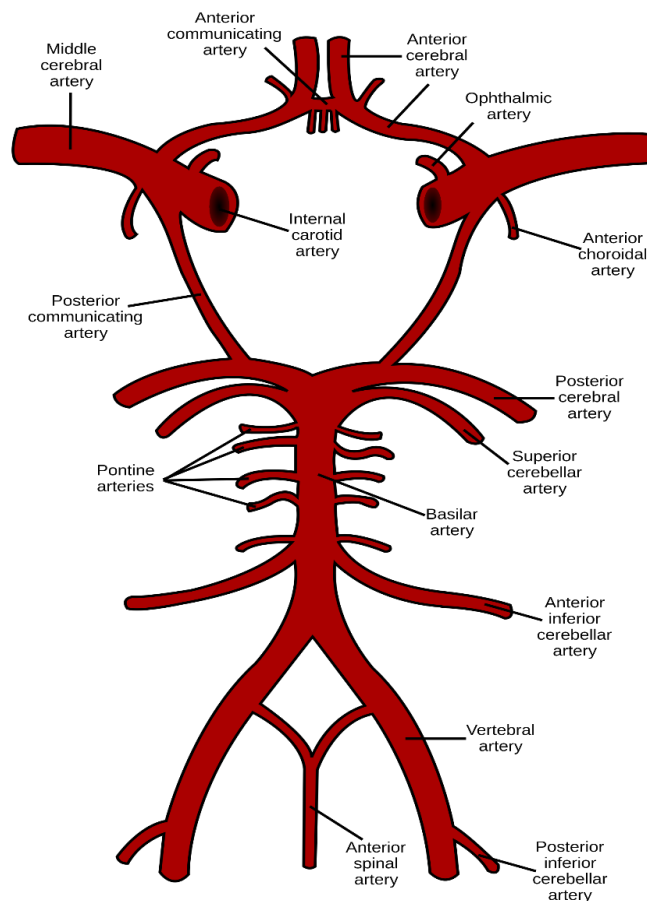
Figure 1. Circle of Willis

Stroke models:

The use of animal models over the years has helped to improve our understanding of the pathophysiology of a disease. Rats are a widely used model to study the effects of stroke. There are several methods of focal ischemia induction in rats which have been described out of which middle cerebral artery occlusion (MCAO) is the most used method. However, MCAO may sometimes injure deep brain structures. Another method

which is used is the thromboembolic method where a thrombus is generated directly in the MCA by the local injection of thrombin. However, such a technique may cause endovascular injuries and unwanted inflammatory processes. Other simpler methods have been developed, one of which involves an intracerebral injection of vasoconstrictor substances like Endothelin-1 (ET-1) adjacent to the MCA causing a lesion due to the reduction in blood flow in that region. Stroke induced by ET-1 is reproducible and causes a lesion which leads to significant motor deficits. Other advantages include a quick procedure and the ability to modify the arterial constriction by altering the dose of ET-1 administered (Casals et al., 2011).

Treatments for stroke:

Stroke not being a lethal disease most of the time, disables more than it kills. Some of the treatments used to combat stroke are surgery to remove the clot, medications to dissolve the clot, rehabilitation or neuroprotective drugs. If the stroke has occurred due to a block in the carotid artery, surgeries like carotid endaterectomy are performed to physically remove the clot. Tissue plasminogen activator (tPA) is the only FDA approved treatment for ischemic stroke. tPA dissolves the blood clot and allows the blood to flow to the parts of the brain which were deprived of blood. Anticoagulants and antiplatelet medicines are also used in stroke recovery. Depending on the severity of the stroke, there are various rehabilitation options which motivate the stroke survivor to relearn basic skills which have been lost. Most of the research for neuroprotection in stroke has been done in animal models. Various neuroprotective treatments have been found to be successful in animal models and human subjects. There are numerous targets for neuroprotection in stroke: inflammation (antagonists of interleukin receptors); oxidative stress (antioxidants to remove free radicals from the body) and excitotoxicity (inhibition of calcium into the cell), among others (Majid, 2014).

Estrogen - an endogenous neuroprotector:

Estrogen has been said to provide females with endogenous protection against cerebrovascular diseases (Gibson et al., 2006). This can be confirmed by the fact that the onset of stroke in women is later than the onset of stroke in men. The incidence of

stroke in pre-menopausal women is lesser as compared to men. However, the incidence of stroke in post-menopausal women is similar to that of men if not more, coincident with the decreasing levels of estrogen and progesterone (Go et al., 2013). This has led to the use of Hormone Replacement Therapy (HRT) in reducing cerebral ischemic injury in women. However, estrogen therapy (both short term and long term) in post-menopausal women has detrimental side effects (Viscoli et al., 2001).

Genistein:
Recent pharmaceutical developments have led to the use of selective estrogen receptor modulators (SERMs) as a replacement for estrogen. SERMs are synthetically prepared to confer the same neuroprotection but without the detrimental side effects (Musa et al., 2007). However, naturally occurring phytoestrogens like isoflavones can also mimic the neuroprotective effects of estrogen without the side effects. Isoflavones, especially genistein (a poly phenolic non-steroidal isoflavone found in soy plant), are being looked into as potential neuroprotectors in ischemic stroke (Whishaw et al., 2008). Genistein has a greater affinity for the estrogen receptor β in the CNS as compared to the peripheral organs which is the main reason why such compounds are the focus of recent research (Cyr et al., 2002). Genistein is responsible for the anti-atherosclerotic benefits of soy (Davignon and Ganz, 2004). Genistein is an antioxidant which neutralizes free radicals and upregulates endogenous antioxidant pathways (Siow and Mann, 2010). It also exhibits neuroprotection against mitochondria dependent apoptosis in ischemic stroke (Qian et al., 2012). A short-term (acute) dose of genistein (10mg/kg administered 1 hour prior to surgery) has been shown to be neuroprotective in Parkinson's disease (Baluchnejadmojarad et al., 2009). Treatment to male rats with a high soy isoflavone diet (10mg/kg every day for two weeks) significantly reduces the cerebral infarct size following permanent middle cerebral artery occlusion (Burguete et al., 2006).

Given that both long-term and short-term administration of genistein confers neuroprotection, we hypothesize that there is a difference in the neuroprotective effects

of long-term and short-term administration of genistein in an Endothelin-1 ischemic stroke rat model.

Further to evaluate the effect of Genistein in Endothelin-1 induced ischemic stroke rats on motor functions, there are several behavioural paradigms. For example, reach to grasp, cylinder test, gait analysis, horizontal ladder, etc. More specifically, reach to grasp test is widely used test to evaluate the skilled motor functions of the rat. Vertebrate skilled movements have been widespread among mammalian taxa suggesting a common evolutionary origin of movement. Rodents, primates and humans have similar skilled reaching movements (Sacrey et al., 2009). This led to the use of rodents as an appropriate model to analyze skilled reaching movements. Some movements used by animals are unique and hardly change with experience. This movements are called action patterns and skilled reaching movements shown by rats have such characteristics. Rat skilled reaching is a highly constrained activity suggesting an innate organization of the motor system (motor cortex) (Metz and Whishaw, 2000). A motor cortex lesion will lead to the loss of the innate action patterns which in turn will lead to the loss of skilled motor movements. However, recovery is possible due to the emergence of compensatory movements (Whishaw, 2000). The reach to grasp task is considered a brilliant parameter to assess the skilled motor movements of rats with ischemic stroke. The cylinder test was used to access the use of the contralateral forelimb before and after stroke. Both gait and horizontal ladder tasks were performed to ascertain the locomotive patterns of the animal.

Histological studies are also carried out to examine the motor neurons and its arborization branching pattern in layer V as this layer has Betz cells whose axons form the corticospinal tract which is the main pathway for voluntary motor control. The arborization pattern and the length of the dendrite can be used to determine if a motor cortex lesion by ET-1 induced ischemia will lead to a structural change in the motor neurons.

Hypothesis

Administration of Endothelin-1 leads to ischemic stroke with deficits in skilled motor functions and atrophy of cortical pyramidal neurons

Short-term (10mg/kg) and long-term (1mg/day for 10 days) intraperitonial administration of Genistein before Endothelin-1 infusion, leads to ischemic stroke with lesser deficits in skilled motor functions and recovery in pyramidal neuron atrophy.

Aim

To validate the neuroprotective effects of short-term and long-term administration of genistein in ET-1 induced ischemic stroke model

Objectives

1) Confirmation of stroke by TTC staining
2) Behavioural (cylinder, gait, horizontal ladder and reach to grasp) analysis
3) Morphological analysis by Golgi-Cox staining methods.

MATERIALS AND METHODS

Experimental materials and methods

<u>Subjects:</u> Sprague Dawley male rats (2-3 months old) bred and raised in the Central Animal Research Facility (CARF), NIMHANS were used throughout the experiment. Sprague-Dawley rats were used because of their excellence in skilled reaching (Nikkhah et al., 1998). The rats were housed in polypropylene cages and were on a controlled diet. Maximum care was taken to minimize the pain and discomfort to the experimental animals during the procedures. The entire experiment was carried out in accordance with the guidelines of the Central Animal Research Facility (CARF), at NIMHANS. Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC).

<u>Surgical procedure:</u> The rats were anesthetized with Ketamine (90-100mg/kg body weight) and xylazine (5-10mg/kg body weight) injected intraperitoneally. The fur on their dorsal skull was shaved and these anesthetized animals were mounted on a stereotaxic device (Stoelting Co., USA). The scalp was incised and cleaned and the head position adjusted to the bregma and lambda in the horizontal plane according to the skull coordinate system (Paxinose & Watson, 2004). Local anesthesia of lidocaine (0.5%) was delivered subcutaneously and a burrhole was drilled into the skull to implant the needle at the following coordinates: anterioposterior (AP) 1.6 mm; mediolateral (ML) 5.0mm and dorsoventral (DV) −7.9 mm below the dura.

Intracerebral arterial occlusion was induced by microinjection of 2 µl Endothelin-1 (Sigma-Aldrich, St. Louis, USA) of (2000 pmol concentration dissolved in saline) using a 10 µl Hamilton Syringe in the vicinity of middle cerebral artery at the above mentioned coordinates. ET-1 was injected at the rate of 1.0 µl/2 min with one minute pause between each 1µl and a 5-min delay before withdrawing the syringe slowly. The injection was given to the side of the brain opposite to the side of the rat which was dominant. After the surgery was complete, the incision in the brain was covered with dental acrylic cement and thinner. The entire surgery took place under the warmth of a lamp.

Genistein injections: Genistein dissolved in propylene glycol (Sigma-Aldrich, St. Louis, USA) was injected intraperitonially at 10mg/kg body weight, one hour (acute effect) prior to Endothelin-1 insult. Genistein was injected intraperitonially at 1mg/kg body weight daily for 10 days (chronic effect) prior to the Endothelin-1 insult. Animals assigned to the control stroke group did not receive any drug. Animals assigned to the ischemic stroke (short term genistein) and ischemic stroke (long term genistein) received 10mg/kg of genistein and 1mg/kg of genistein respectively. (Fig. 2)
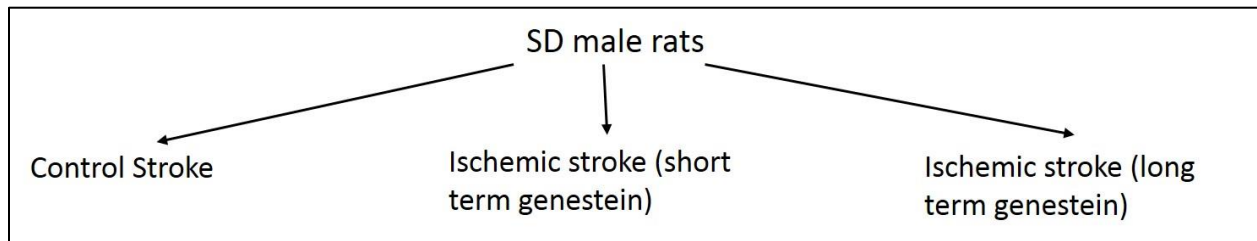

Figure 2. Animal experimental groups

The experiments were done according to the following timelines. Figures 3, 4 and 5 show how the experiments were performed.
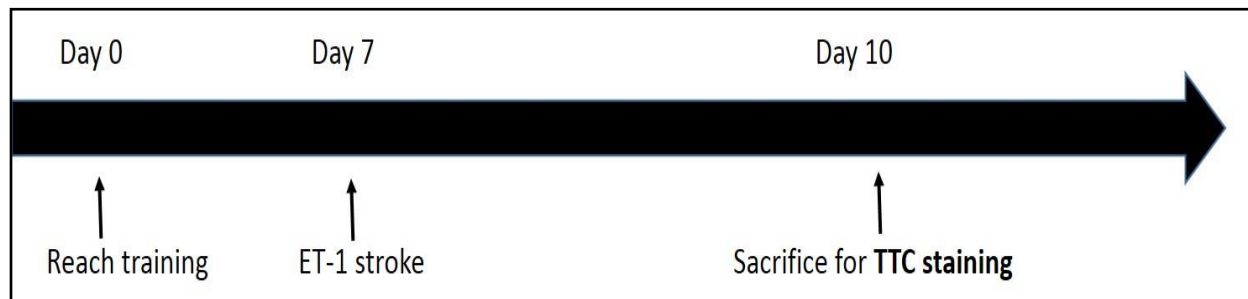
Figure 3. Timeline for TTC staining
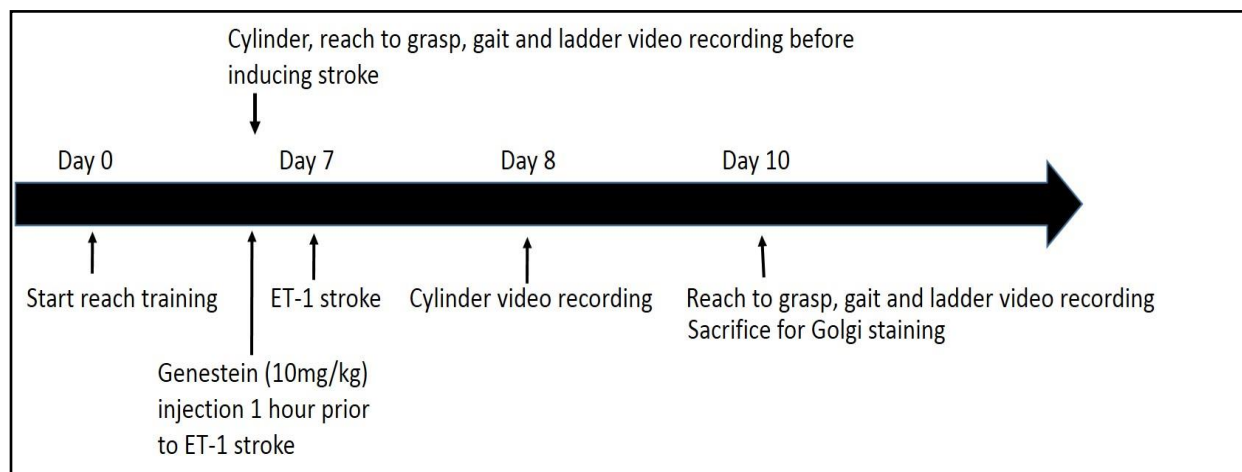
Figure 4. Timeline for short term administration of genistein
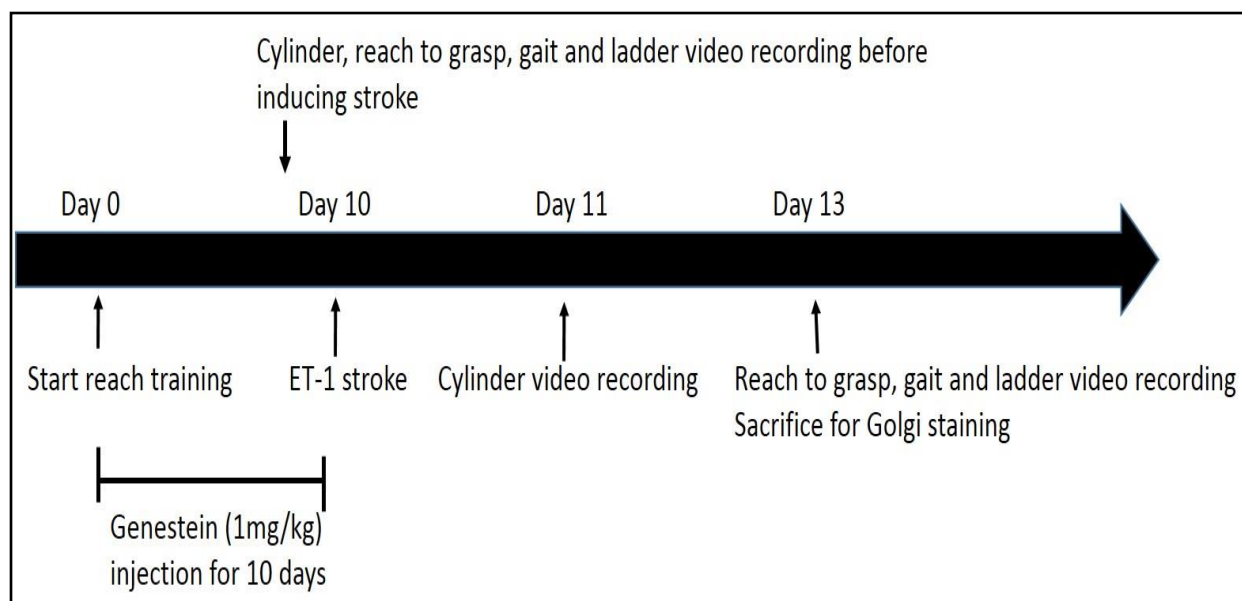
Figure 5. Timeline for long term administration of genistein

<u>Morphological studies:</u> 2,3,5-Triphenyltetrazolium chloride (TTC) staining is a convenient procedure for detection of brain infarcts which stains metabolically active tissues. TTC staining was performed 72 hours after ischemic stroke. The rats were sacrificed and their brains immediately removed and kept in cold PBS solution. Coronal sections of the brain were cut and placed in 0.05% TTC solution for 15 mins at 37°C after which they were photographed. The infarct area and hemisphere area of each section was visualized.

<u>Behavioural studies:</u> To evaluate motor abilities of the rats after ischemic stroke, the rats were tested before and 24 hours (cylinder task) and 72 hours (reach to grasp task, gait and horizontal ladder task) after the surgery. For motivating the animal into performing the reach to grasp task, the rats were placed in a light food-restriction diet. The rats were fed right after the testing session everyday with the total testing session lasting for about 1 week.

1) Reach to grasp task– This task was performed to assess the skilled motor movements. Animals were trained and tested inside a clear Plexiglas box with 40 x 45 x 13.5 cm dimensions. In the center of the front wall was a 1.3 cm wide vertical slit to which a shelf was attached to the outside of the front wall 4 cm above the ground. Two small indentations (5 mm in diameter and 1.5 mm in depth) in the shelf served as wells to hold food pellets. These were aligned with the edges of the slit and were located 1.5 cm from the inside of the front wall of the box. This design prevented rats from retrieving food pellets with their tongues and required them to reach with one forelimb through the slit for a food pellet located in the contralateral food well and then pronate medially over the food to grasp it. The task was successfully completed when the rats were able to grasp the food pellets, skillfully retract their paws and consume the food. Rats were individually trained and allowed to reach with their preferred forelimb for food pellets (Fig. 6). 20 pellets were given to each rat on the contralateral side and the limb movement was analyzed by videotaping the rats. The rats were scored on the percentage of corrects hits (reaching, holding and inserting the food pellet in their mouth on the first try) and the number of reaches required to grasp the pellet. (Whishaw et al., 1991)

   a) Total success-
      A successful reach score was obtained from the formula = <u>Number of hits</u>

                                             Number of reaches

Where hit refers to a single reach movement which results in a successful grasp of the food pellet and transfer to the mouth; and reach refers to any advancement of the forelimb.

b) First trial success-

Success % = $\underline{\text{No of pellets obtained on first advance}}$ x100

                         20

20 refers to the total number of pellets given each time, i.e. the number of reach trials used for scoring.
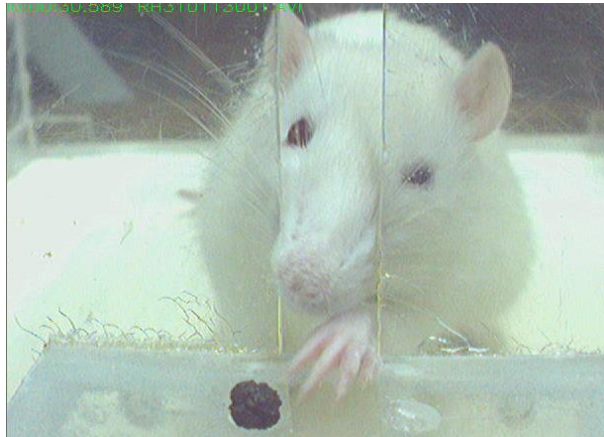
Figure 6. Rat performing the reach to grasp task

2) Cylinder task – This task was used to compare the spontaneous use of the ipsilateral (unaffected) and contralateral (affected) arms before and after stroke. A transparent cylinder 20 cm in diameter and 30 cm high was used to measure the forelimb use during explorative activity. The rats were videotaped for 3–10 min depending on the movement of the rat during the trial (Fig. 7). 2 mirrors were placed behind the rats at an angle such that the forelimb movement could be discerned even when the rat was not facing the video camera. The behavior of the animal to stand up straight using its forelimbs as support on the wall of the cylinder were scored to determine the extent of forelimb-use symmetry displayed by the animal. This behavior consisted of independent use of the left or right forelimb for contacting

the wall and simultaneous use of both the left and right forelimb for contacting the wall of the cylinder. (Bland et al., 2000)

Limb use = $\dfrac{\text{(contralateral + 1/2 both)}}{\text{(ipsilateral + contralateral + both)}}$ x100

Contralateral refers to the affected limb with ET-1 induced into opposite hemisphere.

Figure 7. Forelimb usage assessment in the cylinder task

3) Gait task - The gait analysis was used to check alterations, if any, in stride length and base of support. The bottoms of the rats' forelimbs were painted in red ink and the hindlimbs in blue ink. The rats were then made to walk on a long sheet of plain paper to record the gait pattern (Fig. 8). After identification and labelling of each footprint, the stride length was calculated by measuring the distance between the ball mount region of the footprint in 2 consecutive and the base of support was calculated by measuring the distance between the forelimbs. The average of 4 hindpaw/forepaw strides when the animal was walking continuously at a constant pace was analysed. (Glajch et al., 2012)
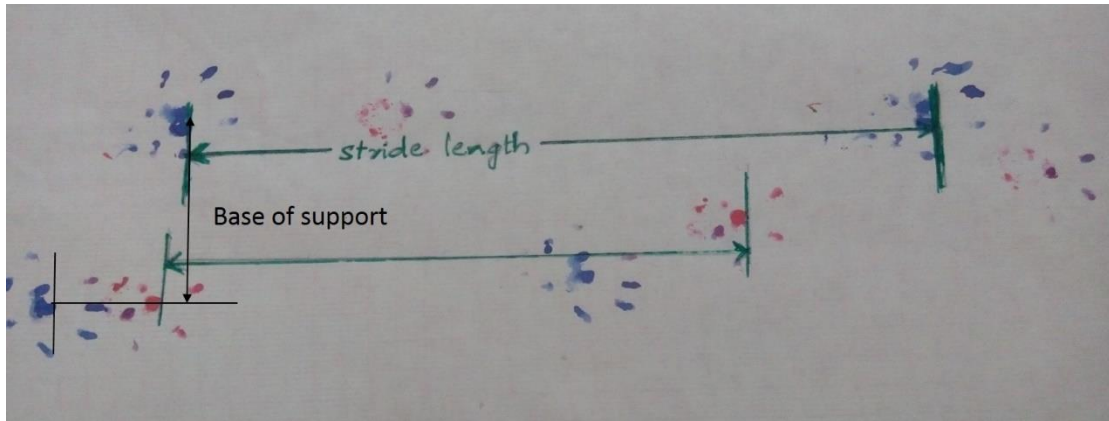
Figure 8. Footprints of rats measured for gait analysis

4) Horizontal ladder – The horizontal ladder task was used to check the sensory-motor integration of the forelimbs when walking on a horizontal ladder. The horizontal ladder rung test apparatus consisted of a ladder of metal rungs 1cm apart with glass vertically on either side. The rat was made to walk on the horizontal ladder after a couple of rounds of training and the movement was videotaped from below (Fig. 9). The grip of each forelimb was scored by analyzing the foot or paw placement on the rung using a 3 point scale (Fig. 10). 0 points were awarded when the paw completely slipped between the rungs and there was no grip, 1 point was awarded when the paw was kept on the rung without the digits holding on to the rung and 2 points were awarded when the midportion of the paw was placed on the rung with full support from the digits. Only the stroke affected limb was analyzed

Figure 9. Horizontal ladder test to access the coordinated motor movement
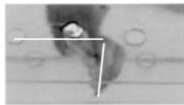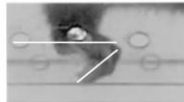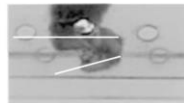
Figure 10. Representative images illustrating the three categories of digit score.(Metz and Whishaw, 2009)

<u>Histology- Golgi-Cox staining:</u> Golgi-Cox staining is a staining technique used to visualize neurons along with the dendrites and dendritic spines fully across all 6 layers of the cortex. This technique is used for observing the cellular geometry, orientation and branching of the dendrites including the arborization.

On the third day of surgery at the conclusion of the behavioural tasks, the rats were deeply anaesthetized with an overdose of halothane anesthesia. Following anesthesia, the rats were transcardially perfused with 0.1M PBS through the left ventricle. At the end of the perfusion, the rats were decapitated and the brains were shelled out carefully. Each brain was cut according Fig. 11 (Ranjan and Mallick, 2010) and only parts A1, A2, B1 and B2 were used for the further experiments. Each tissue block was

separately put in a dark bottle filled with Golgi-Cox solution and incubated for 24 hours at 37°C.
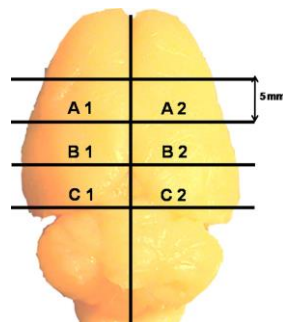
Figure 11. A rat brain showing the four coronal and one sagittal cuts given to obtain six tissue blocks from each brain

Golgi-Cox solution:

    Solution A: 5% potassium dichromate in distilled water (20 parts)

    Solution B: 5% mercuric chloride in distilled water (20 parts)

    Solution C: 5% potassium chromate in distilled water (16 parts)

Mix solution A and B. Mix solution C and 40 parts distilled water. Slowly pour A+B into C while stirring continuously. Store in glass stopped dark bottle for 5 days.

At the end of the incubation period, 250 µm thick sections were prepared using a vibratome in 6% sucrose. The sections were stored in 70% alcohol for maximum 2 days if the processing was not to follow the sectioning immediately. The sections were processed as discussed below:

1) Rinse twice (5 minutes each) in distilled water
2) Dehydrate in 50% alcohol for 5 minutes
3) Keep in ammonia solution (Ammonia:water, 3:1) for 7 minutes (Start from this step if stored in 70% alcohol)
4) Rinse twice (5 minutes each) in distilled water
5) Keep in 5% sodium thiosulfate solution for 10 minutes in dark.
6) Rinse twice (2 minutes each) in distilled water

7) Dehydrate (5 minutes each) in 70%, 80% and 95% ethanol, 99% 1-butanol, cleared in toluene and mounted in DPX on gelatinized slides.
8) Dry at room temperature

The sections were viewed under a microscope at low and high magnifications using the Neurolucida software (MBF Biosciences, USA). Neurons were viewed randomly in layer V of the motor cortex and they had to meet the following inclusive criteria to be selected for tracing: i) the cell body, axons or dendrites should not be affected by stain precipitations, blood vessels, or heavy clusters of dendrites from other cells and ii) neurons must be dark and consistently stained throughout the extent of all dendrites. The morphology of Betz cells was studied in layer V of the motor cortex both ipsilateral and contralateral to the side which received the ET-1 insult. The neurons in the ipsilateral hemisphere were taken as the stroke, short term genistein + stroke or long term genistein + stroke accordingly while the neurons in the respective contralateral hemisphere were taken in the control group. After drawing cells in the above mentioned area using the camera, the total dendritic length and branch numbers were analyzed using Sholl's analysis (Fig. 12). Counting was done blind to the treatment received.
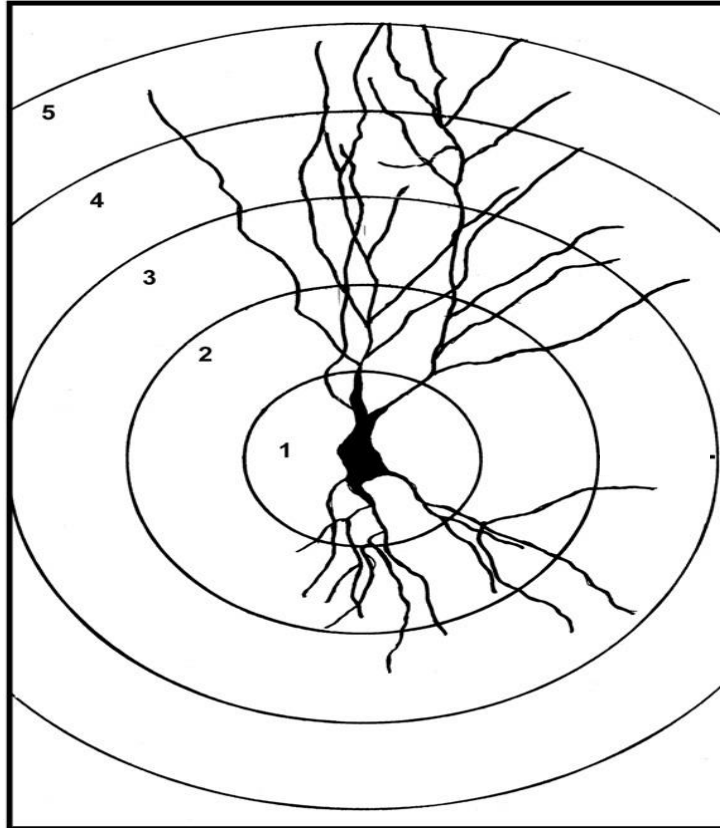
Figure 12. Schematic representation of Scholl's method for quantitative arborization of pyramidal neurons in the motor cortex. The dendritic branching points and intersections were counted in successive radial segments of 10µm, taking the centre of the soma as a reference point and the point at which the dendrites cross the concentric circles was considered the intersection point. (Bindu et al., 2007)

Statistical analysis:

The reach to grasp, cylinder and gait tasks data was expressed as mean ±SD. The horizontal ladder task data was expressed as a percentage of mean. All the behavioural and histological tasks were compared using the one-tailed Student's t-test for paired samples. In all analyses, the null hypothesis as rejected at the 0.01 level.

RESULTS

Results

1) TTC staining-

    We observed an infarct in the brain tissue of the ET-1 infused rat as given by a white patch in the motor cortex shown in Fig. 13. The corresponding brain tissue of the control animals showed no such infarct. The brain tissue of the animals which were administered a dose of genistein 1 hour before surgery also did not show any significant infarct.
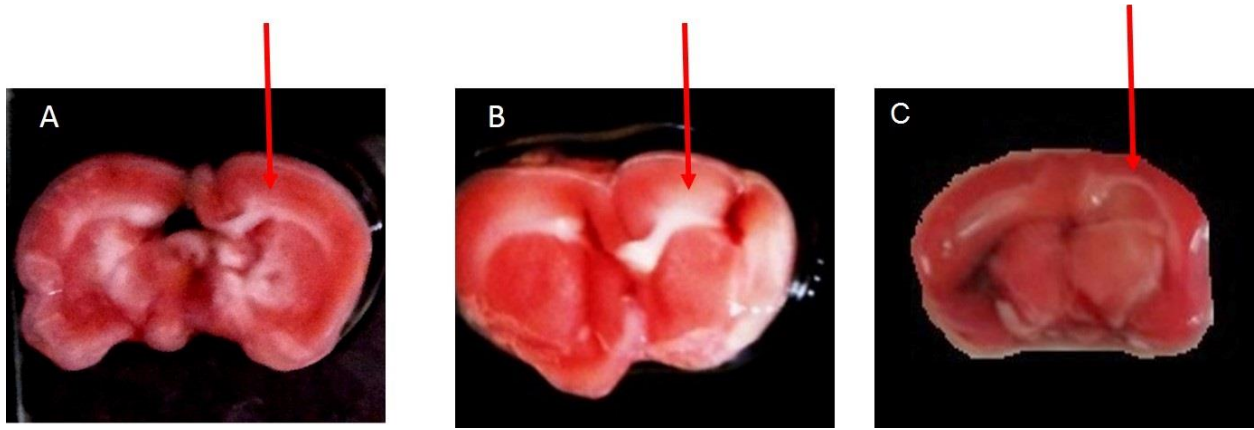
Figure 13. TTC staining for A. Control, B. Stroke and C. Short-term genistein+stroke rats. The arrow points at the region of the motor cortex which has turned white in the stroke rats (B) while remaining pink like the rest of the brain in the control (A) and short-term genistein+stroke (C) rats.

2) Reach to grasp task

a) Total reach success percentage-

*Figure 14. Total reach success percentage represented as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, ***p<0.001.*

b) First hit success percentage-
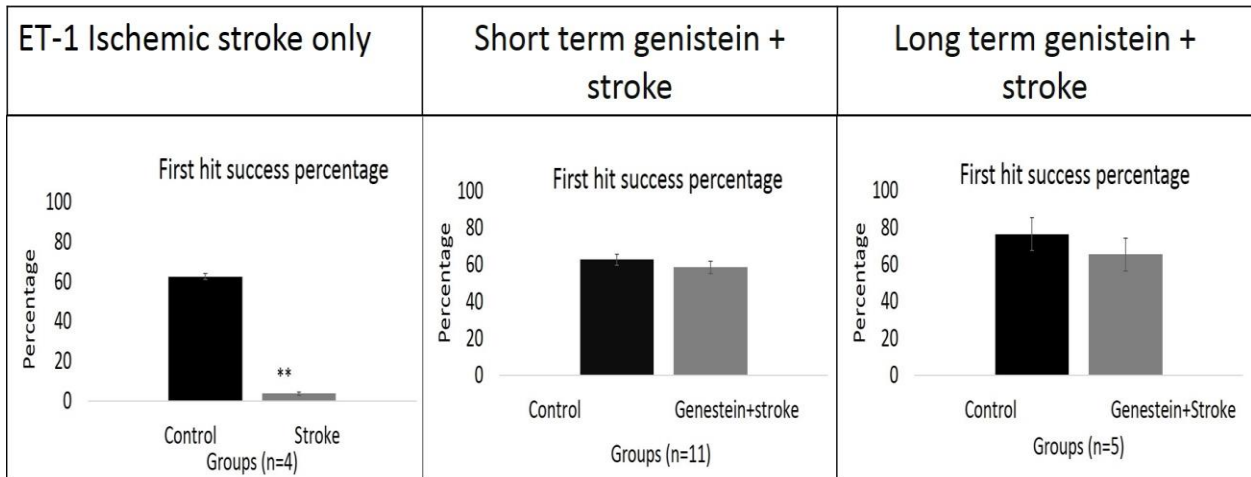
*Figure 15. First hit success percentage represented as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, ***p<0.001.*

3) Cylinder task-
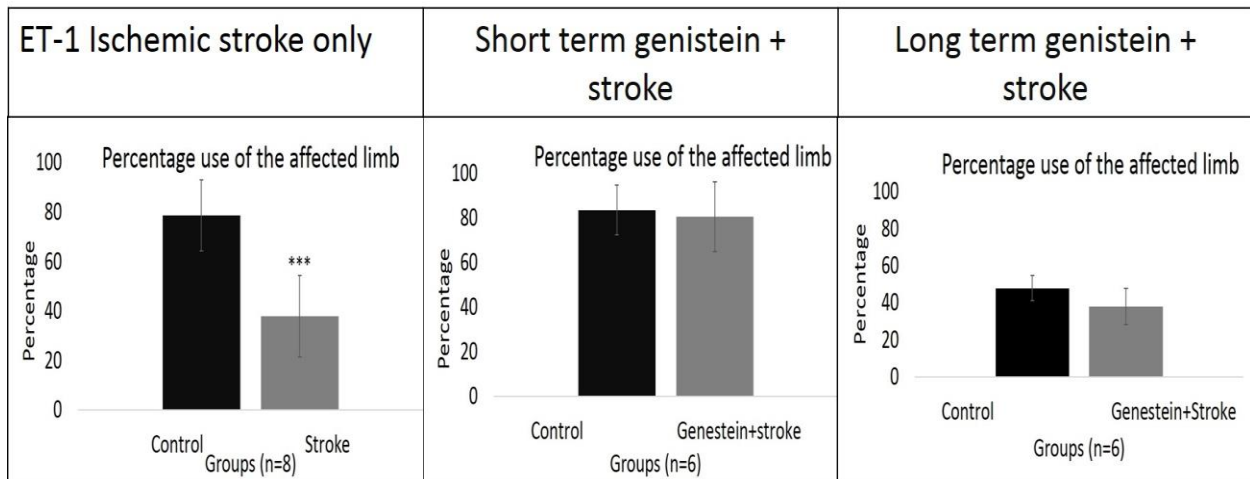
*Figure 16. Percentage use of affected (contralateral) limb represented as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, ***p<0.001.*

4) Gait analysis

a) Stride length-
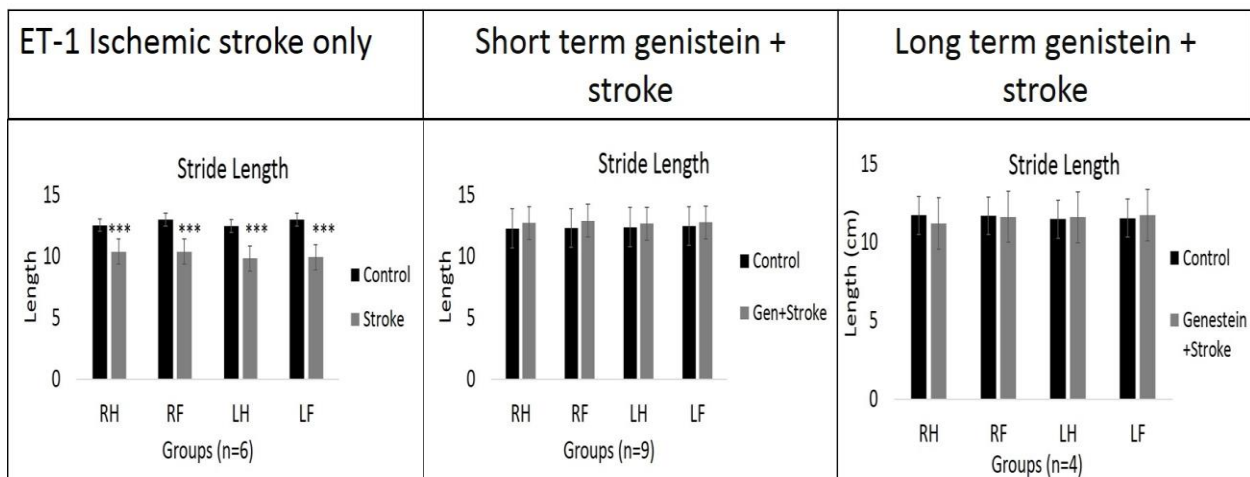
*Figure 17. (RH=Right Hind limb, RF=Right Forelimb, LH=Left Hind limb, LF=Left Forelimb) Stride length for each limb represented as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, ***p<0.001.*

b) Base of support-
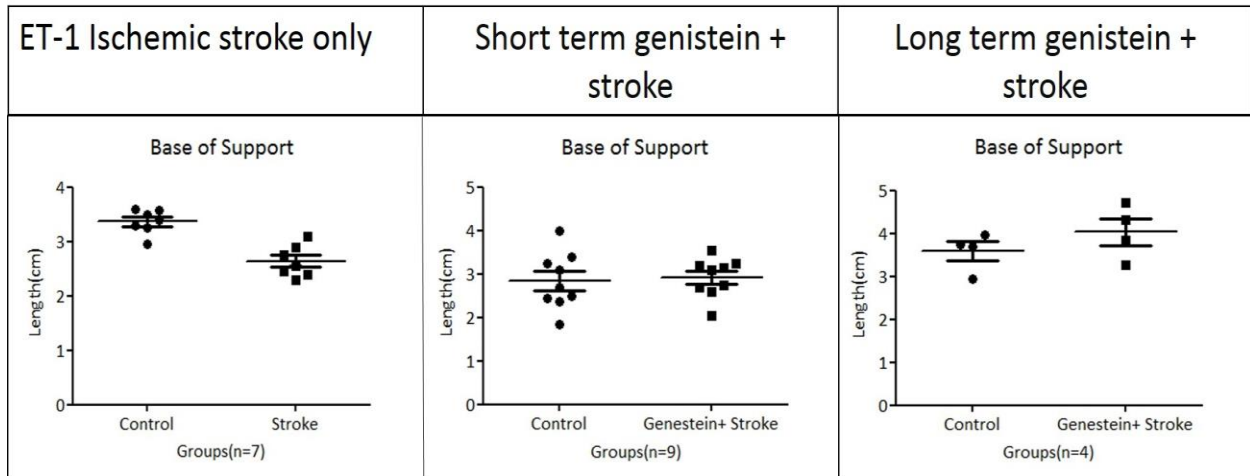
Figure 18. Base of support (Distance between forelimbs) represented as a scatter plot with Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, **p<0.01.

5) Horizontal ladder-

Figure 19. 1 point - partial grip when paw was kept on the rung without the digits holding on to the rung

Figure 20. 2 points - the mid portion of the paw was placed on the rung with full support from the digits.
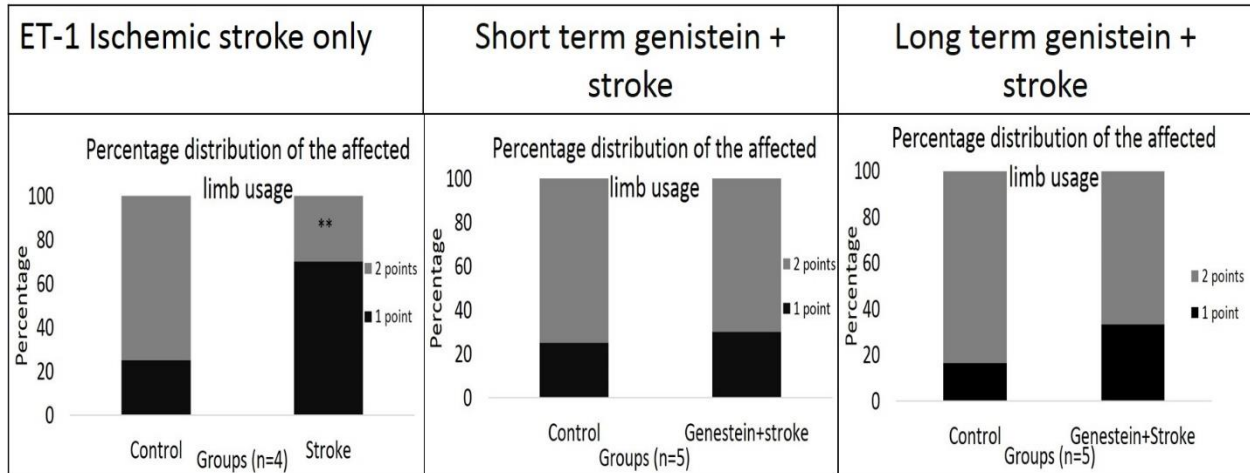
*Figure 21. Points were awarded based on the following parameters: 0 points - no grip and paw slipped through the rungs, 1 point - partial grip when paw was kept on the rung without the digits holding on to the rung and 2 points - the mid portion of the paw was placed on the rung with full support from the digits. Percenta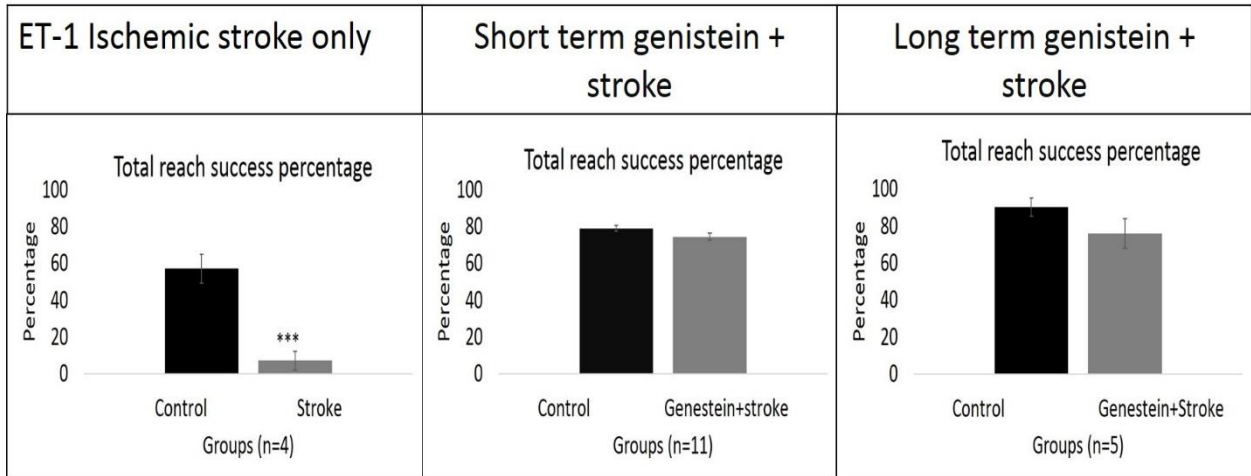ge of paw placement represented as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats. Paired students t-test, ***p<0.001.*

6) Golgi-Cox staining
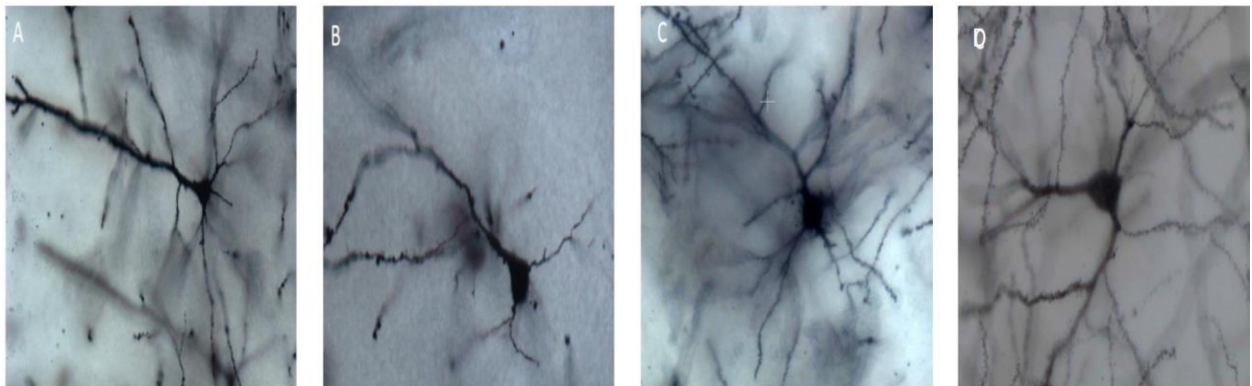
*Figure 22. Representative neuron images after Golgi-Cox staining from A. Control, B. Stroke, C. Short-term genistein + stroke and D. Long term genistein + stroke.*

a) Intersections-
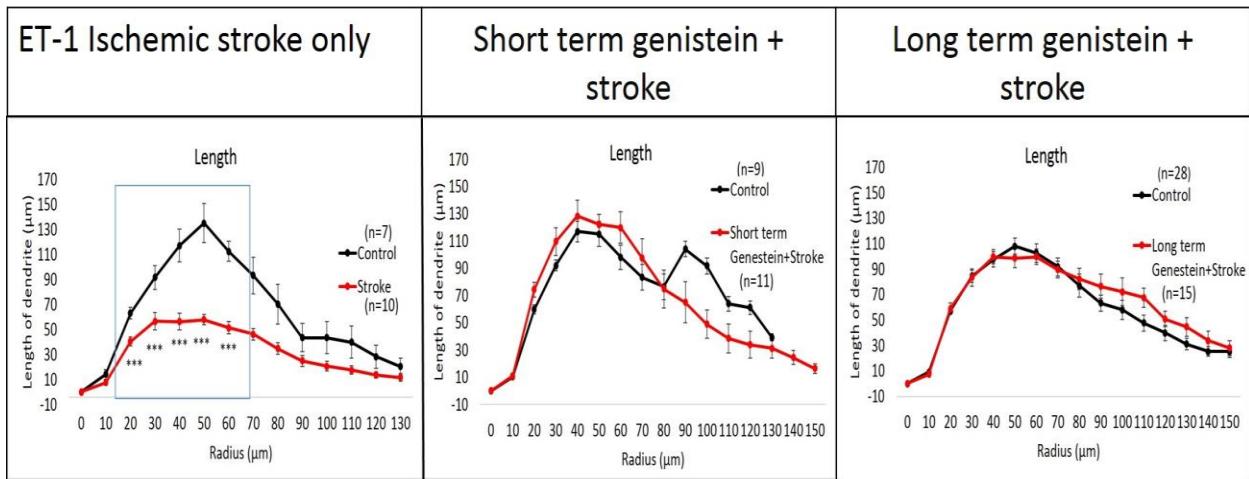
Figure 23. Branching intersections represented in points as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats across the radius of concentric circles as per Sholl's analysis. The box represents the range of radii which show a significant change in intersections. Paired students t-test, ***p<0.001.

b) Length-
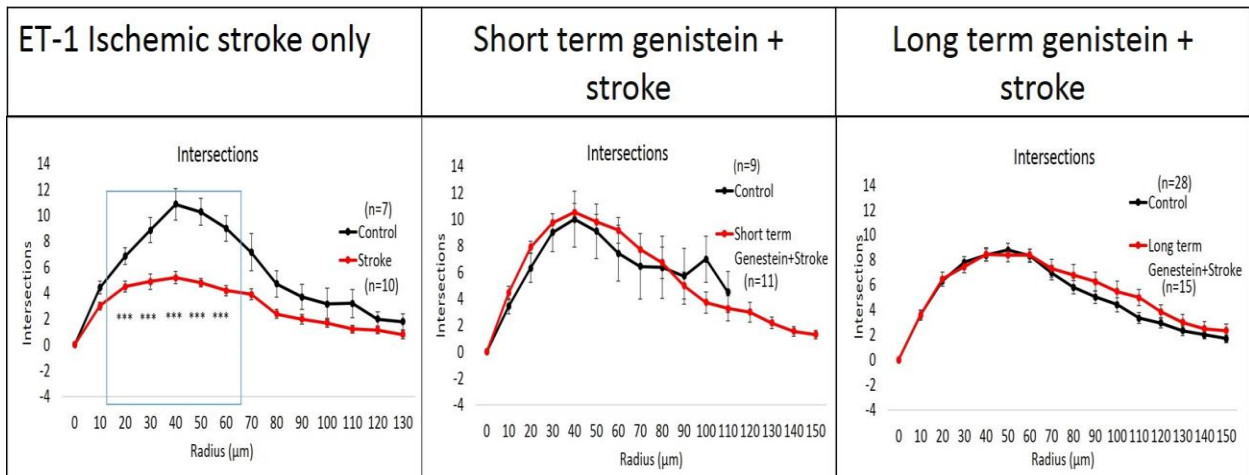
Figure 24. Length of dendrites represented in points as Mean ± SD from control, stroke, short term genistein + stroke and long term genistein + stroke rats across the radius of concentric circles as per Sholl's analysis. The box represents the range of radii which show a significant change in length of dendrites. Paired students t-test, ***p<0.001.

Video recording of the rats was done both pre- and post-stroke, i.e. the same animal was used within a group.

2) Reach to grasp task-

   The reach to grasp task was performed to analyze the skilled motor movements in the animals. Results are as shown below:

   a) Total success-

   A successful reach score was obtained from the formula = $\underline{\text{Number of hits}}$

   $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ Number of reaches

   Where hit refers to a single reach movement which results in a successful grasp of the food pellet and transfer to the mouth; and reach refers to any advancement of the forelimb.

As shown in Fig. 14, the total reach success was significantly lesser in the ET-1 induced stroke animals as compared to the control animals (pre-stroke) going down to almost zero. However, both short term and long term administration of genistein before inducing stroke by ET-1 resulted in a total reach success with an almost equal percentage as the respective control animals (pre-stroke). A paired t-test between the groups showed a statistical significance of $p < 0.001$.

   b) First trial success-

   Success % = $\underline{\text{No of pellets obtained on first advance}}$ x100

   $\qquad\qquad\qquad\qquad\qquad\qquad$ 20

   20 refers to the total number of pellets given each time, i.e. the number of reach trials used for scoring.

As shown in Fig. 15, the first reach trial success percentage was significantly lesser in the ET-1 induced stroke animals as compared to the control animals (pre-stroke), going down to almost zero. However, both short term and long term administration of genistein before inducing stroke by ET-1 resulted in the first reach trial success with an

almost equal percentage as the respective control animals (pre-stroke). A paired t-test between the groups showed a statistical significance of $p<0.001$.

3) Cylinder task-

The cylinder task was performed to compare the spontaneous use of the ipsilateral (unaffected) and contralateral (affected) arms before and after stroke. Results are as shown below:

Limb use = $\dfrac{\text{(contralateral + 1/2 both)}}{\text{(ipsilateral + contralateral + both)}}$ x100

Contralateral refers to the affected limb with ET-1 induced into opposite hemisphere.

As shown in Fig. 16, the percentage use of the affected forelimb was significantly lesser in the ET-1 induced stroke animals as compared to the control animals (pre-stroke), reducing from 80% to 40%. However, both short term and long term administration of genistein before inducing stroke by ET-1 resulted in the usage of the affected forelimb with an almost equal percentage as the respective control animals (pre-stroke). A percentage of 0 meant that limb wasn't used at all while a percentage of 100 meant complete usage of the limb. A paired t-test between the groups showed a statistical significance of $p<0.001$.

4) Gait task –

The gait analysis was used to check alterations, if any, in stride length and base of support during locomotion of the animal. The average of 4 hindpaw/forepaw strides when the animal was walking continuously at a constant pace was analysed.

As shown in Fig. 17, the stride length for each limb was significantly lesser in the ET-1 induced stroke animals as compared to the control animals (pre-stroke). However, both short term and long term administration of genistein before inducing stroke by ET-1 resulted in the stride length to be almost equal to that of the respective control animals (pre-stroke). Similarly, as shown in Fig. 18, the base of support for the ET-1 induced stroke animals was lesser than the base of support for the control animals (pre-stroke). Both short term and long term administration of genistein resulted in recovery of the

base of support of those animals to a level almost equal to the respective control animals (pre-stroke). Both the above gait tests showed that the gait of the rat became smaller both in terms of stride length and base of support. A paired t-test between the groups showed a statistical significance of $p<0.01$.

5) Horizontal ladder task –

The horizontal ladder task was used to check the sensory-motor integration of the forelimbs when walking on a horizontal ladder.

As shown in Fig. 21, the percentage distribution of the affected forelimb usage in the control animals (pre-stroke) shows a greater inclination to score 2 points as compared to 1 point which is in contrast to the ET-1 induced stroke animals. However, both short term and long term administration of genistein before inducing stroke by ET-1 resulted in a similar percentage distribution of the affected forelimb usage as that of the respective control animals (pre-stroke). A paired t-test between the groups showed a statistical significance of $p<0.001$.

6) Golgi-Cox staining-

Golgi-Cox staining was performed to visualize the neurons and observe their morphology. 2 parameters, their arborization (branching intersections) and length of dendrite, were measured using Sholl's analysis to determine any changes in morphology of the neurons and to validate their atrophy. The ipsilateral and contralateral (opposite to the hemisphere which received ET-1 injection) hemispheres of the brain were compared. As shown in Fig. 22, the neurons from the stroke group had reduced dendritic length and number of dendrites while the neurons from the short term genistein + stroke, and long term genistein + stroke group had similar branching patterns as that of the respective controls, showing recovery in atrophy. Only the Betz cells in the motor cortex were used in this experiment.

a) Branching intersections-

As shown in Fig. 23, the branching intersections in the control group (contralateral brain hemisphere) was significantly higher than the branching intersections in the stroke groups (ipsilateral brain hemisphere) between radius 20-60μm. However the branching intersections in the control groups were found to be similar in number to the branching intersections in the groups receiving a short term and long term administration of genistein separately before inducing stroke by ET-1 for all the radius values. A paired t-test between the groups showed a statistical significance of $p < 0.001$.

b) Length-

As shown in Fig. 24, the length of the dendrites in the control groups was significantly higher than the length of the dendrites in the stroke groups between radius 20-60μm. However the dendritic length in the control groups were found to be similar to the dendritic length in the groups receiving a short term and long term administration of genistein before inducing stroke by ET-1 for all radius values. A paired t-test between the groups showed a statistical significance of $p < 0.001$.

DISCUSSION

Discussion

The purpose of this study was to validate the neuroprotective effects of short-term (acute) and long-term (chronic) administration of genistein in an ET-1 induced ischemic stroke model using behavioural and morphological studies.

Stroke is defined as "a neurological deficit of cerebrovascular cause" ultimately causing death and dysfunction of brain cells. The after effects of stroke include motor impairment, on the contralateral side of the brain lesion and memory loss. Currently, the most widely used treatment to combat stroke is the use of a tissue plasminogen activator (tPA). Various neuroprotective strategies have also been found to be successful in human and animal studies. There are many evidences which show that estrogen can induce protection in brain injuries although use of estrogen may have some undesirable side effects. Genistein, an isoflavone, is a naturally occurring phytoestrogen found in soy plant which has recently been found to be a potential neuroprotectors against ischemic stroke. It does not show any side effects and has an ability to cross the blood-brain barrier due to its favourable lipophilic property.

Genistein when given in a high dose (10mg/kg) 1 hour before surgery in rats confers neuroprotection against Parkinson's disease (Baluchnejadmojarad et al., 2009). Another study shows that a continuous treatment of genistein (10mg/kg every day for 2 weeks) in male rats significantly reduced the cerebral infarct size following middle cerebral artery occlusion. The mechanisms by which this occurs is currently not clear and various hypotheses have been proposed. However, behavioural and histological studies are not too many and this study proposes to establish both a behavioural and histological model to quantify the neuroprotection conferred by genistein.

The brains obtained from control, stroke and short-term genistein+stroke rats were stained with TTC solution and stroke was confirmed by visualization of an infarct in the motor cortex. The infarct occurs due to the inability of the dead cells in the ischemic

core region of the brain to take up the TTC dye. Rats that received ET-1 showed infarct in the motor cortex. However when genistein (10mg/kg) was administered 1 hour prior to ET-1 insult, the volume of the infarct region was reduced compared to rats that received only ET-1.

We initiated our behavioural studies with the reach to grasp task. The reach to grasp task was performed to assess the skilled motor movements of the animals. There was a statistically significant difference in both total reach success and first hit success in control animals (pre-stroke) and animals which received ET-1 induced stroke. These results were similar to those observed by Ian Whishaw which suggested that motor cortex lesions lead to an impairment in reaching performances (Whishaw, 2000). However, both short term (10mg/kg) and long-term (1mg/kg every day for ten days) administration of genistein before inducing stroke by ET-1 managed to give neuroprotection to the animals. We observed an almost equal total reach success and first hit success between both the genistein + stroke groups and the control group (pre-stroke). This finding could contribute to the understanding of skilled forearm movements, suggesting that genistein administration might be protective against ET-1 induced ischemic stroke.

The cylinder task was used as a behavioural paradigm to compare the spontaneous use of the contralateral forelimbs before and after stroke. Use of the affected forelimb significantly decreased after ET-1 stroke in the stroke animals as compared to the control animals (pre-stroke). Short-term and long-term treatment of genistein showed a significant improvement in the use of the affected arm as compared to the stroke animals, with the affected arm usage being almost equal to the control animals. These results were similar to those obtained with bFGF (a dendrite growth promoting factor) in an MCAO stroke model (Kawamata et al., 1998). Such type of tests might prove to be useful in screening various neuroprotective drugs for recovery in function after ischemic injury.

We used a basic version of the gait analysis where ink was applied to the paws and animal was made to walk across a sheet of paper. Our results showed that the stride length and base of support of the rats reduced significantly after induction of stroke by

ET-1 as compared to the control animals (pre-stroke). Administration of genistein (short term and long term) increased the stride length and base of support of the rats that had been altered by ET-1 and it was almost similar to that of control animals. Gait analysis increases the understanding of locomotion and is regularly used as a progress measurement in humans undergoing rehabilitation after stroke. (Balkaya et al., 2013)

Accurate placement of the affected limb was examined using the horizontal ladder test which has been shown to be sensitive to chronic movement deficits after stroke. Prior to inducing stroke, the rats showed mostly correct placements of their forelimbs but after stroke, they made more errors in placements leading to a lower score. Exposure to either short-term or long-term genistein before stroke led to the rats making less errors in forelimb placement as compared to the stroke animals thus leading to a better score almost equal to that of the controls (Farr et al., 2006). This task represents a reliable method of gaining insight into the stepping patterns of the rat without any rewards like food or water. It also does not require extensive training before the rat learns to walk.

Golgi-Cox staining is a staining technique used to visualize neurons along with their dendrites and dendritic spines across all 6 layers of the cortex. This can be used to check various parameters but we chose to look at the dendritic length and the branching pattern of the dendrite which are a rudimentary measure of the structure of a neuron. We used the control for each of the 3 groups, stroke, short-term genistein + stroke and long-term genistein + stroke from the same brain. The brain hemisphere which did not receive ET-1 injection was taken as the control. There was a significant difference in the branching intersections and length of the dendrites between the control and stroke sides of the brain within the radius 20-60μm. The radius signifies the distance from the soma of the neuron as given by Sholl's analysis. This suggested that neuronal atrophy occurred at this distance away from the soma. One of the theories which have been suggested to explain neuronal atrophy is the increased release of glutamate by excess glucocorticoids. Increase in glutamate levels leads to cell damage. Another theory states that after ischemia, macrophages in the brain phagocytose viable neurons leading to neuronal atrophy. However, no evident atrophy was seen in neurons in the short-term genistein + stroke groups and the long-term genistein + stroke groups at any

radius. This supported our theory that genistein was useful in recovery of the neurons after stroke.

The results helped us to speculate that genistein could be beneficial in providing neuroprotection to the rats and helped in the process of brain recovery after stroke. Most of the previous studies were conducted by injecting a concentration of 10 mg/kg genistein every day for 2 weeks before inducing stroke. Other studies have been done which checked various doses of genistein (2.5mg/kg, 5mg/kg and 10mg/kg) and proved that genistein even at 2.5mg/kg for 2 weeks also showed neuroprotection against ischemic stroke (Qian et al., 2012). We wanted to check the least concentration at which genistein showed neuroprotection and hence we injected 1 mg/kg of genistein every day for ten days instead of 10mg/kg every day. Along with this, we could also check if a total of 10mg/kg genistein across 10 days had the same effect that an acute dose of 10mg/kg genistein prior to stroke had. The behavioral and histological analyses did not show any significant difference between the brain recovery induced by a short dose of genistein and a prolonged dose of genistein. However, there seems to be a slight difference between the protection conferred by the short term dose of genistein and the long term dose, though not significantly different. Increasing the number of rats could probably lead to a difference in the neuroprotection conferred by short-term genistein and long-term genistein. One of the possible reasons why no difference was seen could be that the concentration of genistein (1mg/kg) used in the long-term administration was not enough to induce complete neuroprotection as seen in the rats which received a dose of 10mg/kg just before inducing stroke although the total genistein injected was the same. Though most of the work done earlier demonstrates that long term administration of genistein is required to confer neuroprotection, we observed that short term treatment of genistein was also sufficient to induce neuroprotection.

The proper mechanism by which genistein acts in the cerebral cortex is not entirely clear. However, there is growing evidence that genistein confers neuroprotection through membrane associated estrogen receptors as well as through the genomic pathway which use intracellular estrogen receptors (Mendelsohn and Karas, 2010). This

could be the reason why short-term administration of genistein is also able to confer neuroprotection. An acute injection of genistein could be triggering the membrane associated estrogen receptors and causing a faster response as compared to triggering a response through the genomic pathway (McEwen et al., 2012). This is validated by studies which show that estrogen also acts through membrane associated ERβ (Walf and Frye, 2008). Various other mechanisms have been proposed which allow genistein to confer neuroprotection to the animal after stroke. Isoflavones, like genistein are said to be responsible for the anti-atherosclerotic benefits of soy. Genistein has anti-oxidative properties and it gives protection against vascular dysfunction by inhibiting lipid peroxidation, prevention of accumulation of free radicals and neutralizing the amount of NO produced by the endothelium which in turn reduces the atherosclerotic plaque (Davignon and Ganz, 2004). Genistein reduces ROS production which reduces mitochondrial dysfunction. This leads to less caspase-3 activation and less DNA fragmentation (Qian et al., 2012). Genistein has estrogen-like biological activity and being a phytoestrogen, it also acts on estrogen receptors. Genistein has a stronger affinity for the ERβ receptor than ERα and it can affect regulation of the transcription of mitochondrial genes.

Inclusion of soy in one's regular diet could lead to protection against stroke in people prone to it. In summary, this study establishes a potential basis for the protective effect of genistein administration with development of therapeutic strategies and this may be put forward as a novel treatment strategy for this disease. However, more work has to be done to elucidate the mechanism behind genistein proving to be neuroprotective. Further studies could be done by raising the concentration of genistein injected every day and checking when the effect is the same as that of genistein administered an hour before inducing stroke. Whether using genistein as a preventive measure after stroke will be useful, still has to be tested.

References

Balkaya, M., Krober, J.M., Rex, A., and Endres, M. (2013). Assessing post-stroke behavior in mouse models of focal ischemia. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism *33*, 330-338.

Baluchnejadmojarad, T., Roghani, M., Nadoushan, M.R., and Bagheri, M. (2009). Neuroprotective effect of genistein in 6-hydroxydopamine hemi-parkinsonian rat model. Phytotherapy research : PTR *23*, 132-135.

Bindu, B., Alladi, P.A., Mansooralikhan, B.M., Srikumar, B.N., Raju, T.R., and Kutty, B.M. (2007). Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions. Neuroscience *144*, 412-423.

Bland, S.T., Schallert, T., Strong, R., Aronowski, J., Grotta, J.C., and Feeney, D.M. (2000). Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats : functional and anatomic outcome. Stroke; a journal of cerebral circulation *31*, 1144-1152.

Burguete, M.C., Torregrosa, G., Perez-Asensio, F.J., Castello-Ruiz, M., Salom, J.B., Gil, J.V., and Alborch, E. (2006). Dietary phytoestrogens improve stroke outcome after transient focal cerebral ischemia in rats. The European journal of neuroscience *23*, 703-710.

Casals, J.B., Pieri, N.C., Feitosa, M.L., Ercolin, A.C., Roballo, K.C., Barreto, R.S., Bressan, F.F., Martins, D.S., Miglino, M.A., and Ambrosio, C.E. (2011). The use of animal models for stroke research: a review. Comparative medicine *61*, 305-313.

Cyr, M., Calon, F., Morissette, M., and Di Paolo, T. (2002). Estrogenic modulation of brain activity: implications for schizophrenia and Parkinson's disease. Journal of psychiatry & neuroscience : JPN *27*, 12-27.

Davignon, J., and Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. Circulation *109*, III27-32.

Farr, T.D., Liu, L., Colwell, K.L., Whishaw, I.Q., and Metz, G.A. (2006). Bilateral alteration in stepping pattern after unilateral motor cortex injury: a new test strategy for analysis of skilled limb movements in neurological mouse models. Journal of neuroscience methods *153*, 104-113.

Francis, J., Raghunathan, S., and Khanna, P. (2007). The role of genetics in stroke. Postgraduate medical journal *83*, 590-595.

Garcia, J.H., Liu, K.F., Yoshida, Y., Chen, S., and Lian, J. (1994). Brain microvessels: factors altering their patency after the occlusion of a middle cerebral artery (Wistar rat). The American journal of pathology *145*, 728-740.

Gibson, C.L., Gray, L.J., Murphy, S.P., and Bath, P.M. (2006). Estrogens and experimental ischemic stroke: a systematic review. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism *26*, 1103-1113.

Glajch, K.E., Fleming, S.M., Surmeier, D.J., and Osten, P. (2012). Sensorimotor assessment of the unilateral 6-hydroxydopamine mouse model of Parkinson's disease. Behavioural brain research *230*, 309-316.

Go, A.S., Mozaffarian, D., Roger, V.L., Benjamin, E.J., Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., *et al.* (2013). Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation *127*, e6-e245.

Guyton, A.C., and Hall, J.E. (2006). Textbook of medical physiology, 11th edn (Philadelphia: Elsevier Saunders).

Jones, T.H., Morawetz, R.B., Crowell, R.M., Marcoux, F.W., FitzGibbon, S.J., DeGirolami, U., and Ojemann, R.G. (1981). Thresholds of focal cerebral ischemia in awake monkeys. Journal of neurosurgery *54*, 773-782.

Kawamata, T., Ren, J., Chan, T.C., Charette, M., and Finklestein, S.P. (1998). Intracisternal osteogenic protein-1 enhances functional recovery following focal stroke. Neuroreport *9*, 1441-1445.

Majid, A. (2014). Neuroprotection in stroke: past, present, and future. ISRN neurology *2014*, 515716.

McEwen, B.S., Akama, K.T., Spencer-Segal, J.L., Milner, T.A., and Waters, E.M. (2012). Estrogen effects on the brain: actions beyond the hypothalamus via novel mechanisms. Behavioral neuroscience *126*, 4-16.

Mendelsohn, M.E., and Karas, R.H. (2010). Rapid progress for non-nuclear estrogen receptor signaling. The Journal of clinical investigation *120*, 2277-2279.

Metz, G.A., and Whishaw, I.Q. (2000). Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behavioural brain research *116*, 111-122.

Metz, G.A., and Whishaw, I.Q. (2009). The ladder rung walking task: a scoring system and its practical application. Journal of visualized experiments : JoVE.

Musa, M.A., Khan, M.O., and Cooperwood, J.S. (2007). Medicinal chemistry and emerging strategies applied to the development of selective estrogen receptor modulators (SERMs). Current medicinal chemistry *14*, 1249-1261.

Nikkhah, G., Rosenthal, C., Hedrich, H.J., and Samii, M. (1998). Differences in acquisition and full performance in skilled forelimb use as measured by the 'staircase test' in five rat strains. Behavioural brain research *92*, 85-95.

Purves, D. (2004). Neuroscience, 3rd edn (Sunderland, Mass.: Sinauer Associates, Publishers).

Qian, Y., Guan, T., Huang, M., Cao, L., Li, Y., Cheng, H., Jin, H., and Yu, D. (2012). Neuroprotection by the soy isoflavone, genistein, via inhibition of mitochondria-dependent apoptosis pathways and reactive oxygen induced-NF-kappaB activation in a cerebral ischemia mouse model. Neurochemistry international *60*, 759-767.

Qureshi, I.A., and Mehler, M.F. (2010). Emerging role of epigenetics in stroke: part 1: DNA methylation and chromatin modifications. Archives of neurology *67*, 1316-1322.

Ranjan, A., and Mallick, B.N. (2010). A modified method for consistent and reliable Golgi-cox staining in significantly reduced time. Frontiers in neurology *1*, 157.

Rothman, S.M., and Olney, J.W. (1995). Excitotoxicity and the NMDA receptor--still lethal after eight years. Trends in neurosciences *18*, 57-58.

Sacco, R.L., Kasner, S.E., Broderick, J.P., Caplan, L.R., Connors, J.J., Culebras, A., Elkind, M.S., George, M.G., Hamdan, A.D., Higashida, R.T.*, et al.* (2013). An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke; a journal of cerebral circulation *44*, 2064-2089.

Sacrey, L.A., Alaverdashvili, M., and Whishaw, I.Q. (2009). Similar hand shaping in reaching-for-food (skilled reaching) in rats and humans provides evidence of homology in release, collection, and manipulation movements. Behavioural brain research *204*, 153-161.

Siow, R.C., and Mann, G.E. (2010). Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? Molecular aspects of medicine *31*, 468-477.

Srivastava, P.V., Sudhan, P., Khurana, D., Bhatia, R., Kaul, S., Sylaja, P.N., Moonis, M., and Pandian, J.D. (2014). Telestroke a viable option to improve stroke care in India. International journal of stroke : official journal of the International Stroke Society *9 Suppl A100*, 133-134.

Viscoli, C.M., Brass, L.M., Kernan, W.N., Sarrel, P.M., Suissa, S., and Horwitz, R.I. (2001). A clinical trial of estrogen-replacement therapy after ischemic stroke. The New England journal of medicine *345*, 1243-1249.

Walf, A.A., and Frye, C.A. (2008). Rapid and estrogen receptor beta mediated actions in the

hippocampus mediate some functional effects of estrogen. Steroids *73*, 997-1007.

Whishaw, I.Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. Neuropharmacology *39*, 788-805.

Whishaw, I.Q., Pellis, S.M., Gorny, B.P., and Pellis, V.C. (1991). The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. Behavioural brain research *42*, 77-91.

Whishaw, I.Q., Whishaw, P., and Gorny, B. (2008). The structure of skilled forelimb reaching in the rat: a movement rating scale. Journal of visualized experiments : JoVE.