2016

Examining Infarct Sizes in Female Sprague Dawley Rats in Response to a Delayed Post-Stroke Pharmacological Treatment in Combination with Physical Rehabilitation

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EXAMINING INFARCT SIZES IN FEMALE SPRAGUE DAWLEY RATS IN RESPONSE TO A DELAYED POST-STROKE PHARMACOLOGICAL TREATMENT IN COMBINATION WITH PHYSICAL REHABILITATION

A thesis submitted in partial fulfillmentof the
requirement for the degree of
Master of Science

By

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2016

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

August 09, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sayali Ravindra Dharmadhikari ENTITLED Examining Infarct Sizes In Female Sprague Dawley Rats In Response To A Delayed Post-Stroke Pharmacological Treatment In Combination With Physical Rehabilitation BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Dharmadhikari, Sayali Ravindra. M.S. Department of Pharmacology and Toxicology, Wright State University, 2016. Examining Infarct Sizes in Female Sprague Dawley Rats in Response to a Delayed Post-Stroke Pharmacological treatment In Combination with Physical Rehabilitation.

In this study, we hypothesize that a pharmacological drug treatment comprised of Fluoxetine, Simvastatin and Ascorbic acid together with physical rehabilitation would reduce infarct sizes.

Over the period of 60 days after stroke-induction, 13 of the 23 rats were administered the drugs beginning 20-26 hours after stroke-induction and the rest were assigned to the control group. Physical rehabilitation exercises were initiated from post-stroke day 8 and continued for 23 alternate days. The rats were tested for functional recovery using Montoya staircase apparatus and were euthanized after post-stroke day 60. The brains sections were analyzed using Nissl stain for infarct volume analysis.

Due to the excess amount physical rehabilitation exercise in comparison to the previous studies conducted in our laboratory, the rats suffered large amount of stress. Stress antagonizing neurogenesis is believed to be the reason for lack of motor functional recovery and lack of any reduction in infarct sizes.

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ACKNOWLEDGEMENTS

These have been two great years for me, thanks to the many wonderful people I have met here in the United State of America. I would like to thank Dr. Adrian Corbett for being such an amazing mentor that words fall short to describe her. She has always been so easy to talk to, may it be about research, books or games. She helped me understand the world of stroke research and taught me from the basics. She never considers any question unimportant and trusts her students immensely. Also, I cannot appreciate enough the lab members of Corbett Lab. Even though we have hardly worked together, we contributed a great deal to each other's work. I am glad to have come across such a happy lab.

I would like to thank my thesis committee members, Dr. Yanfang Chen and Dr. Ji Chen Bihl for being so supportive and stress-relieving. They have always known the power of encouragement. They have always appreciated and acknowledged the hard work.

I would like to thank my friends who have been like my family in the US. We have all grown up a lot in the past two years since our arrival in the US and it was fun growing up with them.

Most of all, I would like to offer my gratitude to my family back in India. They have appreciated every small complement I have received, teaching me to focus on the positives in life. Thank you for always being my confidant and for believing in me. I am truly blessed to have been born in a family that is so caring and loving.

I. INTRODUCTION

Stroke is the fifth leading cause of death in the United States of America. Every year, nearly 795,000 people are affected by stroke, out of which 185,000 have previously suffered one. Stroke is said to kill almost 130,000 Americans every year at the rate of one death every 4 minutes on an average(Writing Group et al., 2016).It costs the US around $68.9 billion dollars every year including the costs of treatments, medications and loss of work(Albright et al., 2010)

Stroke is mostly seen in the age range of 20 to 65 years(Writing Group et al., 2016). From 2009 to 2012, nearly 6.6 million Americans in this age group died of stroke. Ethnicity is also considered to be a factor causing stroke with African Americans, American Indians and Alaska natives being more prone to getting affected by stroke as compared to the other races. Stroke can also be a result of the standards of living as people with lower level of education and unhealthy lifestyle have a higher occurrence rate of stroke(Writing Group et al., 2016). Geography also seems to play a role in the occurrence of stroke. Southeastern United States is comparatively more stroke-affected than the rest of the country. Inherited risk is also a factor in the causation of stroke(Writing Group et al., 2016).

According to recent studies, a very less percentage of people have access to the primary stroke centers within an hour. Only 8.5% of the stroke-affected patients reach the hospital within the 3-hour window for an immediate tPA (tissue plasminogen activator) treatment.

Trained doctors can also make use of a wire-caged equipment called a stent to surgically remove the clot within six hours of stroke. Neurointerventionalists can perform a surgery to remove the blood clot 12 hours after the occurrence of stroke but that completely depends on finding a mis-match between perfusion and diffusion MRIs; however, it does not have a very strong research background (Gravanis & Tsirka, 2008). About 85% of the stroke patients, however, have to settle with an Aspirin treatment to avoid the occurrence of a second stroke and have to begin physical rehabilitation to make up for the neural damage.

To account for the seriousness of this disease, it is important to understand the risks attached to stroke and find ways to reduce them.

Types of stroke:

There are two types of stroke: Ischemic stroke and Hemorrhagic stroke.

Ischemic stroke is characterized by blockage of the blood vessels which supply blood to the brain. In this condition, the fatty tissue gets deposited in the blood vessel, narrowing the diameter of the blood vessel opening and the supply of oxygen to the brain cells is blocked when a clot finally blocks the flow of blood. This causes the death of neurons and hence, stroke. Ischemic stroke accounts for 87% of all the stroke cases. Ischemic strokes are of two types:

a. Cerebral thrombosis where the blood clot develops near the narrowed blood vessel in the brain.
b. Cerebral embolism where the blood clot is formed elsewhere in the body and travels to the site of the narrowed blood vessel(Zaret et al., 1992)

Hemorrhagic stroke, on the other hand, is caused because of a rupture in the blood vessel supplying blood to the brain. Due to the leak, the blood increases pressure on the brain cells and causes their death. Two main reasons for hemorrhagic stroke are, aneurysm-a balloon in the weak regions of the blood vessels which could burst and cause a hemorrhage- and arteriovenous malformation (AVM)-a cluster of abnormally formed blood vessels which could rupture. Hemorrhagic stroke is of two types:

a. Intracerebral hemorrhage where the blood leak is within the brain tissue
b. Subarachnoid hemorrhage where the blood leak is between the brain and the tissues surrounding it(Zaret et al., 1992).

Transient Ischemic stroke (TIA) is another type of stroke, also termed as 'mini stroke'. As its name suggests, it is a kind of ischemic stroke but the blockage of the blood vessels is for a very less amount of time. It usually lasts for only about five minutes; however, it should be taken as a serious indicator of the probable occurrence of a future stroke(Zaret et al., 1992)

Current treatment of stroke:

Administration of recombinant tissue plasminogen activator (r-tPA) intravenously through arm is the only FDA approved treatment for acute ischemic stroke since 1996 (Zivin et.al., 2009; Clark et al., 1999).

In ischemic stroke, a clot is formed in the blood vessels. Plasmin is a protein which is capable of breakdown of fibrins formed during a blood clot. It is an activated form of Plasminogen. tPA catalyzes the activation of Plasmin from plasminogen. This, ultimately,

helps in fibrinolysis; thus, dissolving the blood clot and helping with reperfusion of the affected tissue (Gravanis&Tsirka, 2008).

In the treatment involving the administration of r-tPA, a time window of 3 hours (4.5 hours in an extremely small percentage of eligible patients) in patients is extremely important. The patients must present themselves within 3 hours of the onset of stroke to be treated with tPA. Because of the limitation of time, only approximately 3% to 8.5% of the patients in the US receive this treatment. It is important to follow this time frame because tPA administration and reperfusion beyond 3 hours after the onset of stroke may lead to hemorrhagic transformation (HT). tPA is said to increase the risk of HT by nearly 10-folds(Jickling et al., 2014a, 2014b) and thus, hemorrhage is said to be closely associated with this thrombolytic therapy(Zhao et al., 2004).

The more time it takes to re-perfuse the damaged tissue, more is the rate of hemorrhagic transformation (Liu da et al., 2014) as tPA causes an imbalance between coagulation and fibrinolysis activities resulting in the dissolution of microscopic plugs that prevent hemorrhage (Gravanis & Tsirka, 2008). Apart from this,tPA binds to the low density lipoprotein-like receptor protein(LRP) causing an increase in the plasma levels of matrix metalloproteinases (MMPs), especially MMP-9.They are the enzymes that disrupt the extracellular matrix, degrade the blood brain barrier (BBB) and cause brain edema; thus, leading to hemorrhage(Ishrat, Soliman, Guan, Saler, & Fagan, 2012). Oxidative stress caused by the reperfusion therapy is also believed to elevate the levels of MMP-9, leading to BBB disruption (Ishrat et al., 2012). Further, caspase-8 and degranulation of mast cells are considered to play mediators in t-PA-induced brain damage.

In order to avert the damage caused by the tPA treatment and only utilize its advantages, many combination therapies have been shown to possess synergistic effects (L. Zhang, Zhang, & Chopp, 2012). Administration of tPA with free radical scavengers has shown neuroprotective results against hemorrhagic transformation (Ishrat et al., 2012) and with Minocycline, an antibiotic, has proven effective in suppressing MMP-9 activation (L. Zhang et al., 2012). Similarly, MMP inhibitors and mast cell stabilization have proven benefits against the tPA treatment-induced injury (Ishrat et al., 2012). LRP antagonist is also believed to be of use in combination with tPA(Ishrat et al., 2012).

Endogenous tPA: tPA is also produced by the human body. It is found to be expressed in the developing and mature brains, neurons and microglia and also produced by the endothelial cells (Ishrat et al., 2012). Endogenous tPA comprises of five domains, each with its respective target cell and receptor and thus, their resultant sustained effects (Docagne, Parcq, Lijnen, Ali, & Vivien, 2015). Depending on the target cell type, the resultant effects could be either positive or negative (Docagne et al., 2015). It is believed that post-injury, the sudden increase in tPA levels leads to large amount of fibrinolysis thus, expanding intracerebral hemorrhage (Hijazi et al., 2015).

Methods for measuring Infarct Volume:

Triphenyltetrazolium chloride (TTC): TTC is one of the most commonly used stains for evaluation of infarct volume in ischemic stroke models. The principle of TTC staining comprises of the oxidation of TTC by a dehydrogenase enzyme resulting in the product, formazan. Formazan causes the red stain over the intact tissue of the brain. However, an infarcted tissue lacks the mitochondrial dehydrogenase thus, obstructing the formation of

formazan from TTC and leaving the infracted area un-stained(Isayama, Pitts, & Nishimura, 1991; Schilichting et al., 2004; Tureyen, Vemuganti, Sailor, & Dempsey, 2004). TTC staining, even though vastly used, seems to be incapable of differentiating between infarcted and intact tissue if evaluated at 6 hours as opposed to evaluation at 24 hours and 48 hours after middle cerebral artery occlusion (MCAO)(Isayama et al., 1991).A limitation of TTC staining method is that only native and unfixed tissues can be used (Vogel, Mobius, & Kuschinsky, 1999). Moreover, TTC staining requires thick sections of a tissue (2mm thick)(Isayama et al., 1991)which could limit the number of immunohistochemical tests that can be performed.

Cresyl Violet (Nissl stain): Cresyl Violet is another method of staining the brain sections to determine the volume of infracted brain tissue. Cresyl Violet works by binding to the ribosomes rich in RNA and the nucleoli based on acid-base interaction. It mainly stains the Nissl substance in the endoplasmic reticulum(Tureyen et al., 2004). Cresyl Violet stains the brain tissue in a specific pattern based on the density of cells: more cell density has a darker stain as more nissl substances are available to take up the stain whereas; areas in which cells have died would have a lighter stain. Thus, a necrotic or infracted tissue is stained light blue whereas, an intact tissue is stained in a darker shade of blue(Tureyen et al., 2004).An advantage of Cresyl Violet stain is that it does not mask any critical markers, making them available for immunohistochemistry testing (Rousselet, Kriz, & Seidah, 2012). Furthermore, Cresyl Violet staining procedure does not require thick tissue sections which gives this technique an added advantage over TTC staining procedure as more sections become available for a variety of immunohistochemistry studies.

Stroke Animal models:

The choice of an animal model is one of the most important aspects of stroke studies (Casals et al., 2011) and its importance is attributed to the studies that often call for a surgical procedure, a study of the first few minutes of ischemic insult and for investigation of reperfusion injury and vasculature (Ginsberg & Busto, 1989). Different kinds of animal species like dogs, cats, rabbits and non-human primates are used for studying stroke. However, most commonly used animals are rodents like mice, rats and gerbils (Casals et al., 2011; Ginsberg & Busto, 1989; Mergenthaler & Meisel, 2012). Also, rodents and the procedures performed on them are comparatively less expensive (Ginsberg & Busto, 1989; Howells et al., 2010; Mergenthaler & Meisel, 2012). Moreover, they are ethically more acceptable as compared to non-human primates (Ginsberg & Busto, 1989).

We have used10-12 month old female Sprague Dawley rats in this study as they properly fit in the commercial Montoya staircase apparatus (limited to 400 gram animals) as opposed to male Sprague Dawley rats (generally over 400 grams at this age). They are outbred retired breeder rats with a weight-range of 320 to 385 grams. Age is one of the important factors to be considered in stroke studies as most of the population suffering from stroke isn't young (Howells et al., 2010; Mergenthaler & Meisel, 2012).Even though not fully confirmed, many studies have shown that adult neurogenesis occurs prominently in the sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of the dentate gyrus of an aged brain and is not as active as in a younger one (Nogueira et al., 2014). Thus, we chose the rats of the age that corresponds to an elderly group of humans.

Sprague Dawley rats have a blood collateralization system that is closer to that of humans and hence, produced smaller infarcts than those observed in Long Evans rats.

Brain-derived neurotrophic factor (BDNF):

Brain-derived neurotrophic factor (BDNF) belongs to a family of neurotrophins which are the growth factors distributed in the central nervous system. BDNF binds to the tropomyosin kinase receptor B (TrkB) and activates a series of signal transduction pathways (Binder & Scharfman, 2004), thus, enhancing adult neurogenesis (Schabitz et al., 2007), survival and proliferation of neurons, synaptic plasticity, neuroplasticity (Ploughman et al., 2009) and hence, learning and memory(Binder & Scharfman, 2004). Moreover, it was shown to be an important factor for reducing infarct size and secondary neuronal cell death (Schabitz et al., 2007).

Studies have shown that inducing BDNF or activating BDNF-related pathways leads to an improved motor functional recovery targeting the residual functional deficits after the occurrence of stroke (Kleim, Jones, & Schallert, 2003; Ploughman et al., 2009). Physical rehabilitation leads to enhanced BDNF expression which helps in reorganization of the cortical maps, ultimately, leading to synaptogenesis, enhanced dendritic spine formation and branching and neuronal plasticity and thus, behavioral recovery (Ploughman et al., 2009).

Slight injury to the brain can cause a gradual increase in astrocytic expression of neurotrophins. This increase is believed to be protective of brain damage and enable repair, assisting with motor function recovery (Kleim et al., 2003). Forced rehabilitation has been shown to improve functional recovery and is believed to be doing so by

enhancing the expression of BDNF and thus, neurogenesis (Livingston-Thomas, McGuire, Doucette, & Tasker, 2014).

Stem/Progenitor Cell Proliferation:

Ki-67 is a nuclear and a nucleolar protein (Endl & Gerdes, 2000) which is associated with cell proliferation. It is determined that Ki-67 is expressed only during the active stages of cell cycle like G_1, S, G_2 and mitosis whereas, it remains strictly unexpressed during the resting stage (G_0). Moreover, during interphase, the protein is found in the nuclear region and during mitosis, it moves further up to the perichromosomal surface (Scholzen & Gerdes, 2000). This distribution of Ki-67 from the nucleus to the surface of chromosomes is accompanied by phosphorylation by cdc2 kinase and protein kinase C (PKC) (Endl & Gerdes, 2000).

Because Ki-67 is expressed only during the active phases of cell cycle, it is most useful as a proliferative marker and to understand the growth fraction of various cell populations (Scholzen & Gerdes, 2000). Our lab has studied and utilized Ki-67 as a proliferative marker and detected considerable neurogenesis in the lateral ventricles of the brains of female Sprague Dawley rats after administration of a pharmacological treatment consisting of 5mg/kg Fluoxetine, 1mg/kg Simvastatin and 20mg/kg of Ascorbic acid, combined with physical rehabilitation. The treatment was chronic: over a period of 60 days and was initiated 20 to 26 hours after the induction of stroke.

In addition, measurement of Ki-67 for determination of stem/progenitor cell proliferation is believed to be more beneficial that the traditional 5'-Bromo-2-deoxyuridine (BrdU) immunohistochemical method, particularly in a brain injury model. Studies have reported

that apart from the uncertainty of the diffusion of BrdU on intraperitoneal injection, it also causes mutagenesis and such severe abnormalities of developing tissue. Any injured cell which has DNA damage would normally split the DNA for repair, and this would allow a false BrdU positive for cell proliferation. Moreover, intraperitoneal injection is good source of stress and since, stress is highly detrimental towards neurogenesis, BrdU administration could hamper with the results. Ki-67 being an endogenous protein, is devoid of all these disadvantages and hence, better preferred than BrdU(Kee, Sivalingam, Boonstra, & Wojtowicz, 2002).

Pharmacological treatment:

Antidepressants:

Studies suggest that chronic administration of antidepressant drugs helps in neurogenesis in adult brain hippocampus (Duman, Nakagawa, & Malberg, 2001; Malberg, Eisch, Nestler, & Duman, 2000). It is believed that antidepressants activate the cAMP-CREB cascade and also, induce the expression of BDNF. Both of these factors are important contributors towards neurogenesis(Chen et al., 2015; Duman et al., 2001).

Amongst antidepressants, selective serotonin reuptake inhibitors (SSRIs) are more efficient and tolerable as compared to the tricyclic antidepressants (TCAs) (Lee et.al., 2013).

Selective serotonin reuptake inhibitors, specifically, target a membrane protein, SLC6A4 (The solute carrier family 6, member 4). A free tryptophan is converted in to 5-hydroxytryptophan (5-HT or serotonin) by enzymes tryptophan hydroxylase and aromatic decarboxylase in the neuron. This 5-HT is, then, taken up and stored in the vesicles of a

neuron with the help of a monoamine transporter, SLC18A2. On the generation of an action potential, the stored serotonin is released into the synaptic cleft where it activates pre- and post-synaptic receptors. In normal circumstances, the activation of pre-synaptic receptors would activate a feedback loop and re-uptake of serotonin via SLC6A4; but, on prolonged administration of selective serotonin reuptake inhibitors, SLC6A4 and pre-synaptic receptor activity is blocked. This causes a surplus release of serotonin from the vesicles and effector signals. (Sangkuhl, Klein, & Altman, 2009). The accumulated neurotransmitter, serotonin, in the synapse leads either to the activation of G-protein coupled receptor, in turn activating adenylyl cyclase and cAMP cascade or to the increase in intracellular calcium levels which further activate adenylyl cyclase and increase cAMP levels. The activation of cAMP cascade leads to phosphorylation of cAMP- responsive element binding (CREB) protein and the resultant expression of BDNF gene (Tardito et al., 2006). Further, BDNF activates its receptor Tropomyosin receptor kinase B (TrkB) further eliciting various cellular signaling pathways like mitogen-activated protein kinase/ extracellular signal regulated protein kinase (MAPK/ERK) pathway, phospholipase Cγ and phosphoinositide 3- kinase (PI3K) responsible for neural stem cell proliferation, differentiation and survival (Numakawa et al., 2010).

Fluoxetine:

Studies have suggested that fluoxetine has neuroprotective activity. There have been notable reductions in the post-stroke infarct sizes on chronic administration of fluoxetine (W. L. Li et al., 2009; Lim et al., 2009). It has also proven to be a useful agent for reducing sensory(Gaillard & Mir, 2011) and motor deficits as a result of increased motor cortex excitability, along with increasing neurogenesis (Chollet et al., 2014; Klomp,

Vaclavu, Meerhoff, Reneman, & Lucassen, 2014). On comparison with control, it is found that fluoxetine-treated groups show a nearly 10 fold increase in neurogenesis (Corbett et al., 2015).Furthermore, chronic administration of fluoxetine is said to play an important role in regaining most of the spatial cognition (W. L. Li et al., 2009).

A hypothesis also suggests that fluoxetine causes an increase in neurogenesis and helps with neuroprotection as a result of mediation of vascular endothelial growth factor (VEGF). SSRIs are suggested to be the only antidepressants that increase the expression of VEGF, resulting in the increased efficacy of fluoxetine in reducing infarct size after ischemic stroke (Gaillard & Mir, 2011). Apart from VEGF, fluoxetine also influences the expression of molecules like neurotransmitters and BDNF which play an important part in re-wiring of the brain (Guirado et al., 2012; Kitahara et al., 2016).

In addition to this, fluoxetine has anti-inflammatory properties which contribute to its function of neuroprotection. Nuclear factor kappa B cells (NFκB) is a protein complex that activates a set of enzymes which eventually contribute to the inflammatory process. Fluoxetine reduces the activity of NFκB which ultimately suppresses the inflammatory activity and helps in neuroprotection. Moreover, treatment with fluoxetine decreased the microglia activation-which mediates the inflammatory processes, neutrophil infiltration and expression of inflammatory markers (Lim et al., 2009). Also, Interleukin-10 (IL-10) is believed to mediate the anti-inflammatory effect of fluoxetine leading to decrease in sensory impairments(Blatteau et al., 2015; Nijenhuis, ter Horst, van Rein, Wilffert, & de Jong-van den Berg, 2012).

Apart from this, drug-drug interactions exist between fluoxetine and some other drugs. Studies have suggested that maternal exposure to SSRIs during second and third trimester

lead to an incomplete development of the enteric nervous system because of the inhibitory effect of serotonin re-uptake transporter (SERT) as serotonin is essential for the development of enteric neurons. This causes the child to be excessively dependent on laxatives (Nijenhuis et al., 2012; Warren, 2012). SSRIs are known to block serotonin re-uptake by platelets; thus, inhibiting platelet aggregation and aggravating excess bleeding(Halperin& Reber, 2007; Schelleman, Brensinger, Bilker, & Hennessy, 2011). Ill-timed administration of fluoxetine with warfarin can also aggravate bleeding. Fluoxetine inhibits CYP2C9, a cytochrome P450 enzyme, essential for metabolizing the potent S-enantiomer of warfarin (Schelleman et al., 2011; Teles, Fukuda, & Feder, 2012).A drug combination of fluoxetine and diazepam is believed to cause delirium in patients as fluoxetine inhibits CYP2C19 which is responsible for the metabolism of diazepam; thus, causing an increase in the serum levels of diazepam (Dent & Orrock, 1997).

Statins:

Statins, also termed as 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are mainly used for the reduction in LDL cholesterol levels. They are competitive inhibitors of HMG-CoA reductase enzyme. They reduce endogenous production of cholesterol by inhibiting HMG-CoA reductase which catalyzes the conversion of HMG-CoA to mevalonate. Thus, it acts on the rate-limiting step in the endogenous production of LDL (Hernandez-Perera et al., 1998; Rodriguez Yanez & Davalos, 2008).

Studies have shown with the aid of animal studies that a statin treatment is followed by an improved cerebral blood flow, fibrinolysis and reduced infarct sizes. Also, in

combination with statins' pleiotropic effects such as anti-inflammatory and antioxidant effects, angiogenesis-thus, helping in the increase of cerebral blood flow, plaque stabilization, clot lysis and antithrombotic effects to prevent any further damage by platelets and/or blood cells' accumulation, they are neuroprotective and neuro-restorative (Hong & Lee, 2015; Liao & Laufs, 2005).

Patients treated with statins have been found to have high serum levels of BDNF. With the logic that serum BDNF levels reflect the expression of BDNF in brain, statins seem to help increase the expression of BDNF and thus, may contribute towards brain plasticity and enhancing functional recovery (J. Zhang et al., 2016). In addition to BDNF, VEGF and nerve growth factor expression also increase and together, they contribute towards improved neurological function after intracerebral hemorrhage (Yang, Han, Zhang, Chopp, & Seyfried, 2012).

Moreover, studies have proved that statin administration upregulates endothelial nitric oxide synthase (eNOS). Nitric oxide (NO), on traumatic brain injury, acts as a vasodilator and exhibits antithrombotic and anti-inflammatory effects. Statins upregulate eNOS expression by increasing the half-life of eNOS mRNA which, in turn, produces nitric oxide and helps with increase in the cerebral blood flow and reduction of infarct sizes (Liao & Laufs, 2005; Rodriguez Yanez & Davalos, 2008).

Furthermore, statins inhibit isoprenylation of small GTP molecules such as Rho. By inhibiting the synthesis of mevalonic acid, statins also inhibit the formation of isoprenoids like farnesylpyrophosphate and geranylgeranylpyrophosphate. Rho proteins, generally, on reaction with farnesylpyrophosphate contribute in the degradation of eNOS mRNA. Along similar lines, RhoA, on reaction with geranygeranylpyrophosphate,

destabilizes eNOS mRNA. Inhibition of the formation of farnesylpyrophosphate and geranylgeranylpyrophosphate due to the action of statins, inhibits eNOS mRNA degradation and stabilizes it (Stepien, Tomaszewski, & Czuczwar, 2005). Also, statins have shown remarkable utility by inhibiting vascular remodeling via downregulation of the Rho/Rho-kinase pathway (Ma, Li, Wang, & Guan, 2012).

Combined administration of r-tPA with statins have shown a decrease in matrix metalloproteinase (MMP) levels (Rodriguez Yanez & Davalos, 2008) which could be a possible solution to the activation of MMP on tPA administration after the 4-hour window. Moreover, similar to fluoxetine, statins have proven effective in inhibiting the activity of NFκB, the resultant inflammatory reactions and aiding with neuroprotection (Q. Li, Zhuang, Yang, & Zhang, 2014). Moreover, statins have been shown to increase the mRNA levels and enzymatic activity of endogenous tPA where it is believed to reduce the production of thrombin and the stability of clots (Asahi et al., 2005).

Simvastatin:

Simvastatin can easily cross the blood brain barrier (BBB) because of its lipophilic nature. Its lactone form is transferred via simple diffusion mechanism whereas, it's acidic form is transported via a carrier-mediated system across the BBB (Tsuji, Saheki, Tamai, & Terasaki, 1993; Wood, Eckert, Igbavboa, & Muller, 2010).

Studies have shown that simvastatin induces the expression of vascular endothelial growth factor (VEGF). VEGF on binding with vascular endothelial growth factor receptor-2 (VEGFR-2), the expression of which is also enhanced by simvastatin in the cortex, gets activated and initiates a series of reactions involving the activation tyrosine

kinases and multiple signals responsible for microvascular permeability, endothelial cell proliferation, survival, migration and vascular length. Moreover, simvastatin induces the phosphorylation of eNOS which is one of the mediators of VEGFR-2 and hence, critical towards angiogenesis (Wu et al., 2011).

Simvastatin, further, assists with reducing neuronal excitotoxicity in the brain. On the occurrence of stroke, there is a rise in the extracellular levels of glutamate which over-activates the glutamate receptors and causes an excess of calcium entry in the brain cells via N-methyl-D-aspartate receptors (NMDARs). This leads to neuronal excitotoxicity. NR3A, a member of the glutamate receptor family, on reaction with NR1 & NR2 receptors (subunits of NMDARs), is proven to reduce calcium entry and thus, reduce neuronal excitotoxicity. In addition, PP2A, a family of serine-threonine phosphatase, is known to attenuate the activities of NMDARs. Results have shown that administration of simvastatin can re-inforce the expression of NR3A and PP2A; thus, reducing the brain infarct size (Zhu et al., 2012).

Moreover, simvastatin is known for the increased expression of BDNF and nerve growth factor (NGF) which are responsible for the brain plasticity and neurogenesis (Yang et al., 2012).

Ascorbic acid:

Previous studies in our laboratory have proven that stress is one of the most deteriorating factors in the process of recovery after ischemic stroke injury. Chronic stress on the rat brains eventually leads to oxidative stress. Oxidative stress is an imbalance between the production of free radicals in the body and the body's antioxidant capacity (Moretti et al.,

2012).Oxidative stress and myeloperoxidase which is expressed by microglia are major contributors towards the generation of reactive oxygen species (ROS) on reperfusion after the occurrence of stroke. ROS activate NFκB and the following cascade of inflammatory responses. Thus, an antioxidant like Ascorbic acid is essential to help with neuroprotection and prevent apoptosis (Cherubini et al., 2008; Iwata et al., 2014).

Ascorbic acid is transported through the BBB via two mechanisms. Sodium-dependent vitamin C transporter 2 (SVCT2) is one of the main mediators for the uni-directional (May, 2012)transportation of ascorbic acid through BBB and another mode of transportation is through glucose transporter 1 (GLUT 1) located in the endothelial cells of BBB which bi-directionally (May, 2012)transports dehydroascorbic acid (an oxidized form of ascorbic acid). SVCT2 is believed to generate and maintain high concentrations of ascorbic acid in the body cells (May et.al., 2012) Chronic supplementation of ascorbic acid has shown to increase the expression of GLUT1 and SVCT2; ultimately, re-enforcing antioxidant protection of neuronal cells and alleviating ischemic injury caused due to oxidation within and around the cells(Iwata et al., 2014; May, 2012).

In addition to antioxidant action, myelin formation, synaptic potentiation, ascorbic acid also protects against cerebral excitotoxicity caused by excess expression of NMDA receptors (May, 2012). This occurs because of exhaustion of high-energy phosphate compounds like ATP due to a lack of oxygen and glucose which causes membrane depolarization and excess of calcium influx leading to uncontrolled release of glutamate and excessive activation of NMDARs (Cherubini et al., 2008).

As a result of ischemia and oxidative stress, nitric oxide (NO) bioavailability decreases. Administration of ascorbic acid causes an increase in eNOS expression and thus,

increases NO levels, decreases oxidative stress and as mentioned earlier, assists with angiogenesis and cerebral blood flow (Yan, Tie, & Messina, 2012).

Animal studies have shown that co-administration of ascorbic acid with Fluoxetine, causes a considerable improvement in the recovery from the depressive disorders in animals in addition to being an inexpensive treatment and the absence of any major side effects (Amr, El-Mogy, Shams, Vieira, & Lakhan, 2013). Combining ascorbic acid with Fluoxetine and simvastatin has beneficial effects for both Fluoxetine and simvastatin. Fluoxetine is a selective serotonin reuptake inhibitor and serotonin being highly sensitive to oxidation, administration of ascorbic acid with Fluoxetine is an important step in sustaining the efficacy of Fluoxetine. Moreover, simvastatin enhances the expression of BDNF via stimulation of eNOS which is susceptible to oxidation and ascorbic acid helps with its antioxidant properties to potentiate the action of simvastatin (Corbett et al., 2015).

In addition to this, our laboratory has proven in the past that combined administration of 0.5mg/kg simvastatin, 5mg/kg Fluoxetine and 20mg/kg ascorbic acid 20-26 hours after the induction of stroke for 31 days in 10-12 months old female rats results in 85% recovery of pre-stroke function of skilled grasping in a Montoya staircase apparatus and 90% recovery of pre-stroke function of voluntary exploring using paws in a forelimb asymmetry test; in addition to a ~19 fold increase in neurogenesis compared to control animals (Corbett et al., 2015).

Stroke-induction models:

Middle cerebral artery occlusion:

This is one of the most commonly performed procedures for induction of stroke. It involves the occlusion of the middle cerebral artery, either permanently or temporarily. The blood flow to the cortex and striatum through the middle cerebral artery is blocked by inserting an intraluminal suture in the internal carotid artery by tying the common carotid and making an incision in the external carotid artery. Damage occurs in several parts of the brain depending on the temporary or permanent nature of the middle cerebral artery occlusion affecting the frontal, parietal, temporal and occipital cortex in addition to the thalamus, hypothalamus and striatum. Furthermore, insertions of microspheres for occlusion similar to that of permanent suture occlusion and occlusion with the thromboembolic clots for a temporary effect are also used. However, this method isn't suitable for our experiments because both the type of occlusions, permanent and temporary, do not affect the forelimb motor cortex (Kleim, Boychuk, & Adkins, 2007).

Photothrombosis:

This model of stroke induction involves intravenous injection of a photosensitive dye in the blood stream. This causes an irradiation of the brain tissues by photo-oxidation which leads to an immunity system response causing vascular occlusion and platelet damage. However, it also leads to endothelial damage (Kleim et al., 2007) which turns out to be a disadvantage in our study as our drugs target the endothelial cells. Moreover, it causes major vasogenic edema which is more representative of traumatic brain injury than ischemic stroke (Carmichael, 2005; Kleim et al., 2007).

Devascularization:

Devascularizing the cortex by electro-coagulation of surface vessels is another method of stroke induction. It produces focal ischemia but, it makes the underlying tissue highly susceptible to secondary hemorrhagia and also, prevents reperfusion (Kleim et al., 2007).

Endothelin-1:

Our study makes use of this model of stroke-induction which comprises of intracerebral induction of endothelin-1. Endothelin-1 is a potent vasoconstrictor which rapidly induces stroke, in approximately 30 minutes and sustains it for around 24 hours, thus, producing rapid neurodegeneration (Kleim et al., 2007; Windle et al., 2006). This type of ischemic stroke is similar to that of humans. Moreover, the direct intracerebral injection makes it possible to produce localized infarcts in the desired areas of brain (Windle et al., 2006) which is the forelimb motor cortex, in our case. Thus, this method has the advantages of being easy to perform, reproducible and accurate in producing focal ischemia (Bacigaluppi, Comi, & Hermann, 2010a, 2010b; Kleim et al., 2007)

Hypothesis

We hypothesize that with the help of a delayed pharmacological treatment using combination drugs (Fluoxetine, Simvastatin, Ascorbic acid), with physical rehabilitation, female rats will show reduced infarct size.

Specific Aims

This study will comprise of two specific aims. The first specific aim is to determine the infarct size in the female Sprague Dawley rats after the administration of a daily drug combination given 20-26 hours after the induction of stroke. The second specific aim is to determine the infarct size in female Sprague Dawley rats after the administration of a daily drug combination given 20-26 hours after the induction of stroke, in addition to physical rehabilitation.

All the rats will be trained on the Montoya Staircase apparatus in order to determine their pre-stroke motor functional abilities for one and a half week. Further, stroke will be induced in the forelimb motor cortex of all the rats to cause paralysis in the contralateral limb. A pharmacological treatment of 5mg/kg Fluoxetine, 1mg/kg Simvastatin and 20 mg/kg Ascorbic acid, rolled in a sugar cookie dough, will be administered to the rats belonging to the drug-treated group whereas, the rats assigned to the control group would be given a plain sugar cookie dough. This would be continued daily for 60 days. Both the groups of rats would undergo physical rehabilitation comprising of a peanut butter-containing shelf hung outside their cages in such a way that they would have to use their stroke-affected limb, for 23 days. At the end of 60 days, the rats will be euthanized and their brain sections will be stained with Nissl stain in order to study their infarct volumes. This would help us compare the effect of physical rehabilitation in the drug-treated rats and control rats.

II. MATERIALS AND METHODS

All the studies conducted during this research were performed in accordance with Wright State University Institutional Animal Care and Use Committee (IACUC).

Pre-stroke training:

The animals chosen as the subjects of this study were female Sprague Dawley outbred retired breeder rats;10-12 months old and 260 g- 340 g in weight. The pre-stroke training of rats was conducted using the Montoya staircase apparatus. This apparatus consists of a bilateral set of staircases with a raised platform between them (Figure 1). It is used to determine a rat's motor skills like reaching out with a forepaw and grasping the food pellets placed on the staircase. Each staircase has seven wells, one on each of the steps. Three sucrose pellets were placed in each well, accounting for 21 pellets on each side of the platform.

On the first day after the arrival of the rats in the LAR facility, they were weighed for their weight before restricted diet. Also, to gauge the food eaten by a rat ad lib, the food was weighed on the first day and on the third day of their arrival. Based on those readings, each rat's diet was restricted to 85% of their ad lib intake. To monitor their weight loss, the rats were weighed every third day and their food intake was increased if they lost more than 15% of their initial weight.

The Montoya staircase training began during the dark cycle in accordance with the rats' circadian rhythm. The rats were taken out of their cages and placed in the Montoya staircase apparatus for 15 minutes. Three Montoya staircase apparatus were employed simultaneously and hence; three rats could be trained (one rat in each apparatus) at the same time. This procedure was continued for 13 days. The rats had to be able to retrieve at least 9 pellets from each side to be able to continue in the study. At the end of 13 days of training, the highest number retrieved by each rat was recorded as their pre-stroke function.

Figure 1: Montoya staircase apparatus

Stroke Induction:

Stroke was induced in the right hemisphere of the forelimb motor cortex of all the rats using endothelin-1, a potent vasoconstrictor.

One at a time, the rats were placed in an induction chamber with the supply of 5% isoflurane used as an anesthetic. The concentration of isoflurane was reduced to 2-3% when the surgical plane of anesthesia was reached. A tail-pinch withdrawal symptom was employed to check for the effect of anesthesia; after which, the rat's head was shaved and it was placed in the stereotactic apparatus. Its head was positioned with non-traumatic ear bars and the rat was laid with a heating pad underneath. For maintenance of anesthesia, a gas mask with the supply of 2-3% isoflurane was attached to the stereotactic apparatus.

Puralube eye ointment was used to keep its eyes moist. Further, Povidone iodine is applied on the incision site to clean and avoid skin infection, followed by Ethanol used for cleaning and Povidone-iodine for scrubbing. A straight midline incision was made on the head skin, and analgesic Bupivacaine was applied to the incision site and the fatty tissues & blood was cleaned using a cotton swab. This helped to see the Bregma position on the skull which is at the center of a white cross, representing the suture lines for fusion of the skull plates. It was marked with a fine-tip marker. The micro drill was positioned over the Bregma and its co-ordinates were noted down. Thus, the right hemisphere of the forelimb motor cortex was located relative to the co-ordinates of Bregma.

The first hole was, then, drilled at 0 mm Anterior-posterior and 2.5mm Medial-lateral; followed by the second hole drilled at 1.5 mm Anterior-posterior and 2.5 mm Medial-lateral. The holes were made till they were fully drilled through the skull. Further, the

drill was replaced by a Hamilton syringe consisting of 3µl endothelin. 1.5µl of 400pmoles/µl of endothelin was injected into each hole at a depth of 2.0 mm and at the rate of 0.5µl/ 10 respirations. The syringe was removed from its stand and cleaned with ethanol.

The incision was stitched using sutures & coated again using Povidone-Iodine. The rat was treated subcutaneously with 2ml of Saline and the supply of anesthesia was cut off. The stereotactic apparatus used to position its head was loosened and at the first movement, the rat was transferred to a cage placed on a heating pad. The rat was transferred to its own cage once it regained its consciousness fully. It was, then, given moist chow in its cage.

Pharmacological treatment:

Out of the total 22 female Sprague Dawley rats, 13 underwent the pharmacological treatment.

The Pharmacological treatment was begun 20-26 hours after the induction of stroke and continued for 60 days. The treatment is comprised of three drugs: 5mg/kg Fluoxetine, 1mg/kg Simvastatin and 20mg/kg Ascorbic acid (Vitamin C).

Since, stress can affect the process of Neurogenesis, it was necessary to make use of a stress-free method for administration of drugs (Corbett et al, 2012). Thus, the drugs were measured and placed in a well made in a 3-4g of Pillsbury sugar cookie dough. The dough was rolled, placed in a petri-dish and placed in a rat's cage. This assisted in voluntary ingestion of the cookie dough and hence, the drugs; thus, helping in the stress-free administration of drugs.

Control rats:

The control rats were each given 3-4 g of Pillsbury cookie dough without drugs on a petri-dish for stress-free administration for 60 days.

Physical rehabilitation:

The process of physical rehabilitation was designed for the rats to exercise their contra-lateral (stroke-affected) limb. All the twenty-four rats underwent rehabilitation.

Three days prior to the beginning of rehabilitation, the rats were presented with peanut butter. The rats which did not seem to like peanut butter were transferred to the no-rehabilitation group.

The formalized physical rehabilitation process began on post-stroke day 8. It consisted of a shelf containing peanut butter hung outside their individual cages every other night. These shelves were placed such that the rats would have to use their contra-lateral (stroke-affected) limb to reach the food. The peanut butter was weighed prior to hanging the shelf and after the night of rehabilitation to mark the progress of a rat. This process continued for 23 days.

	Physical rehabilitation
Control Rats	9
Drugs-treated Rats	13
Total Rats	22

Table 1: Number of rats which received physical rehabilitation

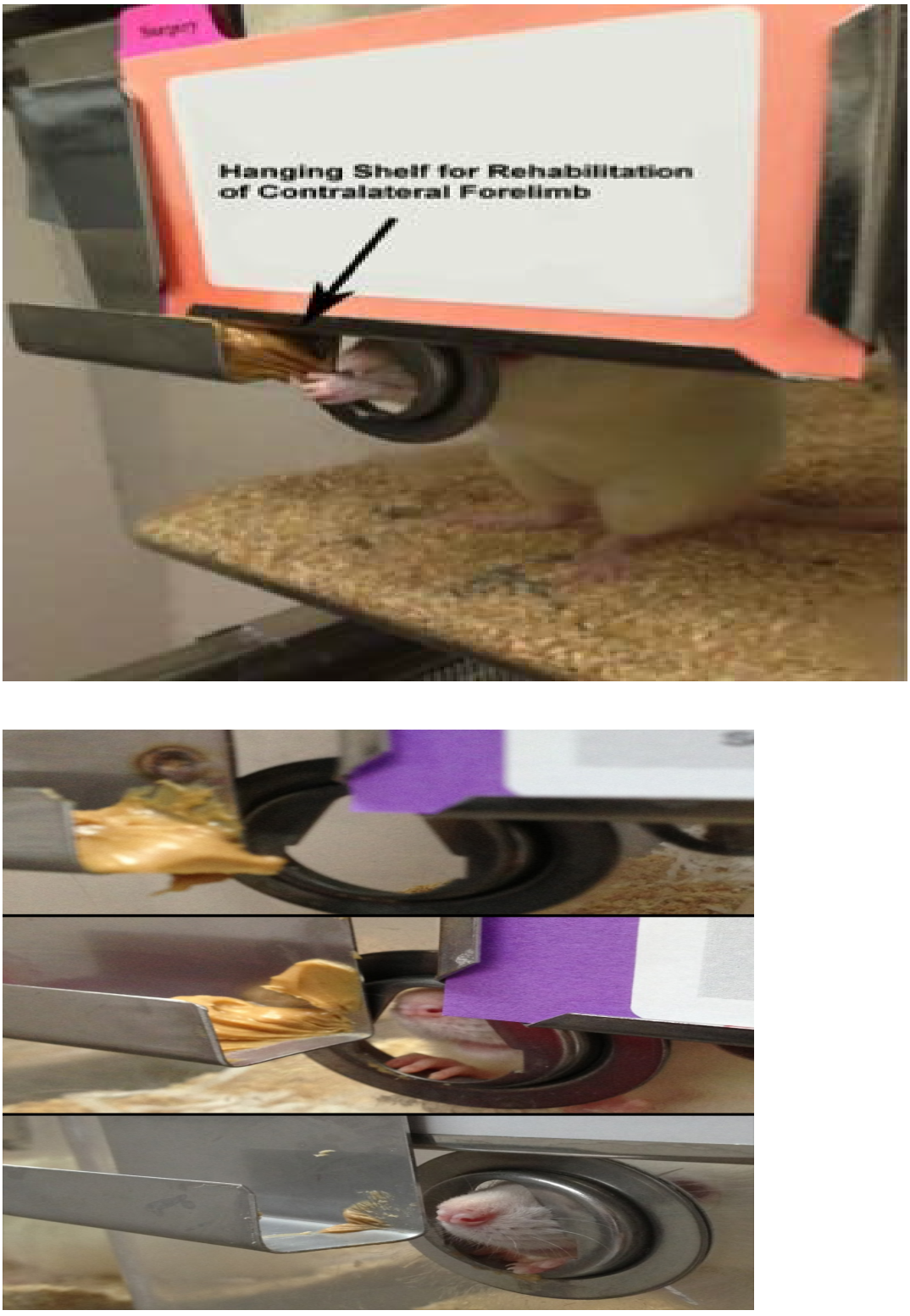


Figure 2: Hanging shelf with peanut butter for physical rehabilitation of contralateral limb

Post-stroke functional recovery analysis:

After the induction of stroke, the rats underwent a series of Montoya trainings to gauge their functional recovery relative to their recorded pre-stroke function.

The Montoya test for baseline deficit were conducted on post-stroke days 3, 4 and 5. The number of pellets consumed by the rats were recorded and the highest number of pellets retrieved was used to calculate the functional recovery and functional deficit of that rat. The highest number of pellets retrieved post-stroke was divided by those retrieved pre-stroke and multiplied by 100 to get the percentage of pre-stroke function recovered by the rat on account of the pharmacological treatment. Post-stroke functional deficit was obtained by subtracting the pre-stroke functional recovery percentage from 100.

Montoya tests for functional recovery were conducted on post-stroke days 28-29-30, 58-59-60, too. Similar calculations were performed and the final functional recovery was recorded.

Infarct analysis:

In the first step towards infarct analysis, the rats were euthanized by cardio perfusion method. They were injected intraperitonially with Euthasol (100mg/kg sodium pentobarbital) to initially, slow and finally, stop the heart-beat.

Once unconscious, the animal was checked for reflexes by the tail-pinch method. An incision was made from the upper abdomen, taking care that heart was exposed but not punctured. The heart was gently lifted with the index finger and the thumb and the apex of the heart was clamped. A blunt-edged cannula with a supply of PBS was inserted in to the left ventricle and the clamp was tightened to hold the cannula in position. The right

atrium was also cut to allow the removal of first blood and then PBS through the body. The tissues were then fixed using 4% paraformaldehyde in PBS to flow through the animal's circulatory system.

Further, the brain was removed and stored in 4% paraformaldehyde in PBS at 5 degrees Centigrade.

Cryostat:

In preparation for cutting, the fixed brain blocks were placed in 30% sucrose for 3 days. Following this, the block of brain tissue was mounted on a cryostat pedestal using o.c.t. compound and frozen at -25 degrees centigrade. When the tissue had equilibrated, 50 micron coronal sections were cut and placed in vials containing PBS. Each vial was used for a different type of staining or immunostaining. In this set of experiments, we had four vials, or four different staining conditions.

Mounting the tissue:

One out of every four coronal brain sections was stained with the Nissl stain, also known as Cresyl Violet and these tissues were placed in a vial labelled for individual rats.

While mounting, these tissues were placed in a petri-dish half-filled with PBS. A gel-coated slide was tilted in the petri dish, wetted with the PBS from the petri-dish and four to six tissues were placed on each slide with the help of thin tipped paintbrush. Using a dissecting microscope, any folds in the brain tissues were straightened up.

Imaging:

Damage to the neurons and the impact of the infarct were captured using brightfield microscopes and the Amscope software. The objective lens was set at 4x and the ocular lens were 10x. Each section was analyzed for the presence of infarct.

Montaging of images:

Some of the brain sections had large infarcts that could not be captured in a single image. Hence, multiple images had to be merged together to create a single large image showing the entire infarct. The multiple images were compiled in a single montage which showed the complete infarct using Adobe Photoshop software.

Calculating infarct areas and infarct volumes:

The infarct area for each rat was calculated using the Image J software. Each brightfield scope had a different calibration using the 4x objective, so we had to set a different number of pixels/mm for each scope used. Please refer to the table below for the calibrations used for each scope in our laboratory.

Pixels	0.1mm	1mm	$1mm^2$
Spot Scope	42	420	176400
Scope 1	61	610	372100
Scope 2	99	990	980100

Table 2: Brightfield microscope calibration

To calculate the infarct volumes, an excel sheet was made with the all the rat IDs and the areas of their individual sections. Each area was multiplied by 0.05 to account for each section's thickness of 0.05mm. This gave the infarct volume for individual brain sections. Further, the volumes of section were added and multiplied by 4 because one of out every 4 sections cut during the process of cryostating was stained with Cresyl Violet. This gave the total infarct volume for each rat.

Statistical analysis:

Statistical analysis was performed to analyze the p-value and to determine if the group of control rats which underwent physical rehabilitation were significantly different than the drugs-treated rats which underwent rehabilitation. This analysis was conducted using the Graph pad prism software. We utilized the t-test with Welch's correction to get a p-value.

III. RESULTS

Our study was comprised of three stages: Pre-stroke training, Stroke induction and Post-stroke analysis.

The pre-stroke training was conducted over a period of 13 days. The female Sprague Dawley rats were trained to grasp sucrose pellets in a Montoya staircase apparatus for 15 minutes daily. The maximum number of pellets retrieved by the rats were recorded as their individual pre-stroke functions.

After the pre-stroke training period, stroke was induced using endothelin-1, a potent vasoconstrictor, in the forelimb motor cortex of the right hemisphere. This procedure induced a stroke in approximately 30 minutes.

The post-stroke treatment included the administration of the pharmacological treatment and physical rehabilitation. The pharmacological treatment was comprised of 5mg/kg Fluoxetine, 1mg/kg Simvastatin and 20mg/kg Ascorbic acid was initiated 20-26 hours after the induction of stroke and was administered daily for 60 days. This treatment was administered to the FSA (drug-treated) group of rats and the rest were categorized as control rats and were administered vehicle. The physical rehabilitation process, including

the use of contralateral limb to eat peanut butter, was begun on post-stroke day 8 and continued every alternate night for a total of 23 days rehabilitation.

In a previous study, rats were given a smaller amount of peanut butter on the hanging shelf used for rehabilitation beginning on post-stroke day 8 and had rehabilitation every other day for a total of 23 days rehabilitation. Then, drug treatment continued for an additional 14 days without rehabilitation. The main difference in these two rehabilitation studies was the following: 1) the amount of peanut butter loaded on the rehabilitation shelves was increased in this study which resulted in increased use of the impaired arm, 2) drugs were initiated 20-26 hours after stroke and included 20 mg/kg ascorbic acid, whereas the previous study only included 5 mg/kg fluoxetine and 1 mg/kg simvastatin (begun 6-12 hours after stroke induction); 3) in this study, the animals had no recovery period without rehabilitation before they were euthanized.

During this process, the rats were tested on the Montoya staircase apparatus for forelimb motor function deficits on post-stroke days 3, 4, 5 and for functional recovery on post-stroke days 28, 29, 30 & 58, 59, 60.

After post-stroke day 60, the rats were euthanized and the brain sections were analyzed for infarct volumes.

Exclusions

In our study, we examined the brain sections of all the rats for significant infarct sizes, barring which they were excluded from the study. Also, we tested if the rat showed less than 20% of contralateral deficit on post-stroke days 3, 4 & 5. We induced stroke using the endothelin-1 injection in the forelimb motor cortex of the right hemisphere at

approximately 2mm depth from the cortical surface. Sometimes, due to human error, the endothelin does not get injected in to the forelimb motor cortex area and instead flows over all the cortical surface damaging it. As this damage is not caused at the forelimb motor cortex and instead is the result of surgical artifact, the animal's brain section does not show an infarct at the forelimb motor cortex and thus, animal exhibits less than 20% of contralateral deficit. Thus, rat 813, a control rat, was excluded from our study for exhibiting less than 20% of contralateral deficit due to the improper injection of endothelin-1.

In addition to this, rat 824 (a drug-treated rat) was considered only for infarct analysis and not for functional analysis because endothelin-1 was injected, due to human error, in to the area adjacent to the forelimb motor cortex as a result of which, we did see an infarct but no contralateral deficit.

A control rat, Rat 827, on the other hand, was excluded from the study as it showed less than 20% contralateral deficit- which is the least expected amount that we expect with this surgery- despite of an ischemic injury to the forelimb motor cortex due to endothelin-1 injection and no significant infarct.

Moreover, when the rats were tested for their pre-stroke function on the Montoya staircase, a criteria was set that the rats should be able to retrieve at least 9 sucrose pellets with each paw. If they were unable to reach this testing criteria, the rats were tested for their pre-stroke function using the Forelimb asymmetry test. Rat 805, a drug-treated rat, was the only rat in our study which did not meet the Montoya staircase criteria and was tested using the Forelimb asymmetry test. Thus, this rat was not considered during the functional analysis for Montoya staircase test.

We also had one death (Rat 806) after the stroke surgery.

Thus, for the final analysis, we had a total of 22 rats, out of which 9 were control rats. The drug-treated (FSA) rats, however differed based on the type of analysis. Because rat 824 was considered only for infarct analysis, the drug-treated (FSA) group was composed of 13 animals. However, for functional analysis, we considered 11 drug-treated animals since, rat 824 (less than 20% contralateral deficit) and rat 805 (Forelimb asymmetry test) did not exhibit the required Montoya staircase deficits.

Rat weights

It is observed that on induction of stroke via the middle cerebral artery occlusion (MCAo) procedure, the rats suffer from a loss of appetite, typically resulting in 10-20% weight loss (Boyko, et al., 2010). Transection of the external carotid artery causes an ischemia of mastication & swallow muscles which cause problems with eating and leads to weight loss. However, stroke induction using a powerful venous and arterial vasoconstrictor, endothelin-1, produces a direct focal ischemia in the targeted area of the cortex; thus, avoiding side effects like prominent weight loss following stroke surgery(Carmichael, 2005).

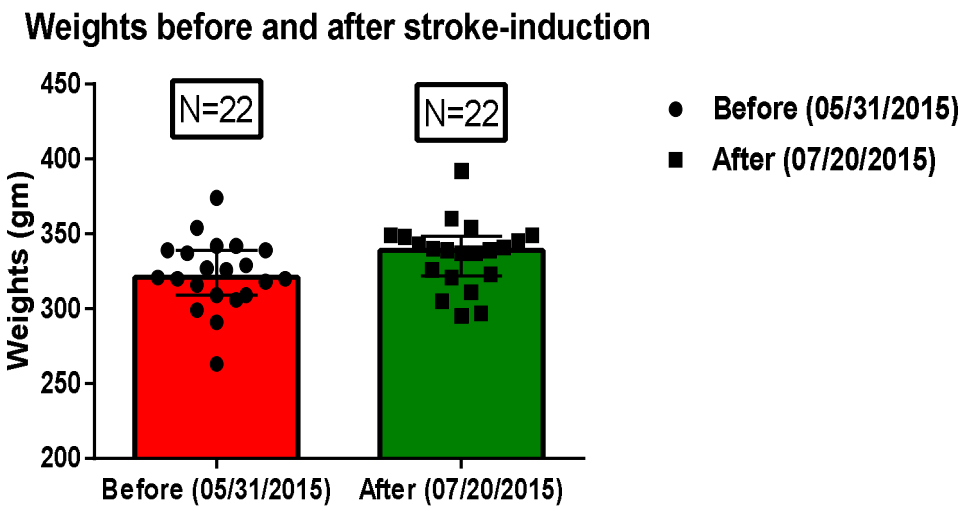


Figure (3): Weights before and after stroke-induction

The 'before' represents the median weight of rats before stroke induction, in red bar graph. The dots (●) indicate the individual weights where the number of rats, N, is 22. The 'after' represents the median weights of rats after stroke induction, in green bar graph. The squares (■) indicate the individual weights where the number of rats, N, is 22. The error bars represent the median with interquartile range. The pre-stroke weight was not statistically significant (P=0.08) from post-stroke weight using the unpaired t-test with Welch's correction.

Figure (3) depicts the median weights (grams) of the rats (drug-treated and control) before and after induction of stroke. The mean ± SEM before the induction of stroke was found to be 322.9 ± 5.13 and that after stroke induction was found to be 335.8 ± 4.88. The difference between the means was 12.86 ± 7.07. The statistical analysis performed using the unpaired t-test with Welch's correction shows that the data is not significantly different with a P value of 0.08 but, this does show a strong trend. The unpaired t-test using Welch's correction is performed to test different populations with statistically different variances, but may also be performed on two populations with equal variances.

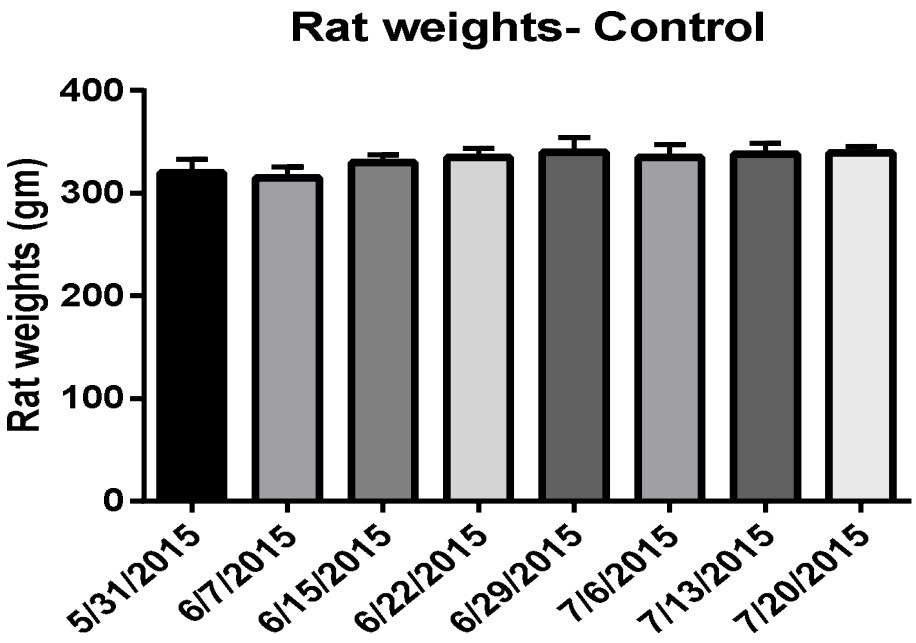


Figure (4): Variations in the weights of control rats

The graph shows median weights of control rats (Number of control rats, N, is 9) on eight different days. The error bars represent the median with interquartile range. Statistically significant difference (P<0.001) was found over time using one- way Friedman repeated measures ANOVA on ranks. Stroke-induction surgeries were performed from 05/30/2015 to 06/06/2015.

Figure (4) depicts the median weights (grams) of the female Sprague Dawley rats belonging to the control group before and after the stroke-induction surgeries. The mean weights of the rats throughout the weights analysis lay in the range of 313.5-338.5 grams and the standard error of the mean was 7.46 and the median of weights ranged from 315-340 grams. According to the One-Way Friedman repeated measures ANOVA on ranks test, there is a statistically significant difference ($P<0.001$) among the median values of weights ranging from 5/31/2015 to 7/20/2015. Post-hoc analysis with Holm-Sidak method revealed the dates on which the weights of rats were significantly different (see the table below). Note that all of the significant differences were between the first two days that the animals were weighed and the later dates. The weights on 5/31 were before stroke surgery and the weights on 6/7 were after stroke surgery.

Comparisons	P-value
6/29/2015 vs. 6/7/2015	<0.001
7/20/2015 vs. 6/7/2015	<0.001
7/6/2015 vs. 6/7/2015	<0.001
7/13/2015 vs. 6/7/2015	<0.001
6/29/2015 vs. 5/31/2015	<0.001
6/22/2015 vs. 6/7/2015	<0.001

7/20/2015 vs. 5/31/2015	<0.001
7/6/2015 vs. 5/31/2015	<0.001
7/13/2015 vs. 5/31/2015	<0.001
6/22/2015 vs. 5/31/2015	<0.001
6/15/2015 vs. 6/7/2015	<0.001

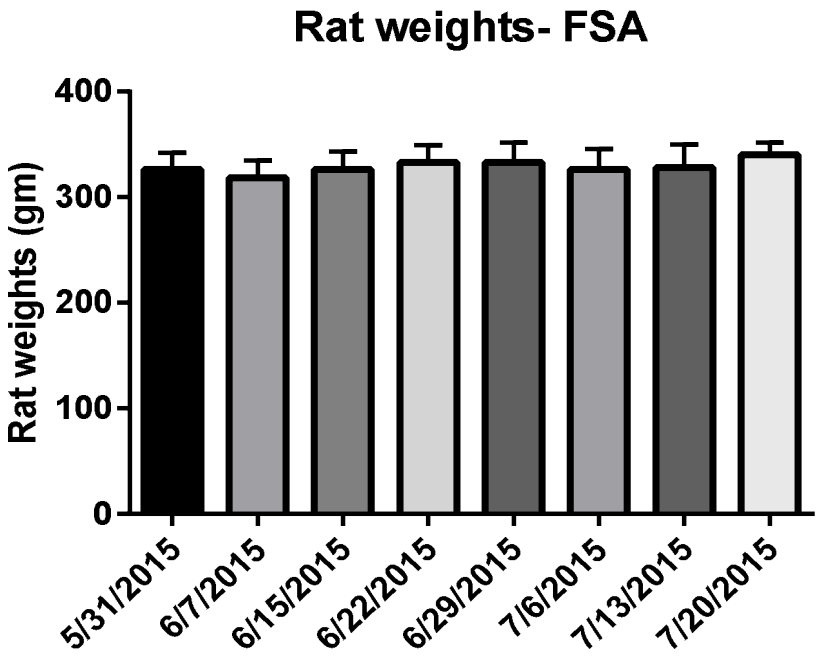


Figure (5): Variations in the weights of drug-treated (FSA) rats

The graph shows median weights of drug-treated (FSA) rats (Number of FSA rats, N, is 13) on eight different days. The error bars represent the medians with interquartile range. Statistically significant difference (P<0.001) was found over time usingone-way Friedman repeated measures ANOVA. Stroke-induction surgeries were performed from 05/31/2015 to 06/06/2015.

Figure (5)depicts the variations in the median weights (grams) of female Sprague Dawley rats belonging to the drug-treated (5mg/kg Fluoxetine, 1mg/kg Simvastatin & 20mg/kg Ascorbic acid) group. The graph gives the median weights of the group of rats from the first day of drug administration to the last day. The mean weights of rats throughout the weights analysis lay in the range of 318.75-334.083 grams and the standard error of mean was found to be 6.807 and the median weights ranged from 318-340 grams. According the results obtained by one-way Friedman repeated measures ANOVA, the median weights of the drug-treated rats show a significant difference ($P<0.001$) over the period from 5/31/2015 to 7/20/2015.

Post-hoc analysis using the Holm-Sidak method revealed the following dates where the weights of rats differ significantly. All of the significant changes occurred soon after stroke surgery, where we see a little weight dip.

Comparisons	P-value
7/20/2015 vs 6/7/2015	<0.001
7/13/2015 vs 6/7/2015	<0.001
6/22/2015 vs 6/7/2015	<0.001
6/29/2015 vs 6/7/2015	<0.001

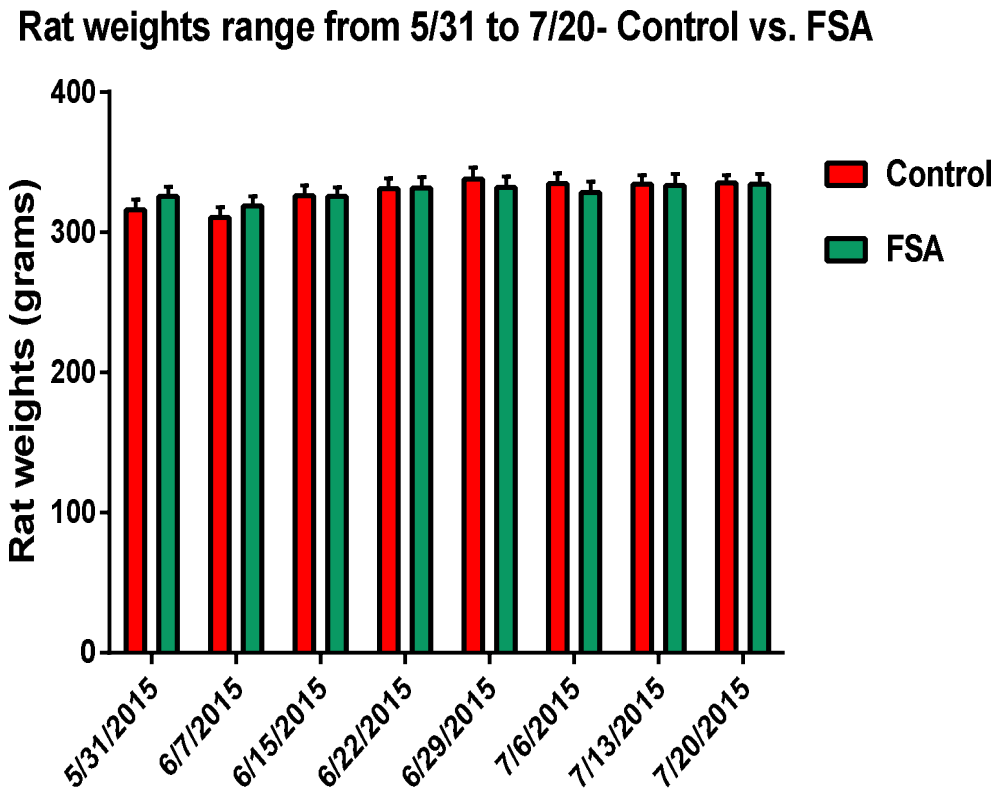


Figure (6): Rat weights from 5/31/2015 to 7/20/2015- Control rats vs. FSA rats.

Red bars depict mean weights of control rats and the green bars depict the mean weights of the drug-treated (FSA) rats. The error bars represent the standard error of the mean (SEM). Mean weights of control rats compared to FSA rats on individual dates are not significantly different (P=0.76), difference between the mean weights of control rats throughout the period of analysis versus the mean weights of FSA rats is not significantly different (P=0.07) using two-way repeated measures ANOVA test.

Figure (6) depicts the mean weights (grams) of the female Sprague Dawley rats over a period from 5/31/2015 through 7/20/2015. Based on the results obtained by Two-Way ANOVA test, the mean weights of control rats and those of FSA on any given individual dates do not differ significantly with a p value of 0.76. Moreover, there is no significant difference between the mean weights of control rats throughout the period and the mean weights of FSA rats over the given period of time (P= 0.07); however, there seems to be a strong trend which could potentially become significant if the N was increased.

Changes in animal weights:

The overall weights of the rats before and after the administration of stroke varies significantly displaying a collective rise in the weights. On precise analysis of the mean weight changes in the control rats on eight different days, we found that the data, collectively, showed a significant differencebetween early timepoints (before and just after stroke) and later time points in July. Comparisons between the mean weights on individual dates also resulted in a considerable number of data being significantly different. Similar results were obtained for the drug-treated rats.

However, comparisons of mean weights of control rats with mean weights of drug-treated rats on each day indicated that the weights of the two groups did not increase or decrease simultaneously over the given period of time. Thus, we can say that the drugs did not have an effect on the animals' weights when administered after stroke surgery. This is different from other work that has been performed in our laboratory, where the drug treated rats showed a decreased weight over time when compared to control rats, but in

the previous experiments the rats did not undergo post-stroke rehabilitation of the impaired arm.

Functional recovery

Montoya staircase test is a skilled grasping test that can effectively analyze even slight impairments in motor skills (Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991). When analyzing the post-stroke deficits and functional recovery, we have tried to extrapolate the results to understand the effects of the drug-treatment and physical rehabilitation on the motor function of the animals.

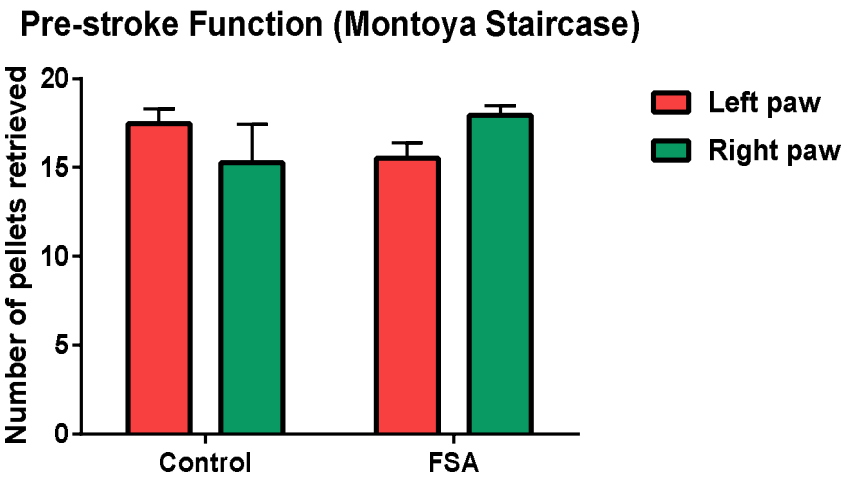


Figure (7): Pre-stroke motor function using the Montoya staircase apparatus.

Red bars represent the mean number of pellets retrieved by the left paw and the green bars represent those retrieved by the right paw. The error bars represent the standard error of the mean (SEM). The N for the number of control rats was 9 and that for the drug-treated (FSA) rats was 11. There was no statistical difference found between the numbers of pellets retrieved by each group (P=0.86)& no significant difference was found between the numbers of pellets retrieved by each paw (P=0.84) using two-way ANOVA test.

Figure (7) shows mean number of pellets retrieved by the rats in pre-stroke training which would be distributed into control and drug-treated FSA groups following stroke induction. The figure gives the mean number of pellets retrieved by the rats by right and left paws on the Montoya staircase apparatus. The mean number of pellets retrieved by control rats with left paw were 17.53 and those by right paw were 15.57; whereas, the number of pellets retrieved by the FSA rats with their left paw were 15.55 and those by the right paw were 17.96. The groups do not differ significantly in pellets retrieval when compared to each other, according to two-way ANOVA ($P=0.86$) indicating that all the rats showed considerable grasping motor function before stroke-induction. Moreover, the number of pellets retrieved by each paw did not show a significant difference ($P=0.84$).

After the intra-cortical induction of stroke with endothelin-1 injected into the forelimb motor cortex, the rats were tested for the motor functional deficit on post-stroke day 3. The rats which exhibited less than 20% of the contralateral deficits were excluded from the study. Thus, rat 813, rat 824 and rat 827 were taken out of further studies.

Furthermore, the rats were analyzed for bilateral deficits and evidence of damage to the corpus callosum.

Groups	Total number of animals	Animals with damage to the corpus callosum	Animals with bilateral deficits	Animals with unilateral deficits	Animals with less than 20% contralateral deficits
Drug-treated (FSA)	13	9	8	4	1
Control	12	7	4	6	2
Total	25	16	12	10	3
Died	1				

Table 3: Numbers of rats with evidences of damage to the corpus callosum, bilateral & unilateral deficits and with less than 20% contralateral deficits

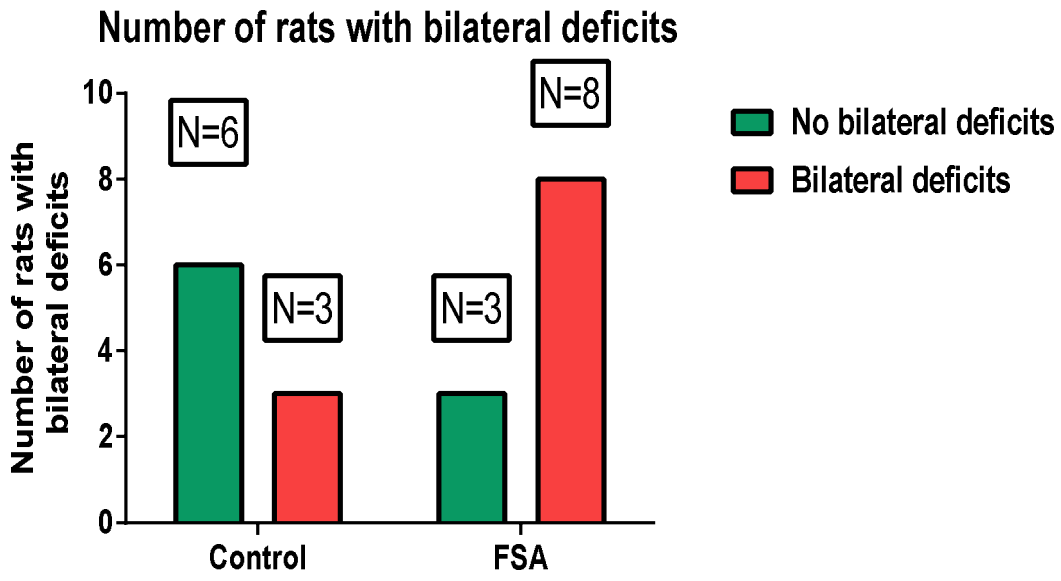


Figure (8): Number of rats with bilateral deficits.

The red bar gives the number of rats with bilateral deficits and the green bar gives those without bilateral deficits. The N for the number of control is 9; whereas that for the drug-treated rats is 11. No statistical difference (P=0.08) was found using Fisher's exact test.

Figure (8) depicts the number of rats from the control and the drug-treated groups with and without bilateral deficits; with the number of rats with bilateral deficits being higher in the drug-treated group. Based on the statistical results obtained via Fisher's exact test, there is no significant difference between the number of rats with bilateral deficits in the control and FSA groups (P=0.08). 3 rats out of 9 from the control group showed evidence of bilateral deficits and 8 rats out of 11 from the FSA group showed evidence of bilateral deficits. The animals were randomly assigned to control or drug groups after stroke induction surgery, so we would not expect to see significant differences in the bilateral deficits between the groups.

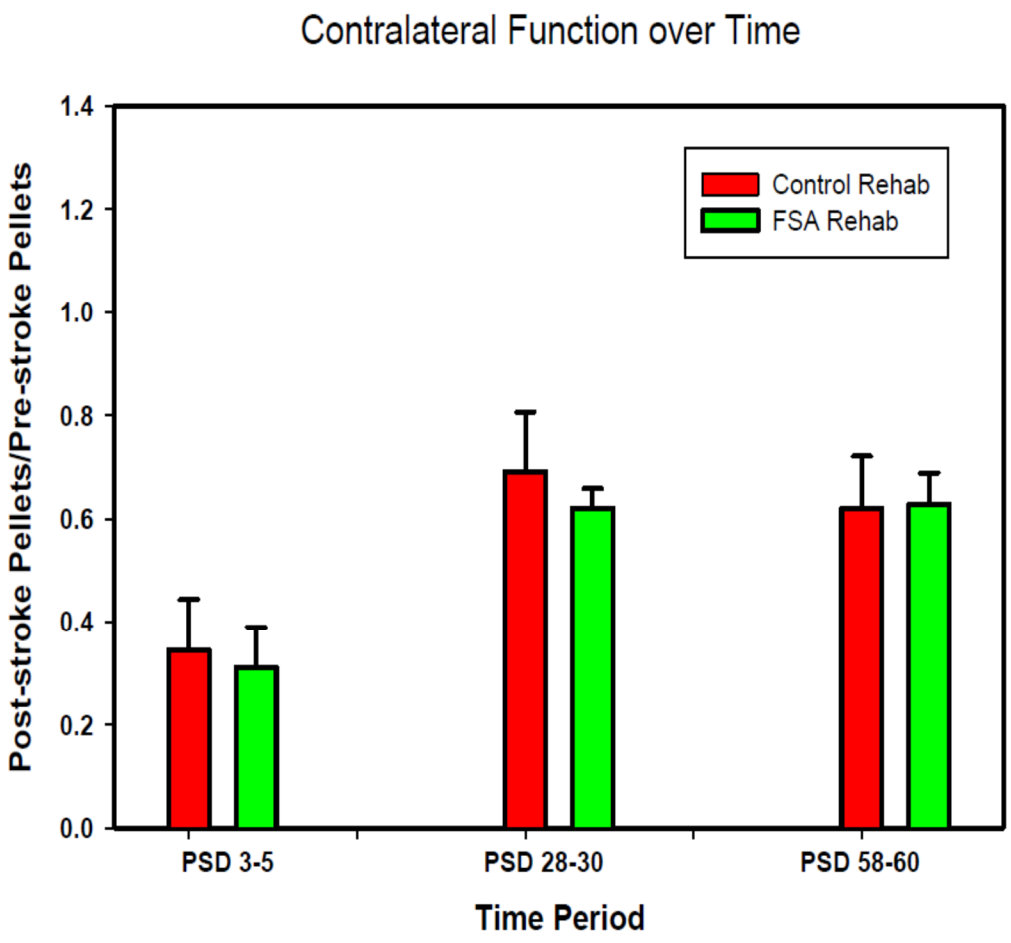


Figure (9): Contralateral function over time.

The red bars represent the control group of rats (number of control rats, N, is 9) and the green bars represent the drug-treated (FSA) group of rats (number of FSA rats, N, 11). The error bars represent the standard error of the mean (SEM). No significant difference was observed between control and FSA rats on any individual day (P=0.73) & a statistically significant difference was found between the results obtained on PSD 3-5 & PSD 28-30, PSD 58-60 (P<0.001) using two-way repeated measures ANOVA.

Figure (9) gives an insight in to the contralateral (stroke-affected) functional recovery achieved over a period of 60 days after the induction of stroke and administration of drugs/vehicle accompanied with physical rehabilitation. From the graph, we observe that the contralateral function tested on the Montoya staircase apparatus is approximately 35% of the pre-stroke function for the control rats and approximately 30% of the pre-stroke function for the drug-treated rats on post-stroke day 3-5. However, the contralateral functional recovery is observed to accelerate from post-stroke day 28 and remain fairly stable till post-stroke day 60, for the both the groups of rats. According to the Two-Way repeated measures ANOVA, the data differs significantly on the given days of Montoya testing with the P value of <0.001. Moreover, detailed post-hoc analysis (Holm-Sidak method) indicate that the contralateral functional recoveries of both the groups differ significantly ($P<0.001$) between post-stroke days 3-5 and 28-30. Similarly, the contralateral functional recoveries of both the groups also, differ significantly ($P<0.001$) on post-stroke days 3-5 and 58-60. However, the p-value for the data between post-stroke days 28-30 & 58-60 was found to be 0.60, thus, resulting in no significant difference between the contralateral recoveries on those two sets of days. In addition to this, the mean contralateral function for the control group of rats on PSD 3-5, PSD 28-30 and PSD 58-60 were found to be 0.35, 0.69 & 0.62, respectively with a SEM of 0.09. Along the same lines, the mean contralateral function for the FSA rats on the same days were found to be 0.31, 0.62 & 0.63, respectively with a SEM of 0.08. Moreover, there seems to be no significant difference between the contralateral function of the control rehabilitation and FSA rehabilitation rats ($P= 0.73$).

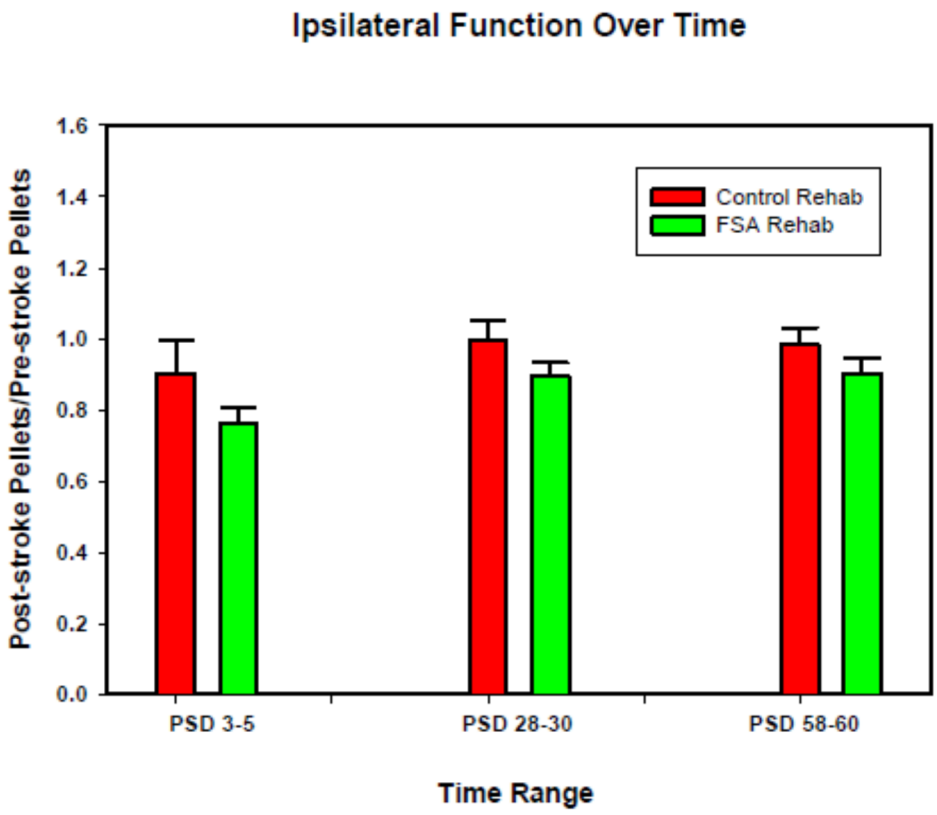


Figure (10): Ipsilateral function over time.

The red bars represent the control group of rats (number of control, N, is 9) and the green bars represent the drug-treated (FSA) group of rats (number of FSA, N, 11). The error bars represent the standard error of the mean (SEM). No significant difference was observed between control and FSA rats on any individual day (P=0.099) & a statistically significant difference was found between the results obtained on PSD 3-5 & PSD 28-30, PSD 58-60 (P=0.007) using a two-way repeated measures ANOVA.

Figure (10)depicts the functional recoveries of the control and drug-treated groups of rats on the ipsilateral side (the non-affected side) over a period of 60 days after the induction of stroke and administration of drugs/vehicle in combination with physical rehabilitation. From the graph, we observe that ipsilateral function on post-stroke days 3-4-5 is approximately 90% of pre-stroke function for the control group and approximately 75% of pre-stroke function for the drug-treated group (both the groups received physical rehabilitation). However, the ipsilateral functional on post-stroke days 28-29-30 & 58-59-60 are slightly higher than that of post-stroke days 3-4-5 & fairly similar to each other, thus, indicating a stable ipsilateral recovery after post-stroke days 28-29-30. According to the two-way repeated measures ANOVA test, the ipsilateral function is significantly different (P=0.007) on the given days of testing. A detailed post- hoc analysis (Holm-Sidak method) reveals that the function of ipsilateral functional recovery on post-stroke days 3-4-5 and 28-29-30 differ significantly with a P value of 0.005. Similarly, the function of ipsilateral functional recovery on post-stroke days 3-4-5 and post-stroke days 58-59-60 differ significantly with a p-value of 0.006. However, those on post-stroke days 28-29-30 and 58-59-60 do not show a significant difference (P=0.92) indicating a stabilized recovery after post-stroke days 28-29-30 up until post-stroke days 58-59-60. In addition to this, the mean function of ipsilateral recovery for control rats on PSD 3-5, 28-30 & 58-60 were found to be 0.90, 0.99 and 0.98, respectively with a SEM of 0.056. Along the similar lines, the mean function of the ipsilateral recovery for the FSA rats on the same days was found to be 0.76, 0.89 and 0.90, respectively with a SEM of 0.051. Moreover, there seems to be no statistical significance between the ipsilateral

function of the control and FSA groups (P=0.099); however, there seems to be a strong trend and a possibility of significant difference if the number of animals subjects be increased.

Total recovery:

The total functional recovery of both the groups of rats was calculated which includes the contralateral recovery and ipsilateral recovery. The calculations were performed as follows.

Example of Rat 802:

Pre-stroke function using Montoya staircase apparatus:

Left paw	Right paw
18	19

Post-stroke day 3-4-5:

These calculations would give us the baseline functional deficit of Rat 802.

Baseline function retained= $\frac{Maximum\ \#\ of\ pellets\ retrieved\ on\ post-stroke\ days\ 3,4,5}{Maximum\ \#\ of\ pellets\ retreived\ pre-stroke}$

1-Baseline function retained= Baseline functional deficit

Left paw	Right paw	Contralateral function	Contralateral deficit	Ipsilateral function	Ipsilateral deficit
11	15	0.61 (61% function retained)	0.39 (39% deficit)	0.79 (79% function retained)	0.21 (21% deficit)

Post-stroke days 28-29-30:

The calculations for establishing a deficit would be performed same as above. In addition to it, the functional recovery would also be calculated as follows:

Functional recovery= Post-stroke day 29 function retained- Baseline function retained.

Left paw	Right paw	Contrala-teral function	Contra-lateral deficit	Ipsilater-al function	Ipsilat-eral deficit	Contrala-teral recovery	Ipsilater-al recovery	Total recovery
10	17	0.56 or 56% function retained	0.44 or 44% deficit	0.89 or 89% function retained	0.11 or 11% deficit	-0.05 or -5 % recovery	0.1 or 10% recovery	0.05 or 5% recovery

Post-stroke days 58-59-60:

The calculations for post-stroke days 58-59-60 would be conducted in the same fashion as mentioned above.

Left paw	Right paw	Contrala-teral function	Contra-lateral deficit	Ipsilate-ral function	Ipsilate-ral deficit	Contralat-eral recovery	Ipsilate-ral recovery	Total recovery
12	18	0.67 or 67% function retained	0.33 or 33% deficit	0.95 or 95% function retained	0.05 or 5% deficit	0.06 or 6% recovery	0.16 or 16% recovery	0.21 or 21% recovery

This would give us the total functional recovery obtained at the end of our study.

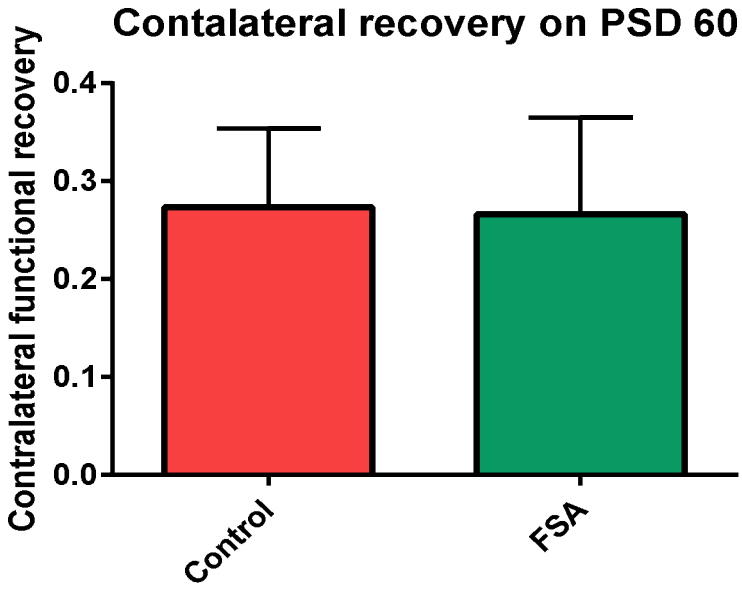


Figure (11): Contralateral recovery on post-stroke day (PSD) 60.

The red bars represent the control group of animals and the green bar represents the FSA group of animals. The error bars represent the standard error of the mean (SEM). No statistically significant difference was found between the contralateral functional recovery of the two groups (p= 0.98) using unpaired t-test with Welch's correction.

Figure (11) depicts the function of the contralateral functional recovery at the end of our study on post-stroke day 60. The control as well as the drug-treated rats seem to have obtained nearly 25% of total recovery tested using the Montoya staircase apparatus. The mean ± SEM for the control rats was found to be 0.26 ± 0.072 and that for the FSA rats was 0.27 ± 0.099. The difference between the means was 0.003 ± 0.12. Based on the statistical analysis performed by the unpaired t test using Welch's correction, the two groups do not show a significant difference with a P value of 0.98.

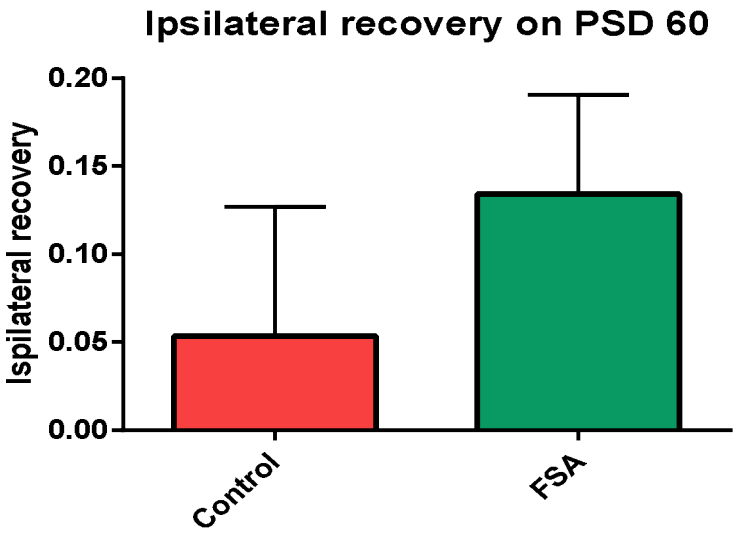


Figure (12): Ipsilateral recovery on PSD 60.

Red bars represent the control rats and the green bars represent the FSA rats. The error bars represent the standard error of the mean (SEM). No statistical significant (P=0.56) was found between the ipsilateral recoveries of the control and FSA groups using unpaired t-test with Welch's correction on post-stroke day 60.

Figure (12) depicts the function of ipsilateral recovery on post-stroke day 60. The control group has obtained a recovery of nearly 7 to 8 % whereas, the drug-treated group does not seem to differ much from the control group and seems to have achieved a recovery of nearly 12 to 13%. The mean ± SEM for the control rats was found to be 0.08 ± 0.08 and that for the FSA rats was found 0.13 ± 0.06. The difference between the means was 0.06 ± 0.09.Moreover, statistical analysis using unpaired t test with Welch's correction do not indicate a statistical difference with a P value of 0.56.

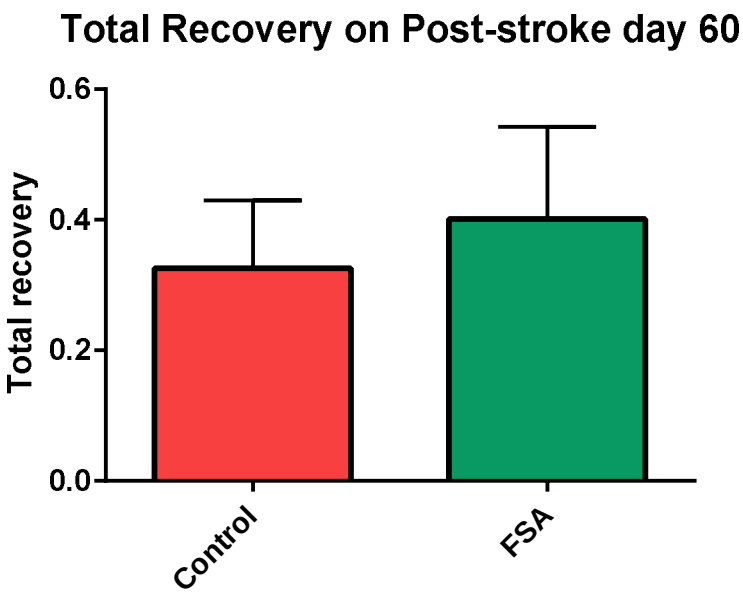


Figure (13): Total recovery on post-stroke day (PSD) 60.

The red bars represent the control animals and the green bars represent the FSA animals. The error bars represent the standard error of the mean (SEM). No significant difference (P=0.69) was found between the total functional recoveries of control and FSA rats using unpaired t-test with Welch's correction on post-stroke day 60.

Figure (13) depicts the total functional recovery achieved by both the groups of rats by post-stroke 60. The total recovery for the control rats seems to be approximately 35% and that for the drug-treated rats seems to be approximately 40%. The mean ± SEM for the control rats was 0.33 ± 0.09 and that for the FSA rats was 0.40 ± 0.14. The difference between the means was0.07 ± 0.17.According to the results obtained on analysis using the unpaired t test with Welch's correction, the total recovery of between two groups of rats does not differ significantly (P= 0.69).

Motor functional recovery over time:

The motor function test performed using the Montoya staircase apparatus has depicted that the control and the drug-treated groups do not differ significantly. An analysis of the contralateral and ipsilateral functional recovery on post-stroke days (PSD) 3-4-5, 28-29-30 & 58-59-60 indicated that the motor function of grasping sucrose pellets from the staircases was regained after the initiation of the delayed pharmacological treatment in combination with physical rehabilitation up until PSD 28-29-30 and was more or less stabilized till PSD 58-59-60. However, it was also observed that the control and the drug-treated groups did not differ from each other significantly. The studies of contralateral, ipsilateral and total recoveries at the end of our study on PSD 58-59-60 also showed that the two groups had no significant differences. Thus, it is a possibility that since the control + rehabilitation group displayed recovery very similar to the drug-treated + rehabilitation group of rats, the drugs were not as effective as they were expected to be. Also, there are chances that the rehabilitation exercises led to stress on the animals thus, antagonizing the effects of drugs and hampering the process of neurogenesis and recovery.

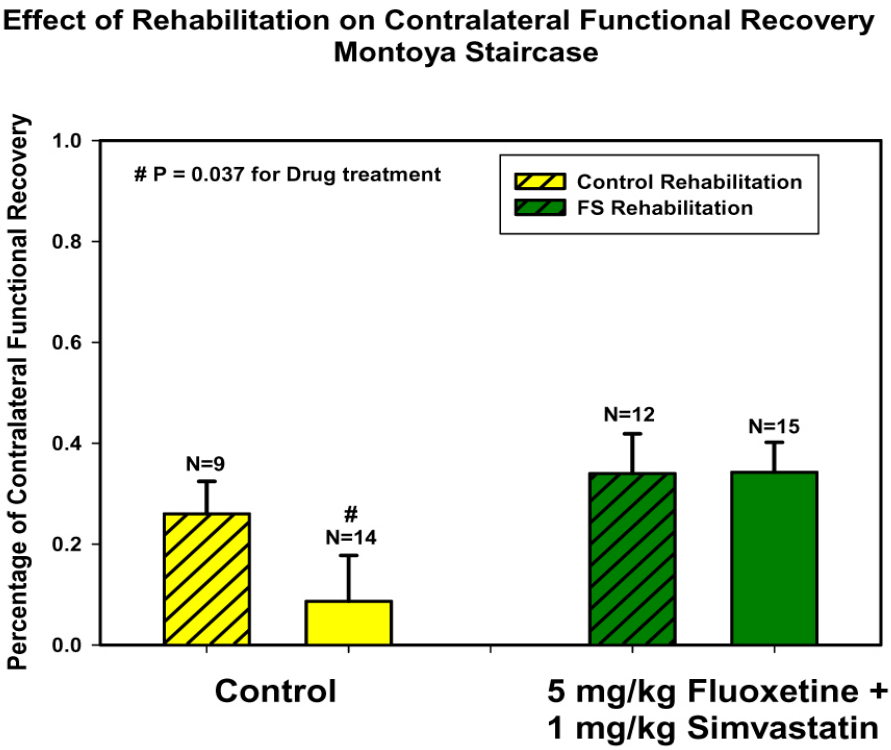


Figure 14: Effect of rehabilitation on contralateral functional recovery using Montoya Staircase

Yellow bars represent animals belonging to the control groups with and without rehabilitation and the green bars represent animals belonging to the drug-treated groups with and without rehabilitation. The hatched bars represent the rats which underwent physical rehabilitation and solid bars represent the animals with no rehabilitation.

Figure (14) consists of the results obtained in the previous works conducted in our laboratory. The drugs administered in this work were 5 mg/kg Fluoxetine and 1 mg/kg Simvastatin, 6-12 hours after the induction of stroke. The data showed a significant difference (P=0.04; Two-Way ANOVA) between the control rats subjected to physical rehabilitation with a contralateral functional recovery of ~26% of the pre-stroke function and those without physical rehabilitation with a contralateral functional recovery of ~8.6%. Moreover, there was no significant difference between the drug-treated rats subjected to physical rehabilitation and those with no rehabilitation with both the groups showing a ~34% of contralateral functional recovery. However, the control and drug-treated groups showed a significant difference overall with a P value of 0.04 calculated using Two-Way ANOVA.

Thus, the previous studies in our lab have proven that the drug treatment alone can help with functional recovery and so does physical rehabilitation, alone or in combination with the drugs.

The differences between the previous and the current studies included the different quantities of peanut butter used during physical rehabilitation, the length of days over which the animals were subjected to physical rehabilitation and the inclusion of Ascorbic acid in the pharmacological treatment. One or more of these differences could be the cause of less than expected functional recovery and neurogenesis in the animals.

Forelimb Asymmetry

The rats which did not meet the training criteria in the Montoya Staircase (at least 9 pellets retrieved with each forepaw) were subjected to Forelimb asymmetry test.This consisted of a cylinder in which the rat was placed and the voluntary exploration of the cylinder was allowed. We calculated the number of times either of the front paws was used as a means of exploration.

We observed that one rat, Rat 805, retrieved no pellets during pre-stroke Montoya staircase training. Thus, it was subjected to the Forelimb asymmetry test and the following results were observed.

Here, l stands for left paw, r stands for right paw, l(f) stands for left fingers and r(f) stands for right fingers. The letters denote the sequence in which the rat touched the cylinder walls and the intervals depict every time that the rat went back to its supine position.

Pre-stroke:

Lr(f)l(f)l rlrlrl(f) rlrlrlrrrl(f)l rlll(f)r
rrllrlrllrlrlrrlrlrlrrlrrlrllrlrrrlrrl(f)l(f)rrlrlrrlllr(f) rllrrl(f)

	Number of times the walls were touched
Left paw	34
Left fingers	7
Right paw	41
Right fingers	2
Total number of times the walls were touched	84

Post-stroke day 4:

Rrrl(f)rrrl(f)rrrl(f)rrl(f)rrrl(f)rl(f)rlrrrrlrl(f)rl(f)rrrrrrl(f)r

	Number of times the walls were touched
Left paw	2
Left fingers	9
Right paw	29
Right fingers	0
Total number of times the walls were touched	40

Post-stroke day 30:

R rlrrrl(f)r r rrrrrrrl(f)rrl(f)r rrl(f)rrrrrrrl(f)rrrrrl(f)r rrrl(f)r rrl(f)r rrrrrrr

	Number of times the walls were touched
Left paw	1
Left fingers	8
Right paw	46
Right fingers	0
Total number of times the walls were touched	55

Post-stroke day 60:

R rl(f) rrrl(f)r rl(f)r rrl(f) l rr r(f) l(f)r rl(f) rl(f) rl(f)l(f)r(f) rl(f) rl(f) rrrlrlrl(f) r rl(f)rr

	Number of times the walls were touched
Left paw	3
Left fingers	13
Right paw	27
Right fingers	2
Total number of times the walls were touched	45

The calculations depict the motor function retained as well as the functional deficit observed at the end of the study.

Calculations:

Pre-stroke function of the left paw =

$$\frac{number\ of\ times\ the\ left\ paw\ touched\ the\ wall}{total\ number\ of\ times\ the\ wall\ was\ touched\ by\ both\ the\ limbs} = \frac{34}{75} = 0.45$$

Post-stroke days 3-5 function of the left paw=

$$\frac{number\ of\ times\ the\ left\ paw\ touched\ the\ wall}{total\ number\ of\ times\ the\ wall\ was\ touched\ by\ both\ the\ limbs} = \frac{2}{31} = 0.07$$

Baseline function retained on post-stroke days 3-5 of the left paw=

$$\frac{Maximum\ \#\ of\ times\ the\ walls\ were\ touched\ on\ post-stroke\ days\ 3,4,5}{Maximum\ \#\ of\ times\ the\ walls\ were\ touched\ pre-stroke} = \frac{2}{34} = 0.06$$

Baseline functional deficit of the left paw= 1- baseline function retained= 1-0.06= 0.94

Post-stroke days 58-60 function of the left

paw=$\frac{number\ of\ times\ the\ left\ paw\ touched\ the\ wall}{total\ number\ of\ times\ the\ wall\ was\ touched\ by\ both\ the\ limbs}$= $\frac{3}{30}$ = 0.1

Functional recovery of the left paw= $\frac{Post-stroke\ function\ on\ days\ 58-60}{Pre-stroke\ function}$ = $\frac{0.1}{0.45}$= 0.22

Functional deficit of the left paw= 1- Functional recovery of the left paw= 1-0.22 = 0.78 (78% deficit)

Similarly, functional deficits on post-stroke days 3-5 & 28-30 were determined and similar calculations were performed to determine the functional deficits of the right paw on pots-stroke days 3-5, 28-30 & 58-60.

	Pre-stroke	Post-stroke day 4	Post-stroke day 30	Post-stroke day 60	Pre-stroke function	Post-stroke day 60 function	Function retained	Functional deficit (%)
Left	34	2	1	3	0.45	0.1	0.22	0.78 (78%)
Right	41	29	46	27	0.55	0.9	1.64	-0.64 (-64%)
Total	75	31	47	30				

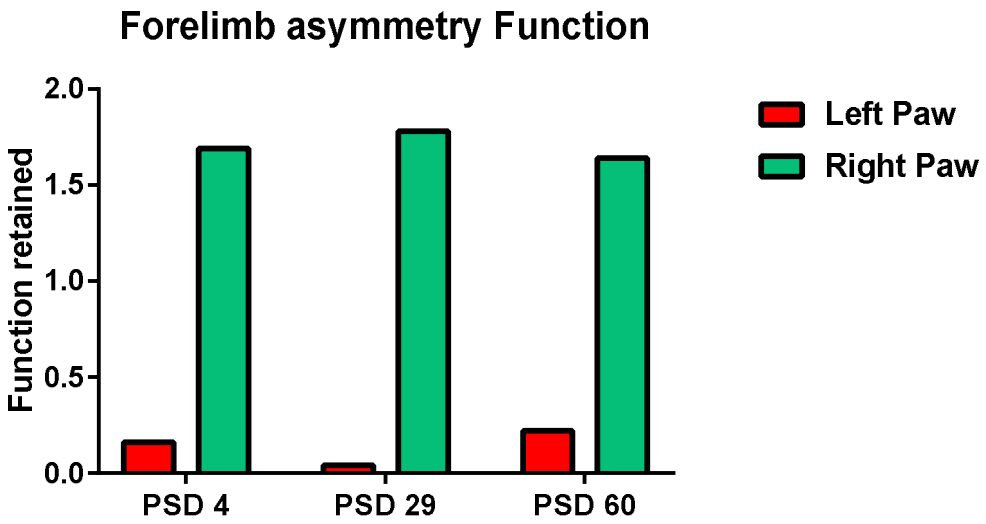


Figure (15): Forelimb asymmetry function.

Red bars represent the function retained by the left paw on PSD 3-5, 28-30 and 58-60 for a single rat. Green bars represent the same for the right paw in the same rat. Significant difference between the function retained by left and right paws on any given individual day (P= 0.004). No significant difference within the function retained by either the left paws or the right paws over the given period of time (P= 0.98) using two-way ANOVA test.

Figure (15) depicts the function retained by Rat 805 on post-stroke days 3-4-5, 28-29-30 & 58-59-60. Here, 1.0 is the pre-stroke function representing 100% functional recovery. The data shows that the function retained by the rat on the contralateral limb was only about 20% of the pre-stroke function over the period of our study as opposed to the ipsilateral limb which retained more function as compared to the pre-stroke function. Statistical analysis using two-way ANOVA indicated significant differences between the contralateral and ipsilateral function throughout (P= 0.004); moreover, no significant difference was indicated (P=0.98) in the case of functional recovery within the contralateral (left) paw results and within the ipsilateral (right) paw results over the given period of 60 days. The function retained by the left paw on PSD 3-5, 28-30 & 58-60 were 0.16, 0.04 & 0.22, respectively and that by the right paw were 1.69, 1.78 & 1.64, respectively.

Functional recovery assessed using the forelimb asymmetry test:

The results show that Rat 805 did not show motor functional recovery over the period of study, despite of the drug treatment. We can see that it displayed a pre-stroke function which comprised of a very similar use of both the fore-paws. Induction of stroke decreased the functionality of the contralateral (stroke-affected) left limb whereas, the use of the ipsilateral right limb increased more than the pre-stroke function on post-stroke days 3-4-5 to compensate for the lack of functionality of the left paw. These results remained uniform on the post-stroke days 28-29-30 & 58-59-60, too. Even though, there were no significant differences between the results, a slight drop in the functional recovery in the left paw on PSD 28-30 and in the right paw on PSD 58-60 were observed. This could be attributed to some effect of drugs and stress on the study animal. This

indicated that the drug-treatment on the rat was not imparting the expected results. There could be a possibility that the stress caused due to physical rehabilitation could have over-powered the effect of the drugs and thwarted neurogenesis.

Infarct Volume

Clinical evidence has suggested that motor deficits after a stroke are affected by the site of cortical damage. The extent of motor and functional recovery are based on the location of the lesion as well as its size(Chen, Tang, Chen, Chung, & Wong, 2000). Thus, we have analyzed the infarct volumes and the infarct locations, analyzed the effect of drugs and tried to compare these results with the observed motor deficits.

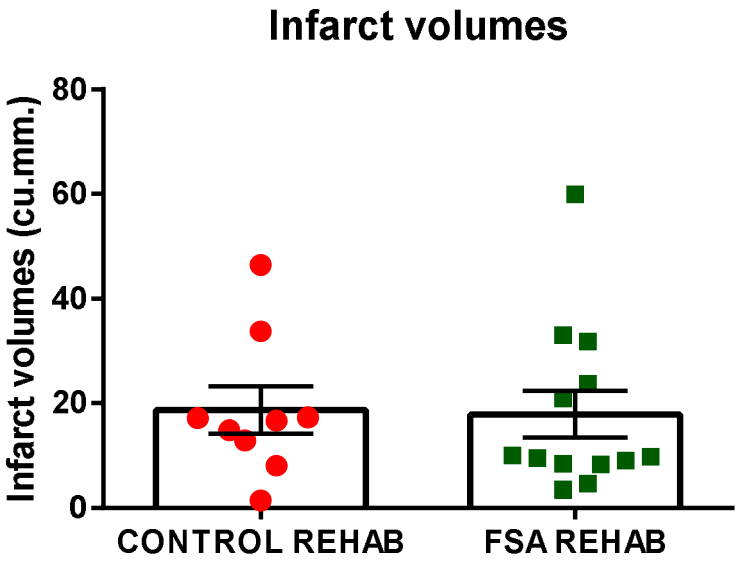


Figure (16): Infarct volumes: Control vs. FSA.

The red dots (●) represent the individual infarct volumes of the control rats (N= 9) and the green squares (■) represent the individual infarct volumes of the FSA rats (N=13). The bar graphs give the mean infarct volumes and the error bars represent the standard error of the mean (SEM). No statistically significant difference (p=0.94) was found between the infarct volumes of the two groups using Unpaired t-test with Welch's correction.

Figure (16) gives the distribution of the infarct volumes (mm^3) in the control and drug-treated rats, both the groups which underwent physical rehabilitation. The mean ± SEM for the control rats was found to be 17.44 ± 4.24 and that for the FSA rats was found to be 17.92 ± 4.43. The difference between the mean infarct volumes of both the groups was 0.49 ± 6.13. Both the groups of rats seem to have approximately similar range of infarct volumes and there seems to be no considerable difference between them. Based on the statistical analysis performed using the unpaired t test using Welch's correction, the infarct volumes observed in the control and drug-treated rats did not differ significantly ($p= 0.94$). Moreover, the data was not significantly different even after disregarding the outlier in the drug-treated group.

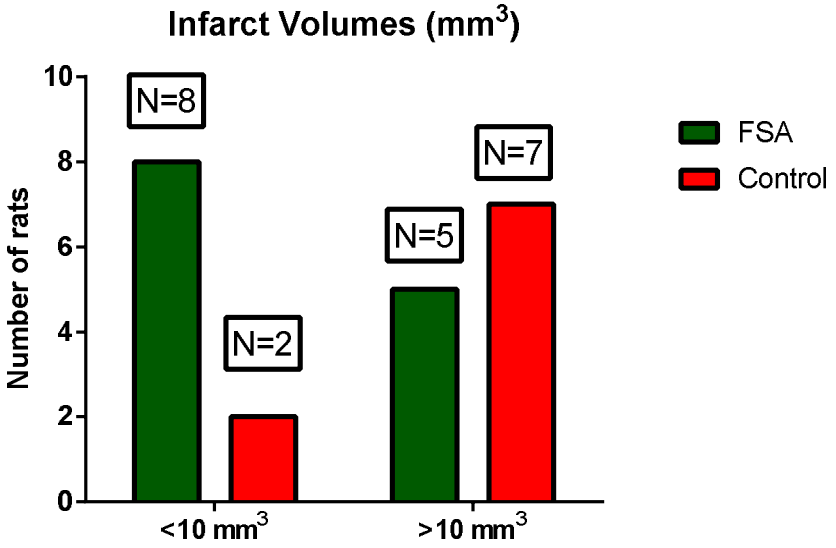


Figure (17): Number of rats with infarct volumes less than 10 mm^3.

The red bars green bars represent the number of control rats with infarct volumes less than or greater than 10 mm^3 and the green bars represent the FSA rats with infarct volumes less than or greater than 10 mm^3. No significant difference (P=0.09) was found between the number of rats in each of the groups with less than or greater than 10 mm^3 infarct volumes using Fisher's exact test.

Figure (17) analyzes the number of rats in both, the control and the drug-treated groups of rats with infarct volumes (mm^3) greater than and less than 10 mm^3. We observed that 8 rats out of 13 from the drug-treated group had infarct volumes less than 10 mm^3 and 7 rats out of 9 from the control group had infarct volumes greater than 10 mm^3. The statistical analysis using Fisher's exact test showed that the number of control rats with infarct volumes greater than or less than 10 mm^3 did not differ significantly and neither did the drug-treated rats ($p=0.09$); however, the p value indicates that the data showed a trend and that there is a possibility of the data being significant had the number of animals subject been increased.

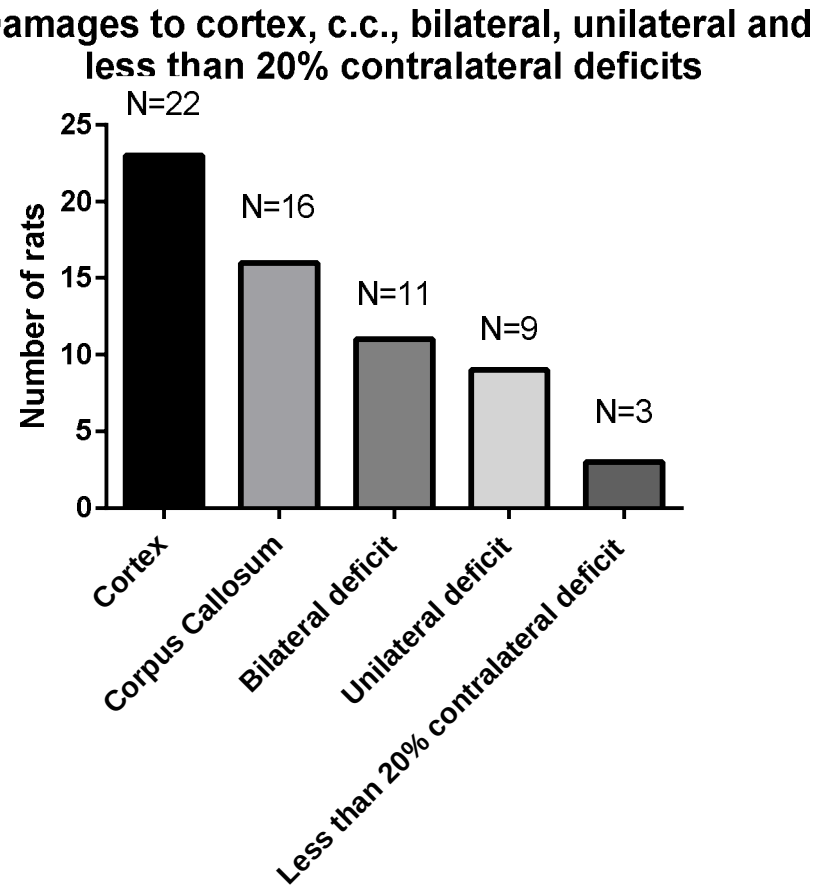


Figure (18): Number of rats with damages to the cortex, corpus callosum (c.c.), bilateral deficits, unilateral deficits and less than 20% contralateral deficits.

In the Figure (18) we give the number of rats that have suffered damage to the forelimb motor cortex in either of the brain hemispheres, damage to the corpus callosum (c.c.), bilateral damages to the brain tissue sections, unilateral damages and damage to the contralateral side of the brain tissue not enough to cause 20 % deficit in the motor function. All the rats in the study (22) suffered damage to the cortex as a result of stroke induced using a powerful vasoconstrictor, endothelin-1. Out of 22 animals, 16 suffered damage to the corpus callosum, 11 exhibited from bilateral deficits during motor function tests as a result of damage to the corpus callosum, 9 exhibited unilateral deficits and 3 showed less than 20 % contralateral deficits.

We analyzed the brain tissue sections with the above mentioned types of damages and found that they correlated with the presence or absence motor function deficits tested using the Montoya staircase apparatus.

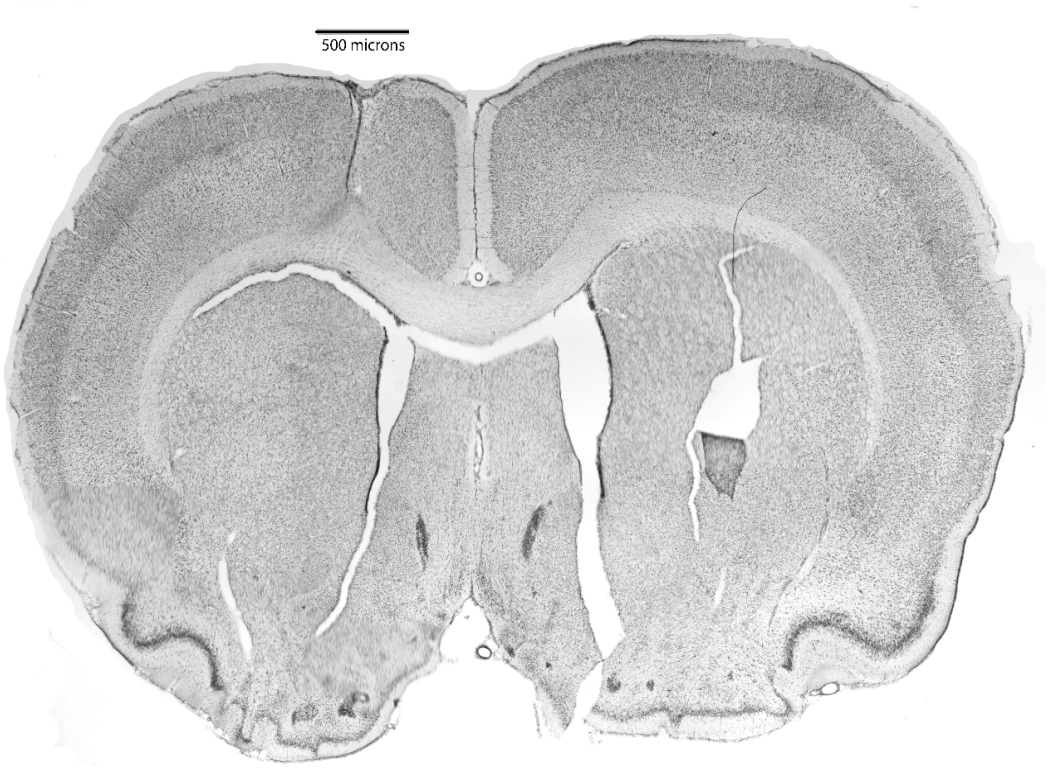


Figure (19): Brain section showing an infarct for Rat 827.

Figure (19) depicts a cartoon image and an actual image of the brain section of Rat 827. This brain section displayed a small infarct and the contralateral functional deficit calculated based on the Montoya staircase test results for Rat 827 was recorded to be less than 20%. Rats with less than 20% of contralateral functional deficit were excluded from the study because the deficit did not meet our inclusion criteria.

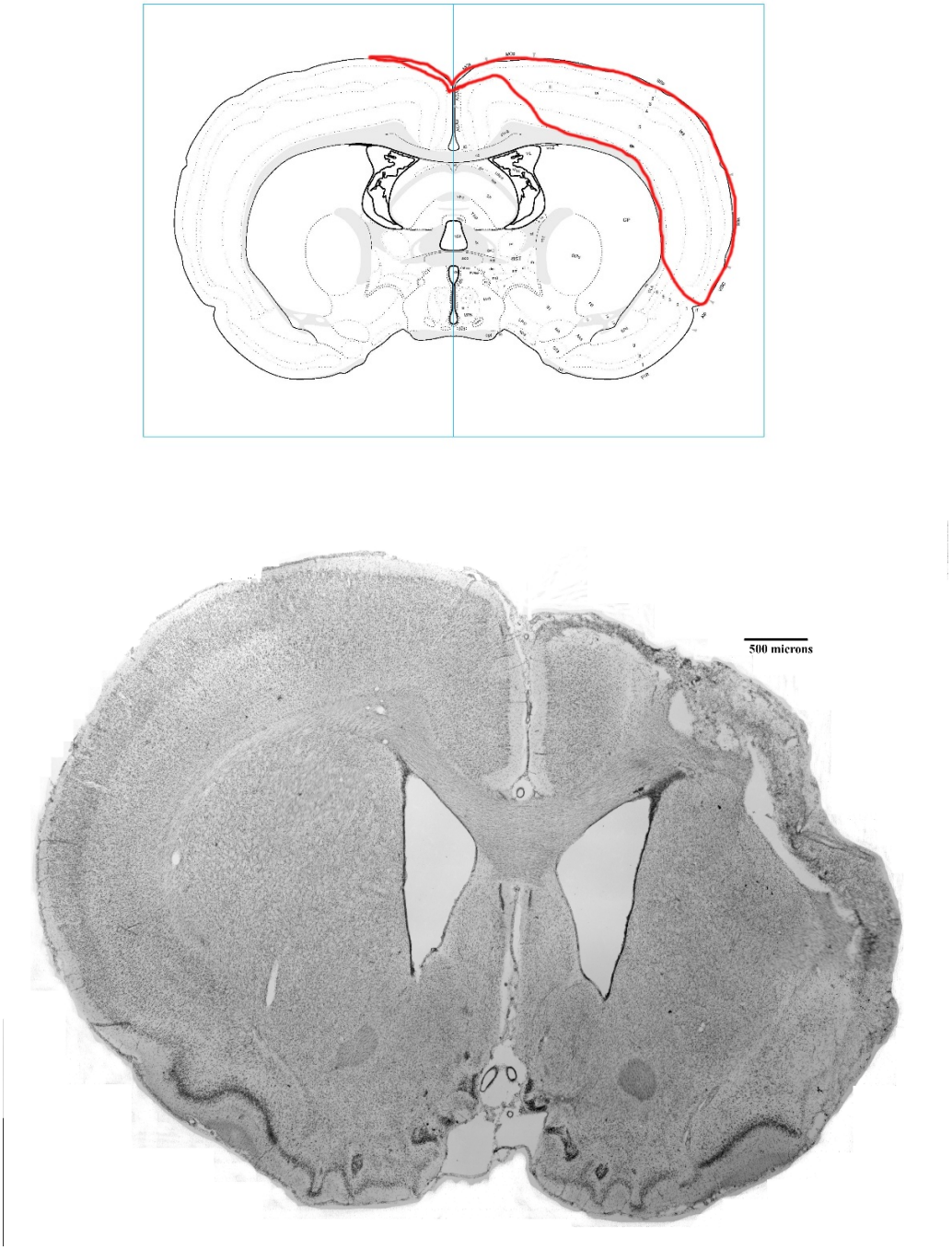


Figure (20): Brain section showing an infarct for Rat 825.

Figure (20) depicts the cartoon image and the actual image of a posterior brain section of a control Rat 825. The brain section displays damage to the corpus callosum which also contributed towards bilateral deficit for Rat 825. We also observe damage to the left hemisphere of the brain section, across the midline.

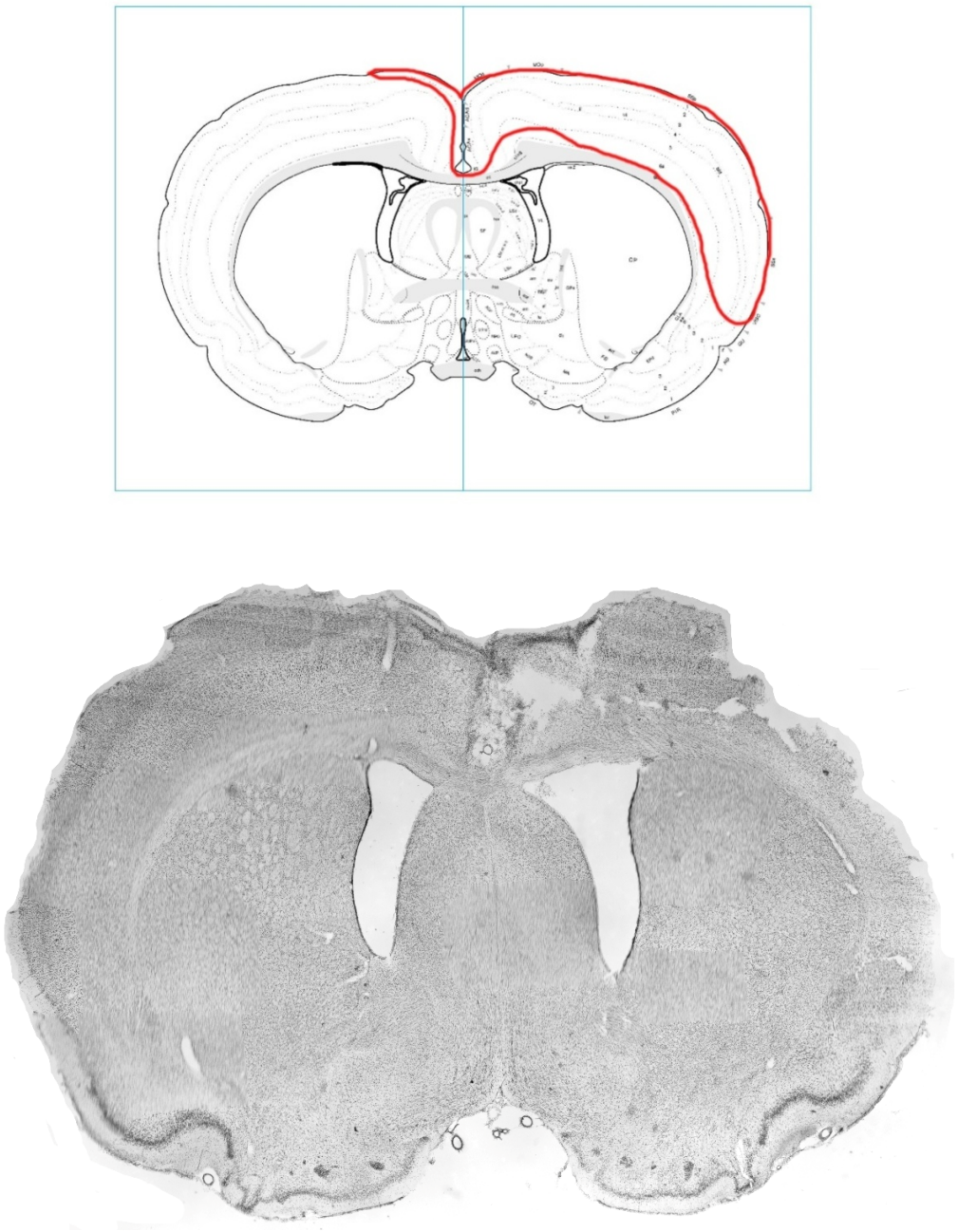


Figure (21): Brain section showing an infarct for Rat 803.

Figure (21) depicts a cartoon image and an actual image of a posterior brain section of a drug-treated Rat 803. The brain section shows damage until the corpus callosum and thus, suggests a bilateral deficit. The functional recovery tests performed using Montoya staircase apparatus confirm that Rat 803 was bilaterally deficient. We also observe the damage to the other hemisphere of the brain section, crossing the midline.

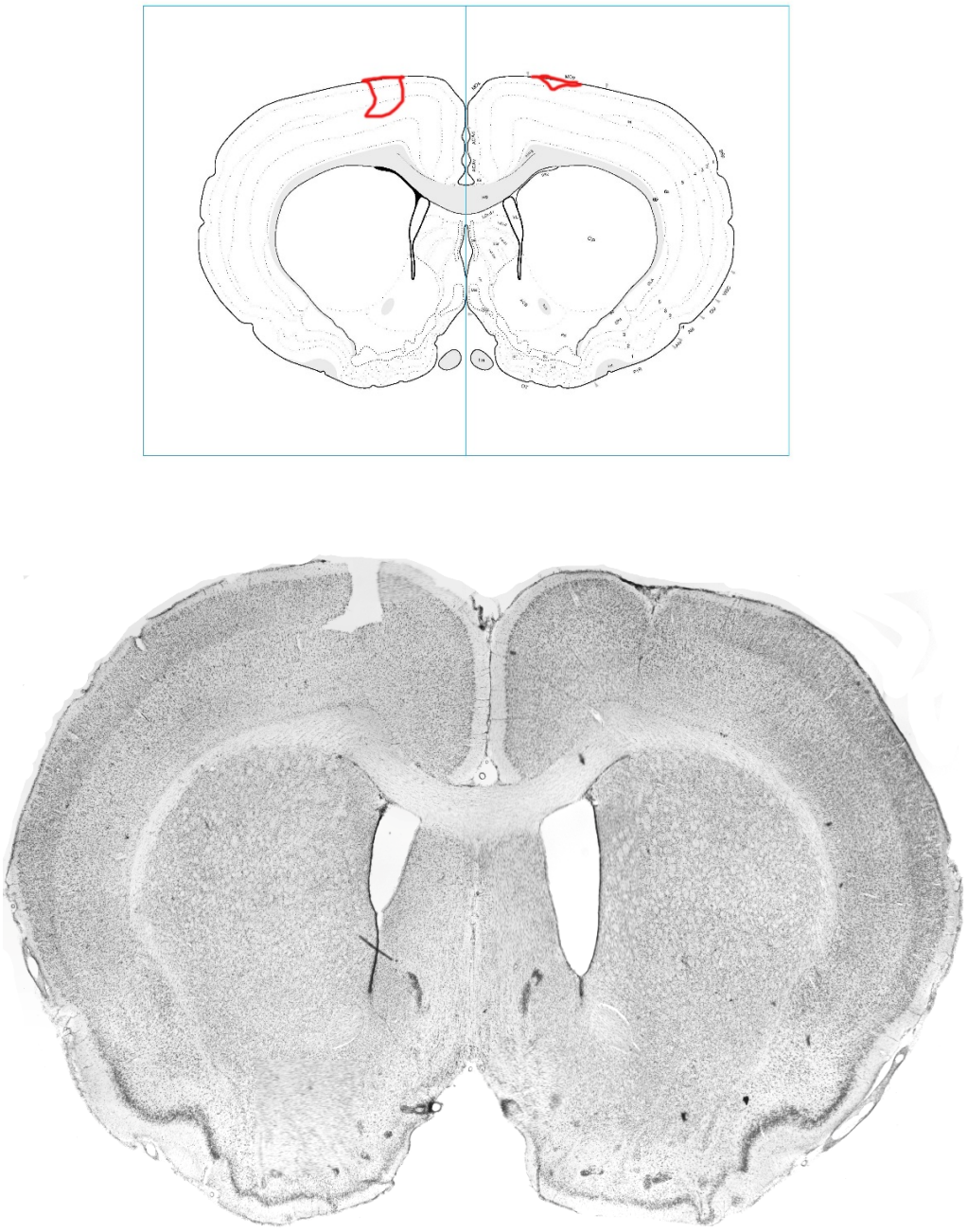


Figure (22): Brain section showing an infarct for Rat 813.

Figure (22) depicts the cartoon image and the actual image of the brain sections of Rat 813. The brain section shows a very small infarct; moreover, the functional deficit calculated based on Montoya staircase test results indicate 11% contralateral deficit and 22% ipsilateral deficit. Thus, this rat exhibited a unilateral deficit but since, it was observed on the ipsilateral side and the contralateral deficit was less than 20%, Rat 813 was not included in the study.

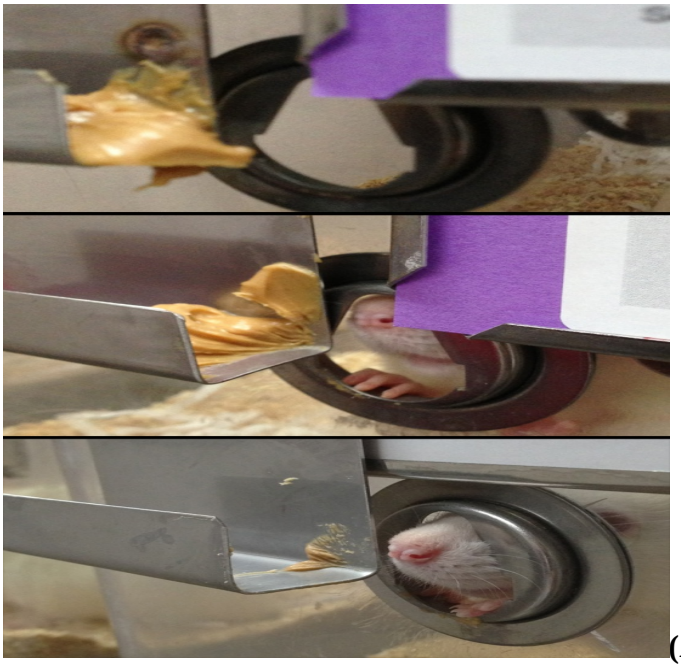
Infarct volume analysis:

Based on the results, we observed that the infarct volumes between the two groups of rats (control & drug-treated) do not differ significantly which could indicate that the drugs did not show the desired effects on the infarct volumes or they were over-powered by the stress caused due to physical rehabilitation. However, it was observed that more of the drug-treated rats had infarct volumes less than 10 mm^3 as compared to the control rats. This could be considered a positive drug-plus-rehabilitation effect on the reduction of infarct volume and functional recovery. In addition to this, the brain sections observed with damage to the corpus callosum and damage to the left hemisphere were found to present bilateral deficits during the Montoya staircase test. The corpus callosum connects the right and left hemispheres of the brain and conducts neuronal transmission. Thus, damage to the corpus callosum in one hemisphere causes a deficit in the functions related to the other hemisphere, too.

IV. DISCUSSION

Our study to examine the effects of a delayed post-stroke pharmacological treatment and physical rehabilitation on the infarct sizes in female Sprague Dawley rats presented the results which indicated that the drug combination of Fluoxetine, Simvastatin and Ascorbic acid administered 20-26 hours after the occurrence of stroke, in combination with physical rehabilitation did not exhibit the expected effects.

Research has shown that an excess and forced use of the impaired limb can lead to a massive increase of the neuronal injury (Kozlowski, James, & Schallert, 1996). In our study, even though the limb use was voluntary, the quantity of peanut butter hung on the shelf outside individual rat's cage lead to an excess use of the affected forelimb. The rats were found to be voluntarily making around 200 attempts towards ingesting the peanut butter every alternate night for 23 such days. This lead to an excess stress, countered the effects of the pharmacological treatment and ultimately, affected the process of neurogenesis and infarct reduction.



(A)

(B)

Figure (23): Peanut butter quantities in (A) Previous study in our lab and (B) Current study in our lab

A comparison of the quantities of peanut butter placed during our previous studies and current study, clearly give an idea of the differences in the amounts of peanut butter. The previous physical rehabilitation studies in our lab had shown positive results on the reduction of infarct sizes and increased functional recovery (Figure 24).

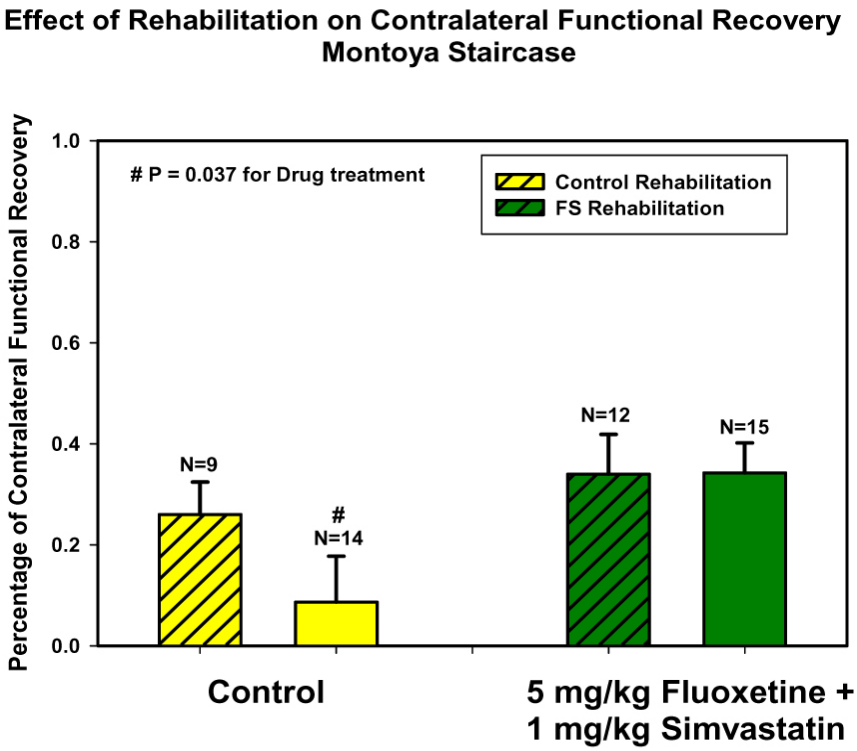


Figure (24): Effect of physical rehabilitation on contralateral functional recovery using Montoya staircase apparatus

The Y-axis depicts the function of contralateral recovery, which when multiplied by 100 would give the percentage of contralateral recovery. Significant difference was found between both groups of drug-treated animals and control animals with no rehabilitation (P=0.037) using a two-way ANOVA test.

The previous study conducted in our lab by Mr. Moner A. Ragas and Ms. Maria HH Balch showed that there existed significant difference between the contralateral functional recovery of drug-treated group of animals with and without rehabilitation and the control animals without rehabilitation. This suggested that the drug treatment was an essential factor in functional recovery after stroke. Moreover, the study showed no significant difference between the control animals with rehabilitation and drug-treated animals with rehabilitation; thus, suggesting that physical rehabilitation alone can be a determining factor in this treatment.

Rat weights

The rats were tested for the differences in their weights before and after the induction of stroke; wherein, all the rats showed significantly different weights within their groups during the period of study. Studies have shown that brain-derived neurotrophic factor (BDNF) causes a decrease in appetite, eventually leading to weight loss (Xu & Xie, 2016). Furthermore, Fluoxetine influences the expression of BDNF (Guirado et al., 2012; Kitahara et al., 2016). Based on our observations, the weights of the rats in the drug-treated group did not decrease. Thus, the increase in the mean weights of the rats from approximately 322 grams to 335 grams was possibly due to their regular food intake and lack of the effect of drugs and thus, of BDNF. We think that excessive physical rehabilitation caused a lot of stress to the animals which antagonized the effects of drugs.

Functional recovery

Significant recovery was observed in both the groups of rats after the initiation of physical rehabilitation exercise till post-stroke days 28-29-30. However, the recovery

plateaued out till the end of the period of our study. In addition to this, the drug-treated rats showed recovery similar to the control rats throughout the given period of study. Thus, the drugs did not play a part in the process of functional recovery. A reason for which could be the generation of excessive stress due to physical rehabilitation which antagonized the efficacies of the pharmacological treatment. This suggestion is also in lines with the research that intense rehabilitation can affect neuroanatomical as well as behavioral effects of physical rehabilitation. Studies support that intense rehabilitation can decrease injury-induced plasticity and reduce neurogenesis and functional recovery (Kozlowski, Leasure, & Schallert, 2013). In addition to this, patients forced to use their contralateral arm 9 ± 4 days after stroke occurrence for 2 weeks for a 90 day study exhibited less functional recovery in comparison to the patients who underwent traditional physical rehabilitation (Dromerick et al., 2009). On the contrary, physical rehabilitation, in our current study, helped the animals recover approximately 35- 40% of their pre-stroke function. Thus, stroke patients with limited or no access to medications can rely on physical rehabilitation to a certain extent for the repair of neural damage.

Infarct volumes

The animals belonging to both the groups had similar infarct volumes at the end of the study. This strengthens our understanding that the pharmacological treatment did not have the expected result on the areas with neural damage. In a previous study in this lab, we had also seen that the combination of Fluoxetine, simvastatin and ascorbic acid had little effect on the infarct volume in female rats, while fluoxetine and simvastatin significantly reduced the infarct volume. However, on detailed testing for the infarct volumes greater than or lesser than 10 mm^3, we found that a greater number of drug-

treated rats had infarct less than 10 mm^3 of volume but, the contingency tests showed a strong trend, but no significant difference.

Research has shown that the location and size of lesion affect the motor and functional recovery (Chen, Tang, Chen, Chung, & Wong, 2000). Thus, we tested for the infarct locations and the corresponding motor deficits. All the 22 rats showed a damage to the cortex; thus, ensuring that the cortex was damaged during the stroke-induction procedure using endothelin-1 injection. The damage also extended to the corpus callosum in the right hemisphere near the site of injection in 16 animals and out of these animals, 11 showed evidence of bilateral deficits during the Montoya staircase testing on post-stroke days 3-5, 28-30 & 58-60. Moreover, 3 rats showed less than 20% contralateral deficits which is the least expected amount we anticipate with this surgery; thus, we may have missed the forelimb motor cortex in these animals or our injections were incorrect. Out of these three rats, 2 were excluded from the study because the size of the infarct indicated an injection error for the endothelin-1; however, one rat (Rat 824) was excluded only from the motor functional recovery studies because it showed evidence of damage to the cortex with a considerable infarct but was slightly off from the forelimb motor cortex so, did not show a motor functional deficit.

Possible reason for the lack of neurogenesis

Stress is one of the most detrimental factors affecting neurogenesis after a traumatic brain injury. In addition to neurogenesis, it can affect metabolism, cardiovascular function, immune function, neurophysiological and endocrine function as well as the inflammatory processes (Corbett, McGowin, Sieber, Flannery, & Sibbitt, 2012). Thus, it was imperative to employ a reliable method of stress-free drug administration. Chronic intraperitoneal

drug administration and oral gavage have been shown to increase the levels of the stress hormone, corticosterone (Aguilera, Kiss, & Sunar-Akbasak, 1995; Corbett et al., 2012; Pung, Zimmerman, Klein, & Ehrich, 2003). Hence, we utilized a method developed by our lab for stress-free and voluntary oral administration of the drug combination. This method has been tested and tried to ensure that the sugar cookie dough we use as a vehicle does not interact with the drugs and lessen their efficacy. Moreover, the previous studies in our lab have shown considerable functional recovery in groups administered the drug combinations in the same amounts without physical rehabilitation. Thus, we can rule out the possibility that the drugs did not have the required efficacy or that the mode of drug administration was a source of stress.

We also subjected the rats to pre-stroke and post-stroke training to gauge their grasping abilities by placing them in the Montoya Staircase apparatus. However, these training periods lasted for only 15 minutes a day and moreover, they were conducted only on post-stroke days 3-5, 28-30 and 58-60. Thus, this procedure cannot be deemed stressful either.

Lastly, the rats underwent physical rehabilitation which comprised of swiping peanut butter from the shelves hung outside their cages. Comparison between the quantities of peanut butter placed in the shelves during the previous studies and the current study in our lab exhibits a vast difference. Our current study consisted at least 4 times the amount of peanut butter in each shelf in comparison to that in our previous study. Despite of being a voluntary exercise, the animals used their paws nearly 200 times every other night to eat the peanut butter. Moreover, the duration of physical rehabilitation was longer than that during the previous studies. This caused a lot of physical stress on the

rats which antagonized the effects of drugs and proved to be a reason for less functional recovery.

Effect of stress hormone on neurogenesis

Corticosterone and Cortisol are the corticosteroids which are also termed as ‘stress hormones’ in rodents and humans, respectively. They are the primary hormones responsible for stress management and for maintaining the homeostasis in the body. In rodents, corticosterone binds to either type I (mineralocorticoid) receptors or type II (glucocorticoid) receptors. Even though mineralocorticoid receptors have a higher binding affinity towards corticosterone, glucocorticoid receptors are considered to be the key components in neurogenesis and synaptic plasticity (Lau et al., 2007).

Adult neural stem/progenitor cells in the sub-ventricular zone and in the sub-granular zone of the dentate gyrus of the hippocampus play an important role in memory and psychological disorders. Along with olfactory bulb, sub-granular zone (SGZ) and sub-ventricular zone (SVZ) have been found to be the main sites of adult neurogenesis. (Abdanipour, Sagha, Noori-Zadeh, Pakzad, & Tiraihi, 2015). Stress, on the other hand, has been proven to be detrimental to the process of cell proliferation and neurogenesis (Corbett et al., 2012; Schoenfeld & Gould, 2013). Studies have proven that even though glucocorticoids at basal levels are essential for neuronal plasticity and development (Abdanipour et al., 2015), elevated levels of stress and hence, high levels of glucocorticoids hinder neurogenesis in the hippocampus and in the sub-ventricular zone (SVZ) (Lau et al., 2007). Moreover, research has shown that high doses of cortisol administered in-vitro to the adult neural stem/precursor cells excessively inhibited cell viability and proliferation (Abdanipour et al., 2015). In addition to this, stress also

decreases the expression of BDNF and around 18% decrease in cell proliferation was observed in the SVZ in a study of rats treated with corticosterone (Lau et al., 2007).

Since, the positive and negative effects of exercise depend upon the intensity and duration of stressor (Russell, Zigmond, Dimatelis, Daniels, & Mabandla, 2014), the excessive exercise that the animals went through during physical rehabilitation, in our study, could have caused excess amount of stress, leading to a rise in the corticosterone levels in the brains and inhibiting cell proliferation and neurogenesis.

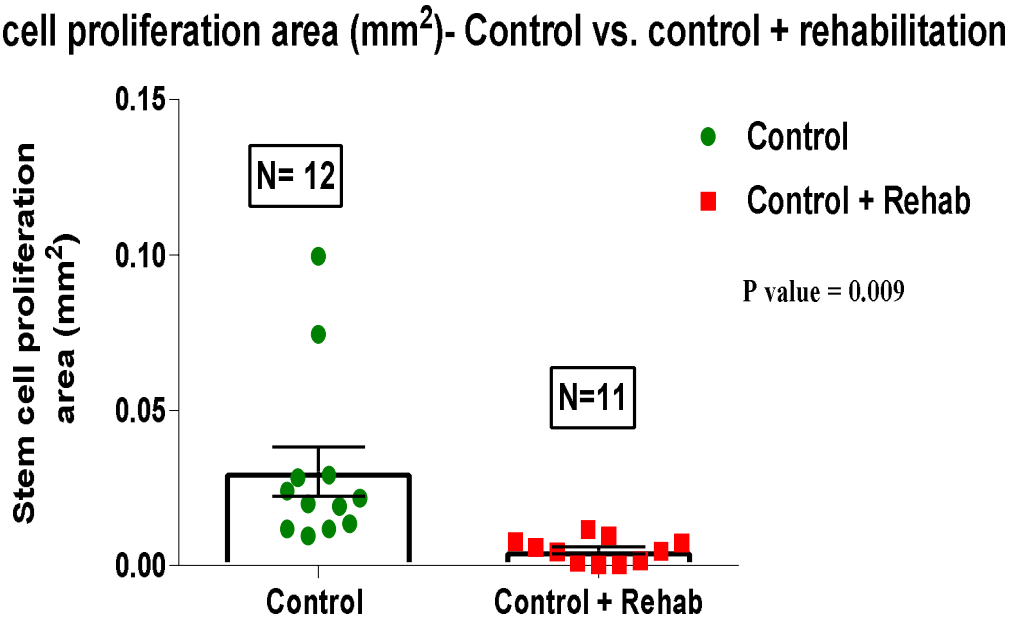


Figure (25): Stem cell proliferation area (mm^2) – Control rats versus Control rats with rehabilitation.

The Y-axis indicates the area of the sub-ventricular zone with stem cell proliferation marked using Ki-67 biomarker. The bars indicate the mean areas of the sub-ventricular region occupied due to stem cell proliferation for all the rats in each of the groups. The green dots (●) represent individual areas for each control rat and the red squares (■) represent those for individual control rats which were also subjected to rehabilitation. The error bars represent the standard error of the mean (SEM). Significant difference (P= 0.009) was observed between the stem cell proliferation areas of the control and control + rehabilitation groups using the unpaired t-test with Welch's correction.

The parallel work in our laboratory by Ms. Devi Priyanka Nagarajan analyzed the effect of the physical rehabilitation on functional recovery. The study utilized a biomarker for neurogenesis, Ki-67. Since, Ki-67 is expressed only during the active stages of cell cycle (Scholzen & Gerdes, 2000), it was utilized as a biomarker to examine stem cell proliferation in the sub-ventricular zone. Figure (25) gives the area of the sub-ventricular zone with Ki-67 expression between the control group without physical rehabilitation and the control group with physical rehabilitation. Analysis with unpaired t-test with Welch's correction suggests that the two groups showed significant difference in stem cell proliferation with a P value of 0.009. The mean ± SEM for the control group without rehabilitation was 0.03 ± 0.008 and that for the control group with rehabilitation was 0.005 ± 0.001. The area covered by stem cells during proliferation in the control group without physical rehabilitation ranged from 0.009 mm^2 to 0.09 mm^2 and that for the control group with physical rehabilitation ranged from 0.0004 mm^2 to 0.01 mm^2.

Thus, the study proves that the control group without physical rehabilitation had better stem cell proliferation and thus, resultant neurogenesis and motor functional recovery in comparison to the group of control rats which underwent physical rehabilitation. These results, altogether, support our suggestion that the stress caused due to excessive physical rehabilitation proved detrimental towards neurogenesis and motor functional recovery.

A plausible new pathway affecting infarct reduction and recovery post-stroke

The utilization of biomarker Ki-67 proved that, in our studies, neurogenesis occurs in the sub-ventricular zone (SVZ) which is closest to the corpus callosum. To reduce the infarct size and improve the motor functional recovery in the cortex region, a factor contributing towards the release of growth factors would be essential. The orexin network is observed in the cortex (Figure 27) and a parallel study conducted in our laboratory observed a correlation between the upregulation of orexin receptors and the reduction in infarct sizes. We have not determined the entire orexin pathway yet and haven't tested the functional recovery correlating to the upregulation of orexin receptors. However, this is a new direction of exploration to further understand the contributing factors towards reduction of infarct sizes and improvement of motor functional recovery.

To study the source and exact pathway of BDNF which plays a pivotal role in infarct reduction and recovery, it was essential to study gene expressions. A parallel study conducted by Mr. Moner Ragas showed a considerable upregulation of the orexin receptor 1 in male Sprague Dawley rats and orexin receptors 1 and 2 in female Sprague Dawley rats.

Orexin, also termed as hypocretin, is a neuropeptide produced mainly by neurons located in the lateral hypothalamic area, responsible for arousal, sleep/ wakefulness. Studies have shown that it is categorized in to isoforms, orexin A and orexin B based on the differences in their amino acid residues. Orexins work by activation of two G-protein coupled receptors, orexin receptor 1 and orexin receptor 2. Orexin A has affinities for orexin receptor 1 (ORX1R) & for orexin receptor 2 (ORX2R), whereas orexin B has affinity only for orexin receptor 2 (ORX2R). Over the time, studies have proven the

positive roles of orexin post-stroke. Orexin has been shown to decrease the infarct volume after stroke occurrence by increasing the cerebral blood flow, orexin A has been proven to exert neuroprotective effects and reduce oxidative stress along with enabling learning and cognition by promoting BDNF production and neurogenesis. Orexin has been proven to help improve depression-like behavior by increasing BDNF production. (Song, Kim, Kim, Song, & Lee, 2015).

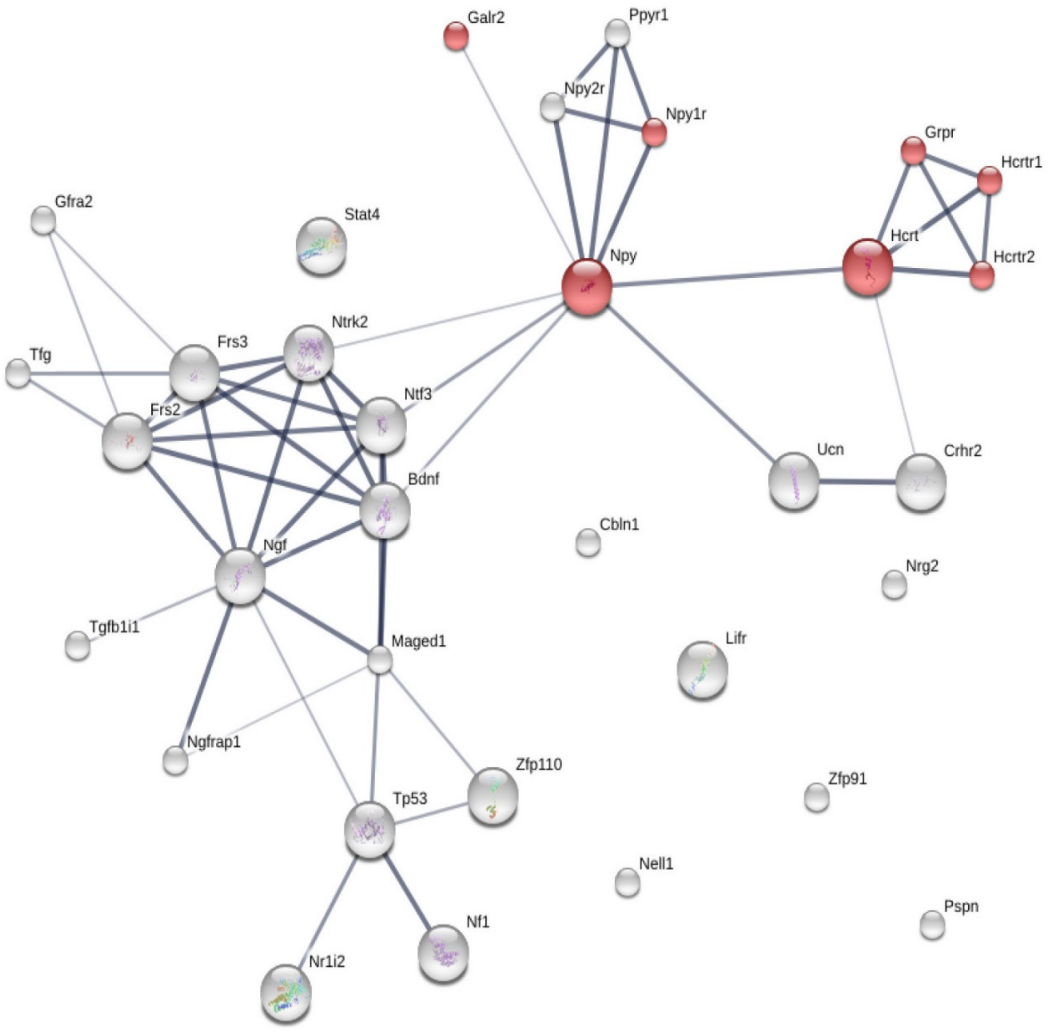


Figure (26): Orexin Pathway- Gene expressions

Figure (26) depicts a detailed understanding of the enhanced gene expressions following the interactions of orexin neuropeptides (hypocretin, Hcrt) with orexin receptors 1 & 2 (hypocretin receptor 1, Hcrtr1 and hypocretin receptor 2, Hcrtr2). Orexin seems to enhance the expressions of BDNF and nerve growth factor (NGF) via the Neuropeptide Y (Npy) gene expression. The thicker the lines, stronger are the evidences of direct interactions.

	Orexin	Orexin 1 receptor	Orexin 2 receptor	Infarct volume
Female FSA		Up 2.07 fold		No change
Female FS		Up 2.07 fold	Up 2.93 fold	reduced
Male FSA	Up 10 fold	Up 1.2 fold	Up 1.4 fold	reduced

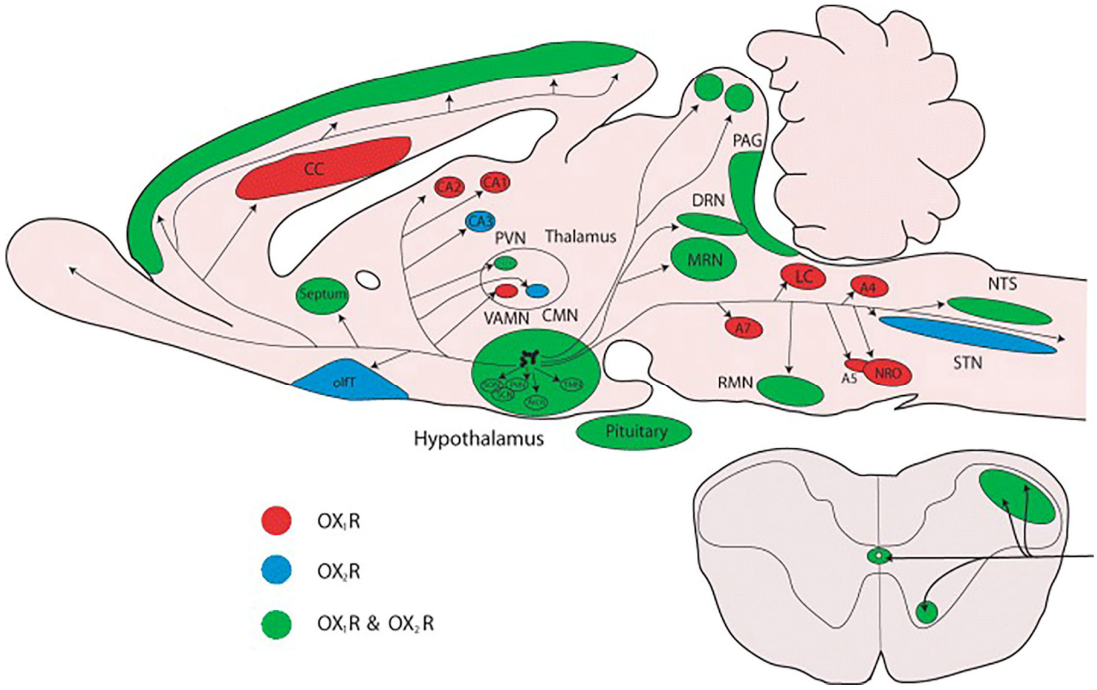


Figure (27): Orexin receptors distribution in the brain

Further, figure (27) gives the distribution of orexin receptors in the brain. The cortex shows a strong distribution of both the orexin receptors, ORX1R and ORX2R. Moreover, the previous studies conducted in our laboratory exhibit a strong upregulation of orexin receptors in the drug-treated animals. Thus, there is a possibility that the orexin receptors expressed in the cortex could play a major role in the increase of BDNF in the cortical region, leading to infarct reduction and functional recovery of cortical injury.

However, a comparison between the drug-treated and control female Sprague Dawley rats showed nearly 2 fold increase in Orexin receptor 1 expression in drug-treated rats when the drug combination used was Fluoxetine, Simvastatin and Ascorbic acid whereas, the comparison between the two group showed an upregulation of orexin receptor 1 (2.07 folds) and orexin receptor 2 (2.93 folds) in the drug-treated rats when the drug combination used was Fluoxetine and Simvastatin. Thus, the results showed that inclusion of Ascorbic acid in the drug treatment depleted the expression of orexin receptor 2, thus, downregulating the total receptor expression from nearly 5 folds to nearly 2 folds. This correlates well with what we see in infarct reduction.

On the other hand, the male drug-treated rats in comparison to control male rats showed an upregulation of orexin receptor one by nearly 9 folds. It was a considerable difference as compared to that seen in female groups of drug-treated rats with and without Ascorbic acid.

While acute stress increases the activation of orexin neurons, chronic stress has an opposite effect. Studies have found that voluntary exercise can help increase orexin system function after chronic exposure to stress, only in male rats; whereas, female rats have not shown any improvement in the anxiety- and depression- like behaviors after the

similar type of voluntary exercise. Thus, the exercise-induced corticosterone secretion could influence the orexin system more in females as compared to males and ameliorate the behavioral and neural deficits only in a sex-dependent fashion, benefiting the males (James et al., 2014).

Studies suggest that higher levels of orexin A are related to hypoestrogenism (El-Sedeek, Korish, & Deef, 2010). On comparison with the Ki-67 expression levels of post-menopausal rats, the lower levels of Ki-67 expression in our current rats proved that our female rats were pre-menopausal and hence, had higher estrogen levels and lower orexin levels. Studies also suggest that the orexin levels decrease on occurrence of stroke (Song et al., 2015). Thus, our drugs which could upregulate the orexin receptors on occurrence of stroke, by up to 5 folds in females and up to 9 folds in males, could be helpful in alleviating a number of neurodegenerative disorders following stroke.

This study opens up a whole new avenue for proper understanding of the genes contributing towards enhancement of BDNF expression and the ability to recover from neurodegeneration. This is especially important since the major diseases still remaining in the brain without effective treatment are neurodegenerative in nature.

Future experiments

Corticosterone levels

Stress played a major role in antagonizing the efficacies of the drugs in our pharmacological treatment. We assume that the excessive physical rehabilitation exercises performed by the animals in our study was a source of excess stress. In the future, it would be helpful to test for the corticosterone levels in the brain in order to be sure about the extent of physical rehabilitation exercises that could be beneficial either alone or in combination with a pharmacological treatment.

Ascorbic acid

A parallel study showed marked reduction in infarct sizes in male Sprague Dawley rats administered Fluoxetine, Simvastatin and Ascorbic acid 20 to 26 hours after stroke induction in comparison to control male rats. On the contrary, the female rats administered the same pharmacological treatment showed infarct sizes similar to control female rats. Thus, a study of the effect of estrogen on ascorbic acid or vice versa would help us understand the gender difference in the susceptibility of stroke and if the treatments should be designed based on the gender of the patient.

Estrogen

Mammalian estrogen, 17-β estradiol, is known to provide robust neuroprotection and enhance the process of neurogenesis in the SVZ. Moreover, it also contributes towards enhancing post-stroke behavioral recovery (Li et al., 2011). Thus, it would be a good source of information if we could perform more tests, in the future, to confirm if the female rats utilized in our studies are pre- or post-menopausal.

Orexin pathway

High levels of orexin A are associated with low levels of estrogen like those seen in post-menopausal women (El-Sedeek et al., 2010). This could be a compensatory mechanism to try to maintain normal cognition in the cortex when estrogen is no longer around. It would be more important to look at how the interactions might change with aging since neurodegeneration is always most highly associated with aging. Hence, it would be interesting to study the estrogen-orexin relation in the female rats. Moreover, the mRNA expression of orexin receptors showed a vast difference between the male and female rats treated with Fluoxetine, Simvastatin and Ascorbic acid. Thus, we assume that estrogen could have a role to play and an interaction between estrogen and orexin could lead to such differences.

Furthermore, the differences in the expression of orexin receptor 2 observed in the upregulation of female rats on including Ascorbic acid in the treatment suggests that Ascorbic acid could be affecting the mRNA expression of orexin receptor 2. Further study of the orexin pathway could be an aid in understanding the detailed orexin pathway.

Conclusion

Even though physical rehabilitation has been proven to help with motor functional recovery and reduction of infarct size, excess of exercise as a part of physical therapy would hamper with the motor functional recovery. Moreover, the resultant excessive stress would antagonize the pharmacological treatment meant to improve neurogenesis and reduce the infarct size after the occurrence of stroke. Thus, it is essential to understand the extent and duration of physical rehabilitation exercise that would be helpful in recovery after stroke.

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Appendix

Control plus Physical rehabilitation group of rats

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
801	1	1	0.13	0.01		
		2	0.06	0.00		
		3	0.18	0.01		
		4	6.36	0.32		
	2	2	0.09	0.00		
		3	0.10	0.01		
		4	4.72	0.24		
	3	1	0.07	0.00		
		2	0.16	0.01		
		3	0.10	0.01		
		4	4.24	0.21		
	4	1	0.13	0.01	3.71	14.82
		2	0.10	0.01		
		3	3.93	0.20		
		4	43.40	2.17		
	5	1	0.23	0.01		
		2	4.95	0.25		
		3	0.71	0.04		
		4	0.67	0.03		
	6	1		0.00		
		2	1.99	0.10		
		3		0.00		
		4	1.75	0.09		
804	1	1	2.21	0.11		
		2	3.46	0.17	3.23	12.93
		3	2.58	0.13		
		4	2.97	0.15		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
	2	1	2.54	0.13		
		2	10.20	0.51		
		3	1.17	0.06		
		4	11.95	0.60		
	3	1		0.00		
		2	1.77	0.09		
		3	3.62	0.18		
		4		0.00		
	4	1	1.30	0.07		
		2	11.59	0.58		
		3	4.76	0.24		
		4	4.50	0.23		
814	1	1	5.09	0.25		
		2	6.96	0.35		
		3	2.18	0.11		
		4	6.09	0.30		
	2	1	6.79	0.34		
		2	0.00	0.00		
		3	4.69	0.23		
		4	0.00	0.00		
	3	1	5.93	0.30		
		2	7.27	0.36	4.29	17.18
		3	1.09	0.05		
		4	6.10	0.30		
	4	1	4.52	0.23		
		2	2.31	0.12		
		3	2.49	0.12		
		4	6.35	0.32		
	5	1	7.18	0.36		
		2	0.97	0.05		
		3	4.44	0.22		
		4	5.46	0.27		
817	1	1	0.00	0.00		
		2	4.45	0.22	8.45	33.79
		3	2.12	0.11		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		4	15.41	0.77		
	2	1	2.15	0.11		
		2	11.51	0.58		
		3	2.72	0.14		
		4	10.77	0.54		
	3	1	0.00	0.00		
		2	1.52	0.08		
		3	8.17	0.41		
		4	15.04	0.75		
	4	1	3.94	0.20		
		2	8.02	0.40		
		3	15.59	0.78		
		4	5.11	0.26		
	5	1	7.23	0.36		
		2	5.73	0.29		
		3	2.26	0.11		
		4	12.66	0.63		
	6	1	5.56	0.28		
		2	6.16	0.31		
		3	6.49	0.32		
		4	16.34	0.82		
820	1	1	2.53	0.13		
		2	3.31	0.17		
		3	0.00	0.00		
		4	2.80	0.14		
		5	0.00	0.00		
		6	2.13	0.11		
	2	1	2.36	0.12	2.03	8.13
		2	3.12	0.16		
		3	3.77	0.19		
		4	1.23	0.06		
		5	0.00	0.00		
		6	0.00	0.00		
	3	1	0.00	0.00		
		2	1.82	0.09		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		3	2.54	0.13		
		4	1.45	0.07		
		5	1.10	0.06		
		6	0.55	0.03		
	4	1	0.00	0.00		
		2	1.96	0.10		
		3	0.00	0.00		
		4	4.00	0.20		
		5	2.55	0.13		
		6	3.41	0.17		
825	1	1	14.85	0.74		
		2	9.46	0.47		
		3	14.64	0.73		
	2	1	15.28	0.76		
		2	14.69	0.73		
		3	13.37	0.67		
		4	14.85	0.74		
	3	1	7.49	0.37		
		2	6.58	0.33		
		3	11.70	0.58	11.61	46.43
		4	15.48	0.77		
	4	1	12.39	0.62		
		2	13.63	0.68		
		3	10.08	0.50		
		4	13.21	0.66		
	5	1	10.54	0.53		
		2	8.58	0.43		
		3	11.45	0.57		
		4	13.89	0.69		
826	1	1	0.06	0.00		
		2	0.32	0.02		
		3	4.37	0.22	4.33	17.33
		4	5.37	0.27		
	2	1	0.00	0.00		
		2	1.24	0.06		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		3	0.00	0.00		
		4	0.00	0.00		
	3	1	0.09	0.00		
		2	0.06	0.00		
		3	3.66	0.18		
		4	1.85	0.09		
	4	1	0.12	0.01		
		2	0.90	0.04		
		3	3.89	0.19		
		4	1.29	0.06		
	5	1	3.25	0.16		
		2	3.71	0.19		
		3	3.72	0.19		
		4	2.19	0.11		
	6	1	4.30	0.22		
		2	4.79	0.24		
		3	5.19	0.26		
		4	4.75	0.24		
	7	1	5.19	0.26		
		2	0.00	0.00		
		3	0.00	0.00		
		4	4.88	0.24		
	8	1	1.15	0.06		
		2	5.30	0.27		
		3	5.51	0.28		
		4	2.34	0.12		
	9	1	4.42	0.22		
		2	0.00	0.00		
		3	2.60	0.13		
		4	0.15	0.01		
835	1	1	0.47	0.02		
		2	0.33	0.02		
		3	0.47	0.02	0.36	1.43
		4	0.74	0.04		
		5	0.13	0.01		
		6	0.62	0.03		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
	2	1	0.00	0.00		
		2	0.13	0.01		
		3	0.17	0.01		
		4	0.13	0.01		
		5	0.57	0.03		
		6	0.00	0.00		
		7	0.78	0.04		
	3	1	0.00	0.00		
		2	0.00	0.00		
		3	0.00	0.00		
		4	0.00	0.00		
		5	0.00	0.00		
		6	0.55	0.03		
	4	1	0.68	0.03		
		2	0.44	0.02		
		3	0.00	0.00		
		4	0.36	0.02		
		5	0.58	0.03		
		6	0.00	0.00		
837	1	1	0.00	0.00		
		2	0.00	0.00		
		3	2.56	0.13		
		4	0.00	0.00		
	2	1	7.91	0.40		
		2	3.96	0.20		
		3	1.75	0.09		
		4	9.29	0.46		
	3	1	0.00	0.00	4.17	16.70
		2	0.00	0.00		
		3	7.80	0.39		
		4	0.00	0.00		
	4	1	7.86	0.39		
		2	5.21	0.26		
		3	7.20	0.36		
		4	0.00	0.00		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05= Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
	5	1	5.73	0.29		
		2	4.92	0.25		
		3	6.17	0.31		
		4	8.05	0.40		
	6	1	5.08	0.25		
		2	0.00	0.00		
		3	0.00	0.00		
		4	0.00	0.00		

Drug-treated (FSA) rats plus Physical Rehabilitation group of rats

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
802	1	1	2.90	0.14		
		2	3.54	0.18		
		3	3.92	0.20		
	2	1	2.20	0.11		
		2		0.00		
		3	3.72	0.19		
		4	4.03	0.20		
	3	1	1.27	0.06		
		2	0.26	0.01		
		3		0.00		
		4	3.72	0.19		
	4	1	2.08	0.10	2.52	10.06
		2	1.43	0.07		
		3	3.43	0.17		
		4	4.51	0.23		
	5	1	3.15	0.16		
		2	2.45	0.12		
		3		0.00		
		4	4.27	0.21		
	6	1		0.00		
		2	1.66	0.08		
		3	1.79	0.09		
		4		0.00		
803	1	1	16.48	0.82		
		2	6.90	0.35		
		3	16.04	0.80	14.97	59.90
		4	23.39	1.17		
	2	1	14.98	0.75		
		2	15.28	0.76		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		3	9.48	0.47		
		4		0.00		
	3	1	23.29	1.16		
		2	*	*		
		3	10.88	0.54		
		4	18.27	0.91		
	4	1	12.63	0.63		
		2	31.16	1.56		
		3	2.97	0.15		
		4	21.89	1.09		
	5	1	21.73	1.09		
		2	25.94	1.30		
		3	4.73	0.24		
		4	23.45	1.17		
805	1	1	5.10	0.26		
		2	7.12	0.36		
		3	10.12	0.51		
		4	4.51	0.23		
	2	1	10.09	0.50		
		2		0.00		
		3	9.46	0.47		
		4	8.38	0.42		
		5	8.66	0.43		
	3	1	8.86	0.44		
		2	0.44	0.02	5.94	23.78
		3	8.96	0.45		
		4	2.24	0.11		
	4	1	0.33	0.02		
		2	5.80	0.29		
		3	10.13	0.51		
		4	0.97	0.05		
	5	1	0.18	0.01		
		2	3.42	0.17		
		3	5.78	0.29		
		4	8.34	0.42		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
807	1	1	4.52	0.23		
		2	8.88	0.44		
		3	5.66	0.28		
		4	6.06	0.30		
		5	8.17	0.41		
	2	1	6.47	0.32		
		2	6.11	0.31		
		3	3.24	0.16		
		4	4.95	0.25		
	3	1	6.48	0.32		
		2	7.68	0.38	5.22	20.88
		3	6.37	0.32		
		4	4.88	0.24		
	4	1	2.69	0.13		
		2	1.21	0.06		
		3	0.31	0.02		
		4	1.84	0.09		
	5	1	1.77	0.09		
		2	4.34	0.22		
		3	7.37	0.37		
		4	5.39	0.27		
809	1	1	9.99	0.50		
		2	4.78	0.24		
		3	10.33	0.52		
		4	6.49	0.32		
	2	1	10.03	0.50		
		2	8.74	0.44		
		3	10.42	0.52		
		4	5.19	0.26	7.95	31.80
	3	1	9.06	0.45		
		2	9.59	0.48		
		3	7.98	0.40		
		4	9.86	0.49		
	4	1	1.87	0.09		
		2	7.78	0.39		
		3	9.69	0.48		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		4	6.35	0.32		
	5	1	9.10	0.46		
		2	10.04	0.50		
		3	5.30	0.26		
		4	6.42	0.32		
816	1	1	0.00	0.00		
		2	0.00	0.00		
		3	4.57	0.23		
		4	5.60	0.28		
	2	1	0.00	0.00		
		2	0.00	0.00		
		3	2.79	0.14		
		4	0.00	0.00		
	3	1	0.00	0.00		
		2	3.67	0.18		
		3	2.34	0.12		
		4	0.00	0.00	2.10	8.39
	4	1	4.89	0.24		
		2	3.03	0.15		
		3	6.00	0.30		
		4	0.00	0.00		
	5	1	0.18	0.01		
		2	0.00	0.00		
		3	0.00	0.00		
		4	1.14	0.06		
	6	1	3.34	0.17		
		2	0.00	0.00		
		3	0.00	0.00		
		4	4.39	0.22		
818	1	1	3.40	0.17		
		2	4.91	0.25		
		3	0.00	0.00	2.13	8.51
		4	0.00	0.00		
	2	1	1.13	0.06		
		2	0.00	0.00		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		3	1.39	0.07		
		4	1.63	0.08		
	3	1	0.00	0.00		
		2	4.29	0.21		
		3	2.40	0.12		
		4	0.00	0.00		
	4	1	2.79	0.14		
		2	0.72	0.04		
		3	0.00	0.00		
		4	0.00	0.00		
	5	1	2.93	0.15		
		2	2.86	0.14		
		3	0.88	0.04		
		4	0.00	0.00		
	6	1	3.34	0.17		
		2	0.00	0.00		
		3	1.06	0.05		
		4	2.66	0.13		
	7	1	2.68	0.13		
		2	2.01	0.10		
		3	1.47	0.07		
		4	0.00	0.00		
819	1	1	9.88	0.49		
		2	9.96	0.50		
		3	10.38	0.52		
		4	7.86	0.39		
	2	1	10.60	0.53		
		2	9.14	0.46		
		3	10.02	0.50	8.25	33.00
		4	5.22	0.26		
	3	1	3.01	0.15		
		2	6.35	0.32		
		3	2.75	0.14		
		4	6.67	0.33		
	4	1	4.56	0.23		
		2	7.12	0.36		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		3	2.58	0.13		
		4	8.18	0.41		
	5	1	8.65	0.43		
		2	7.58	0.38		
		3	10.21	0.51		
		4	6.81	0.34		
	6	1	2.77	0.14		
		2	3.70	0.18		
		3	5.22	0.26		
		4	5.80	0.29		
824	1	1	2.99	0.15		
		2	0.67	0.03		
		3	1.26	0.06		
		4	0.87	0.04		
	2	1	3.36	0.17		
		2	2.67	0.13		
		3	5.50	0.27		
		4	6.00	0.30		
	3	1	2.22	0.11		
		2	1.32	0.07	2.46	9.86
		3	4.49	0.22		
		4	3.78	0.19		
	4	1	0.35	0.02		
		2	3.95	0.20		
		3	0.71	0.04		
		4	0.84	0.04		
	5	1	1.28	0.06		
		2	2.57	0.13		
		3	1.64	0.08		
		4	2.82	0.14		
828	1	1	1.08	0.05		
		2	1.26	0.06		
		3	1.25	0.06	1.15	4.60
		4	0.00	0.00		
		5	0.71	0.04		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
	2	1	0.37	0.02		
		2	0.00	0.00		
		3	1.33	0.07		
		4	0.42	0.02		
	3	1	1.39	0.07		
		2	1.41	0.07		
		3	1.31	0.07		
		4	1.29	0.06		
	4	1	0.16	0.01		
		2	1.91	0.10		
		3	0.00	0.00		
		4	0.00	0.00		
	5	1	1.09	0.05		
		2	2.22	0.11		
		3	0.91	0.05		
		4	1.06	0.05		
	6	1	0.47	0.02		
		2	0.22	0.01		
		3	1.67	0.08		
		4	1.50	0.07		
830	1	1	1.23	0.06		
		2	0.00	0.00		
		3	0.00	0.00		
		4	0.00	0.00		
		5	1.83	0.09		
	2	1	0.00	0.00		
		2	0.00	0.00		
		3	0.15	0.01	0.87	3.48
		4	2.26	0.11		
	3	1	2.94	0.15		
		2	2.51	0.13		
		3	4.19	0.21		
		4	0.34	0.02		
	4	1	0.00	0.00		
		2	1.96	0.10		
		3	0.00	0.00		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		4	0.00	0.00		
834	1	1	0.00	0.00		
		2	0.52	0.03		
		3	4.05	0.20		
		4	0.62	0.03		
	2	1	4.48	0.22		
		2	5.24	0.26		
		3	3.77	0.19		
		4	0.79	0.04		
	3	1	4.05	0.20		
		2	0.00	0.00		
		3	0.00	0.00		
		4	0.00	0.00		
		5	0.54	0.03		
	4	1	0.00	0.00		
		2	0.00	0.00	2.40	9.59
		3	0.00	0.00		
		4	0.00	0.00		
	5	1	0.00	0.00		
		2	4.53	0.23		
		3	1.09	0.05		
		4	0.00	0.00		
	6	1	4.45	0.22		
		2	4.25	0.21		
		3	1.18	0.06		
		4	1.84	0.09		
	7	1	5.55	0.28		
		2	0.00	0.00		
		3	0.00	0.00		
		4	0.98	0.05		
836	1	1	5.78	0.29		
		2	2.96	0.15		
		3	3.84	0.19	2.27	9.10
		4	0.00	0.00		
	2	1	0.00	0.00		

Rat	*Slide*	*Section*	*Area (mm^2)*	*Area*0.05 = Volume (mm^3)*	*Sum of the given volumes (mm^3)*	*Total volume of infarct (mm^3)= Sum of the given volumes*4*
		2	3.84	0.19		
		3	1.07	0.05		
		4	3.23	0.16		
	3	1	2.27	0.11		
		2	0.00	0.00		
		3	2.84	0.14		
		4	2.31	0.12		
	4	1	2.42	0.12		
		2	4.23	0.21		
		3	0.43	0.02		
		4	1.89	0.09		
	5	1	3.59	0.18		
		2	3.46	0.17		
		3	0.00	0.00		
		4	0.00	0.00		
	6	1	0.00	0.00		
		2	0.00	0.00		
		3	0.40	0.02		
		4	0.00	0.00		
		5	0.00	0.00		
		6	0.00	0.00		
		7	0.91	0.05		