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Facilitating the recovery of function following stroke: the efficacy of inosine

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FACILITATING THE RECOVERY OF FUNCTION FOLLOWING STROKE: THE EFFICACY OF INOSINE

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FACILITATING THE RECOVERY OF FUNCTION FOLLOWING STROKE: THE EFFICACY OF INOSINE

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ABSTRACT

Despite years of research, an effective therapy for treatment of ischemic stroke has yet to be found. Survivors of stroke may suffer debilitating and permanent motor dysfunction for the remainder of their lives. Current treatments are limited to physical therapy and tissue plasminogen factor (tPA), a thrombolytic medication with a time-window of efficacy up to only three hours after symptom onset. Clinical studies and animal models have shown that partial recovery of motor function occurs with or without pharmacological interventions due to adaptive plasticity and reorganization in the brain. The precise mechanisms, though unclear, have become a major focus of stroke research. In the following study, we investigated inosine, a naturally occurring purine nucleoside that stimulates axonal growth, as a potential long-term stroke treatment. Following controlled cortical ischemia in the motor cortex of rhesus monkeys, recovery of dominant hand function was monitored through NHP Upper Extremity Motor Dysfunction Scale ratings for two weeks post-operation and through performance on two motor tasks, the Hand Dexterity Task (HDT) and the Digit Coordination Task (DCT). Results of cage-side assessment ratings demonstrated a trend towards greater recovery in the group treated with inosine for functional strength in the dominant hand on 12-14 days after surgery. The suggested trend is enough evidence to pursue research on the use of inosine as a therapeutic agent in post-stroke functional recovery.

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LIST OF ABBREVIATIONS

AAALAC……..Association for Assessment & Accreditation of Lab Animal Care

BUMC……..Boston University Medical Campus

CIMT……..Constraint-Induced Movement Therapy

CNS…….Central Nervous System

DCT……Digit Coordination Task

FDA……..Food and Drug Administration

fMRI……Function Magnetic Resonance Imaging

GAP……Growth Associated Protein

HDT……..Hand Dexterity Task

IACUC…….Institutional Animal Care and Use Committee

LASC…….Laboratory Animal Science Center

M1……..Primary Motor Cortex

MRI……Magnetic Resonance Imaging

NIH……...National Institutes of Health

NHP…….Non-Human Primates

PD……..Parkinson's Disease

ROS……...Reactive Oxygen Species

T1W……..…….T1 weighted

tPA…….Tissue Plasminogen Activator

UE……Upper extremity

INTRODUCTION

Global measurements show that over 15 million people suffer either a new or recurrent stroke each year worldwide. Approximately 6 million of these people die while about 5 million are left permanently disabled (World Health Organization, 2004). Within the United States, where stroke is the fourth leading cause of death, about 800,000 Americans survive annually (Go et al., 2014). Most of these strokes are found to be ischemic and result in a significant percentage of affected adults living with serious, long-term disability, substantial economic burden, and decreased quality of life (American Heart Association, 2012).

Previous research has focused on preventing stroke by targeting several known risk factors. The probability of stroke has been found to increase when patients present with one or multiple of the following: prior stroke, high blood pressure, high blood cholesterol, diabetes mellitus, poor diet, lack of physical activity, obesity, kidney disease, alcohol abuse, drug abuse, cigarette smoking, or a genetic predisposition (American Heart Association, 2012). Age has also been recognized as a significant variable associated with higher risk, with the median age for stroke patients being 75 years old for females and 71 years for males (Go et al., 2014). Another focus of previous research has been neuroprotection and understanding how to limit the series of biochemical reactions, known as an ischemic cascade, that is initiated in the brain following inadequate blood

supply so that the extent of injury may be reduced in brain tissue (Sahota & Savitz, 2011; Ceulemans et al., 2010).

Neuroprotection is the broad term for pharmacological and non-pharmacological treatments used to stop deleterious cellular events that occur in an ischemic cascade (Hinkle, 2007). Although successful in studies with animals, these treatments have shown to have little benefit clinically as they must be administered within a few hours of symptom onset (Dobkin, 2003). Current treatments have, thus, been limited to thrombolytic medications and physical therapy. One example, tissue plasminogen activator (tPA), the only Food and Drug Administration (FDA) approved thrombolytic medication for ischemic stroke, has a time window for effectiveness of only three hours after symptom onset (Cronin et al., 2013). In most cases, residual impairment occurs with or without treatment suggesting a need for a therapeutic agent with a longer time window that can also facilitate functional recovery following partial brain damage (Zai et al., 2009).

During ischemic stroke, a clot or thrombus interrupts the blood supply to the central nervous system (CNS) cutting off access to oxygen and glucose. Hyperexcitability, derived from low glucose and oxygen tension, leads to excitotoxicity through excess release of neurotransmitters, such as glutamate, from synaptic terminals and glutamate receptor overactivation (Hinkle & Bowman, 2003). Changes that occur in the brain are dependent on the severity and duration of deprivation. These cellular events take place in the area of the lesion and include necrosis, apoptosis, and free radical

formation. Neurons and glia, which require a careful balance in their environment and nutrition, begin to die. Simultaneously, cytokines are released by activated microglia, and free radicals start to undergo autocatalytic reactions. These mediators initiate inflammation and oxidative stress to help protect against stroke but may cause even more neuronal damage when in excess (Felberg, 2000).

Several neuroprotective mechanisms counteracting excessive inflammation and oxidative stress have been explored, including ion channel mechanisms, excitatory amino acids, and oxygen radicals, to minimize secondary injury but have shown little to no therapeutic effect clinically (del Zoppo, 2000). Ischemia in the brain ceases when there is reperfusion of the cerebral vasculature, but this process may cause a rapid build up of reactive oxidative species (ROS) and more oxidative stress adding to the cell death that has already occurred in the region (Cherubini, 2005). Often, what is left is a core region of dead cells surrounded by hypoperfused tissue that is functionally impaired but viable (Sims et al., 2010). Rescue of the hypoperfused area, known as the penumbra region, has been the focus of most recent research but must be carried out within a relatively short period (a few hours) of stroke onset or can result in permanent tissue damage (Muir et al., 2006).

Restoration of brain structure and function after tissue damage has become the new direction of many studies and is referred to as brain repair (Kalladka et al., 2014). Several clinical studies and animal models have demonstrated that partial or spontaneous recovery in the absence of treatment occurs due to adaptive plasticity and reorganization

of undamaged cortical areas (Gonzalez et al., 2004; Ward, 2004; Frost et al., 2003; Nudo & Friel, 1999). Reorganization in the brain following ischemia has been found to involve the following: dendritic remodeling (Buga et al., 2008; Jones & Schallert, 1992), high levels of presynaptic growth-associated proteins (GAP) like GAP-43 (Juan et al., 2013), increased synaptophysin (Benowitz & Carmichael, 2010; Buga et al., 2008; Stroemer et al., 1998; Benowitz & Routtenberg, 1997), axonal sprouting (Buga et al., 2008; Li & Carmichael, 2006) and angiogenesis in the peri-infact regions (Beck & Plate, 2009; Carmichael & Chesselet, 2002).

For a long period following a stroke, the cortex experiences the aforementioned pathophysiological and restorative changes particularly in the area of the lesion. The precise mechanisms behind these processes remain unclear, but their role in spontaneous recovery provides convincing evidence that a treatment maximizing neural reorganization and, thus, plasticity may have the potential to improve functional outcome as late as weeks and months following a stroke (Nouri & Cramer, 2011; Benowitz & Carmichael, 2010; Stroemer et al., 1998; Benowitz & Routtenberg, 1997). Several agents promoting regrowth, restoration, and rewiring in the brain are currently being studied (Zai et al., 2009; Papadopoulos et al., 2009; Chen et al., 2002).

Inosine, a naturally occurring purine nucleoside, is a therapeutic agent of interest for treatment of stroke. Formed by the deamination of adenosine, inosine is released by cells in response to metabolic stress (Conta & Stelzner, 2008). Not only does inosine increase urate, a potent endogenous antioxidant, the nucleoside has also been found to

diffuse across the cell membrane in the brain through facilitated diffusion and enhance the intrinsic growth state of neurons (Kawamata et al., 1997). Due to its neuroprotective and plasticity enhancing properties, inosine has previously been studied as a potential treatment in multiple sclerosis and Parkinson's Disease (PD), two neurological disorders, (Markowitz et al., 2009; Schwarzschild et al., 2014). By activating Mst3b, a purine-sensitive protein kinase that plays a central role in the cell-signaling pathway that regulates the expression of several genes involved in axon growth (Benowitz et al., 1998; Irwin et al., 2006), inosine enhances axon extension *in vitro* and improves the ability of undamaged neurons to form axon collaterals *in vivo* following brain damage (Zai et al., 2009). After stroke, inosine-treated neurons have been shown to sprout into denervated areas of the spinal cord (Chen et al., 2002). Inosine administration has also been shown to increase protein indicators of synaptogenesis, such as synaptophysin, and GAP-43, a marker for axonal growth (Dachir et al., 2014). Synaptogenesis is one of the previously mentioned types of processes associated with brain reorganization (Chen et al., 2002). Therefore, a reasonable degree of evidence has been gathered that inosine could be used as potential treatment to promote brain repair and functional recovery in areas surrounding lesions in the brain.

Inosine and several other pharmacological interventions being studied to enhance fiber growth in the adult CNS have been applied in experimental stroke models. Most experiments have been carried out on young animals such as juvenile rodents (Lipsanen and Jolkkonen, 2011; Papadopoulos et al., 2009) or lissencephalic species of non-human

primates (NHPs) (Frost et al., 2003; Nudo, 2003), where stimulation demonstrated that areas surrounding cortical injury partially take over functions affected by damage (Gooddy & McKissock, 1951; Glees & Cole, 1949; Cole & Glees, 1951). Pre-clinically, recovery from motor deficits following stroke have been shown to improve with physical training. Clinically, only a few studies have shown a connection between structural changes in the brain and behavioral/functional changes. All of these functional changes have been training related, including those reported in a study of constraint-induced movement therapy (CIMT), which proved to be very effective in enhancing recovery in the brain after stroke (Kunkel et al., 1999; Taub et al., 1999). Forced use of the impaired limb through CIMT led to full functional recovery, and was associated with the growth of an intact corticospinal tract into denervated region of the spinal cord (Maier et al., 2008).

Objectives. The objective of this study was to determine if inosine might provide a long-term pharmacological treatment option that along with personalized rehabilitation and physical therapy could vastly improve functional recovery following stroke. Monkeys were randomly assigned into control or inosine treatment groups and tested on two different motor tasks assessing dexterity and coordination to determine functional capacity prior to a having a surgically induced stroke lesion. After surgery, animals in the treatment group received a daily dose of inosine for 12 weeks of post-operative (post-op) testing and their performance recovery was compared to that of animals in the control group on the same tasks.

Treatments that exist for stroke are limited in number and efficacy. There has been growing consensus among scientists that an association exists between adaptive plasticity in the brain and repair after stroke. Thus, inosine, one of the several agents that seem to have a role in brain reorganization, has become a main focus in stroke research. The following study seeks to obtain more clinically relevant data than has previously been collected by using middle-aged gyrencephalic rhesus monkeys as an animal model to assess the efficacy of inosine in facilitating long-term recovery following stroke.

METHODS

Overview of subjects and procedures. Eight middle-aged male rhesus monkeys (*M. mulatta*) ranging in age from 14-18 years were obtained from Alpha Genesis Inc. (Yemassee, SC), the National Institutes of Health (NIH), and the Caribbean National Primate Research Center (Sabana Seca, PR). There were several reasons for the age range chosen: these ages were determined to be equivalent to a human age range of 42-54 years (Tigges et al. 1988) when strokes most commonly occur and when there is evidence of age-related differences in concentrations of nucleosides in the human brain (Mecocci et al., 1993). Also, previous studies had demonstrated that middle-aged monkeys showed greater impairment after ischemic damage than young monkeys and, therefore, there would be a greater range for functional recovery in which to observe the therapeutic effect of inosine (Clarke et al., 2014).

The monkeys were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited Laboratory Animal Science Center (LASC) at the Boston University Medical Campus (BUMC). All testing and procedures during this experiment were carried out in accordance with the guidelines of the NIH Committee on Laboratory Animal Resources and with approval from the Institutional Animal Care and Use Committee (IACUC) at BUMC. Prior to being enrolled in the study, health records were screened and animals with a history of malnutrition, chronic illness, diabetes, or neurological disease were excluded. The

animals were then given medical examinations by the LASC veterinary staff, including hematology, serum uric acid analysis, and tuberculosis (TB) testing.

After quarantine procedures were completed, animals were trained on two motor dexterity tasks using a motor testing apparatus (Figure 1A) and their dominant hand was

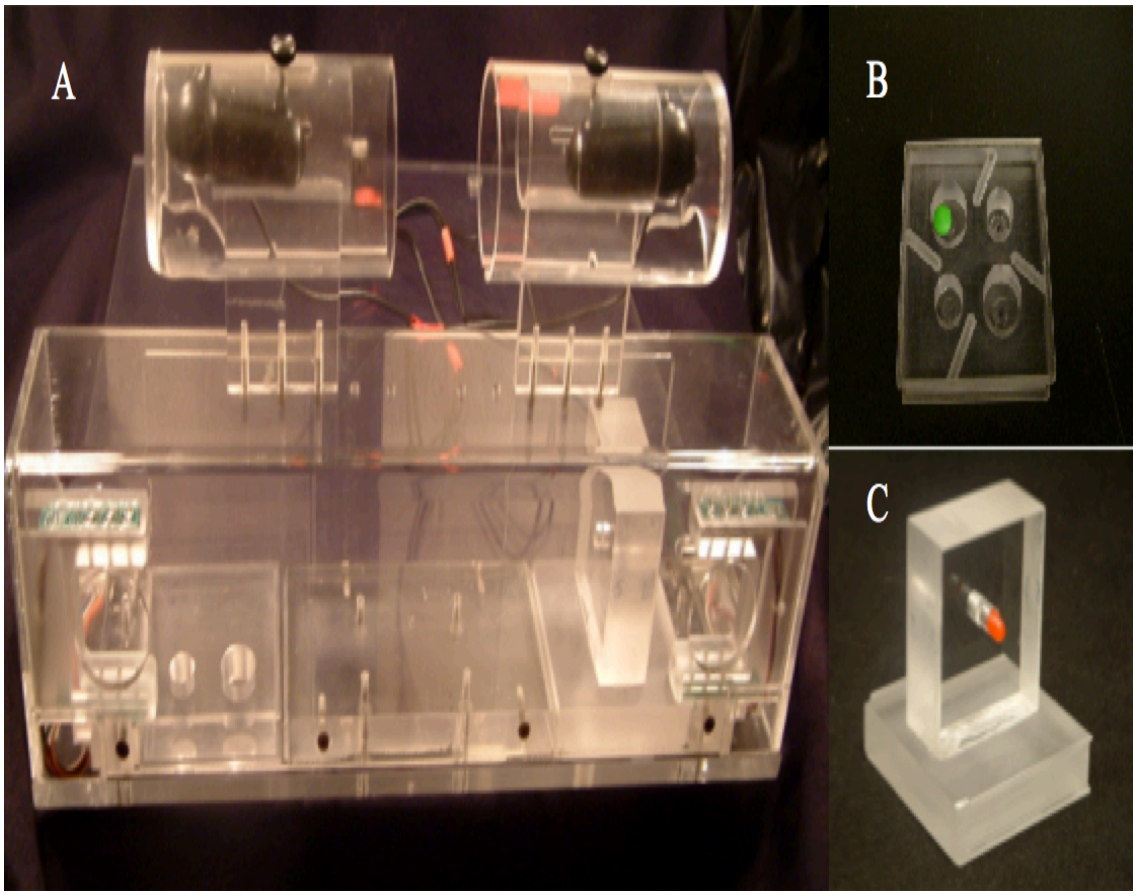

Figure 1: Motor function testing. **(A)** The motor testing apparatus used for HDT and DCT pre-operative and post-operative testing. This photo shows the apparatus as seen by the animal being tested. Examples of the HDT (left) and DCT (right) inserts can be seen in more detail in Figures B and C. **(B)** The insert used in the motor testing apparatus on HDT testing days (modified version of the Kluver board). An example of the food reward used is seen in green and located in the upper left well in this photo. **(C)** The vertical insert used in the motor testing apparatus on DCT testing days (modified version of bimanual testing apparatus). An example of the food reward used is seen in red in this photo.

determined (See Moore et al. 2013 for complete details). After 20 days of pre-operative (pre-op) training, baseline serum urate levels were determined and monkeys were randomly assigned to either the control or inosine treatment group based on testing performance. Monkeys in the inosine group received daily doses of inosine of 500mg. This dosage was based on doses currently being used in human clinical trials of inosine as a treatment for PD (Parkinson's Disease Study Group, 2009). Animals received treatment for 3 days before inosine administration was stopped for a minimum of 2 weeks. Animals were given initial magnetic resonance imaging (MRI) scans during this time to ensure no obvious brain abnormalities and establish a baseline for later post-op analysis.

Following the washout period, monkeys underwent brain surgery to map the motor cortex in the dominant hemisphere and create an ischemic lesion in the area of the dominant hand representation in the primary motor cortex (M1) (See Moore et al. 2013 for complete details). Administration of inosine (500mg/day) was recommenced the day following surgery. All subjects were re-tested post-operatively on both motor dexterity tasks for a total of 60 days. Behavioral testers were blind to treatment assignments throughout the entire duration of the study. Performance of both hands on each post-op testing day was videotaped for future assessment of grasp quality and observation of hand mechanics.

Upon completion of testing, monkeys received a second set of MRI scans to observe changes in lesion size and to identify any changes in the cortex after several weeks of motor testing. Finally, immediately prior to euthanasia, monkeys completed 1 hour of testing with the impaired hand to activate regions supporting the recovered function. Animals were then euthanized by exsanguination during transcardial perfusion of the brain. Following euthanasia, tissue sections were collected to be processed for immunohistochemical and histopathological assays.

Pre-operative training in fine motor function testing. The Hand Dexterity Task (HDT) and the Digit Coordination Task (DCT) were the 2 motor tasks used in this study. The monkeys were trained in the HDT on Mondays, Wednesdays, and Fridays for a total 4 weeks and in the DCT on Tuesdays and Thursdays over the same time period. The HDT, a modified version of the Kluver board (Kluver 1935), requires precise control of the digits, particularly apposition of the finger and thumb, to retrieve food rewards from two wells with varying diameters (Figure 1B). The large well was 25 mm wide while the small well was 18 mm wide. Both the large and small well were measured to be 1 cm deep.

The monkeys were tested on 32 trials divided evenly but pseudo-randomly between both their dominant and non-dominant hands to eliminate potential order effects. Each monkey was given 30 seconds to complete a trial, which consisted of picking up the

food reward from the well while the tester observed to check that the correct and precise fingers and hand form were used (Figure 2).

Figure 2: HDT assessment of grasp recovery. Precise control and use of two digits by the animal, as seen in the figure above, to retrieve the food reward from either the large or small well during HDT testing was the primary requirement for a trial to be considered successful by the tester. Dragging or dropping the food reward during a 30-second trial was recorded as a misgrab.

The exact time to retrieve was captured by photocells. The tester recorded successes or mistrials on a testing observation sheet. If the trial automatically terminated without the monkey making any move for the food reward or if the monkey was

unsuccessful, one additional opportunity was provided. If the monkey failed both opportunities, the animal's difficulties were recorded on the testing observation sheet and the next trial was initiated (Moore et al. 2013). The HDT has been used in assessing fine motor function of the hand and digits in previous studies of young and middle-aged monkeys and in this NHP model of cortical ischemia (Moore et al, 2012).

The DCT uses a modified version of a bimanual test apparatus (Lacreuse et al. 2007) that consists of two vertically standing plexiglass boards, one for each hand, with small holes to be filled with cylindrical-shaped candy (i.e. Mike & Ikes, Just Born Inc., Bethlehem, PA) (Figure 1C). The holes were 10 mm wide and about 1.0 cm deep. Monkeys were required to grab the food reward and pull sideways out of each hole. Each testing day consisted of 16 trials, 8 trials per hand. Similarly to the HDT, the monkey was given two attempts and the time required to remove the treat was recorded for each hand by photocells as well as the tester. Monkeys were trained pre-operatively on both tasks to asymptote levels.

Hand preference. Once the asymptote was determined for both the HDT and DCT during pre-operative testing, the "preferred" or dominant hand was assessed by baiting and allowing both sides of the motor testing apparatus to be accessible by both hands. The dominant hand was later targeted while making an ischemic lesion to ensure that monkeys would be motivated to use their impaired hand during post-operative testing.

Pre-operative inosine administration. Following pre-op training, the monkeys were assigned in a balanced but random fashion to either the inosine treatment group or placebo group. An inosine dose of 500mg per day was used. The inosine dose was mixed in with a favored food treat, in this case, yogurt. Powdered sugar was used as the placebo, also mixed with yogurt. The tester verified that the monkeys ingested the same dose each day after post-op testing. The tester and all other personnel involved in the study, including the preparer of the treatment cups, were blind to the animals' assigned group.

MRI Scanning. Pre-operative scans were carried out to ensure that monkeys were neurologically normal and to serve as a baseline for observation following the ischemic lesion. Scanning included structural T1 weighted (T1W), functional resting state MRI (fMRI) and clinical scans (Black Blood, Flair, T2*). This protocol for scanning was developed in previous studies of normal aging in the monkey to assess age related changes in the brain (Makris et al, 2010; Koo et al, 2012). A second set of scans was conducted at the completion of post-operative testing.

Electrophysiological Mapping of the Motor Cortices and Lesion Production. To create reproducible cortical ischemic damage and motor impairment, an ischemic lesion was localized surgically to the dominant hand region in the M1. The following steps were taken and carried out under sterile conditions. First, the animals were sedated using ketamine hydrochloride (10 mg/kg) and anesthetized intravenously using sodium

pentobarbital (15-25 mg/kg). The monkey's head was then secured in a stereotactic apparatus, an incision was made down the midline, and the temporalis muscle was reflected bilaterally. A bone flap above the frontal and parietal lobes was removed in one piece before making an incision in the dura to expose the motor cortex. A photograph of the central and arcuate sulci was then taken and printed. Using a small surface electrode to deliver electrical stimulation, the dominant hand area of M1 was determined and mapped onto this calibrated photograph.

Stimulus pulses lasting 250 μsec were applied at the surface of the pia once per second either singly or in a series of four and delivered at a rate of 100 Hz/40 msec. Sites with no response were further tested with a 200 Hz series of 4-8 pulses each lasting 2-msec and delivered over 20-40 msec period respectively. Current intensity was varied from 500 μA to 3 mA to determine the lowest threshold that brought about a response. Responses were graded based on intensity of movement upon stimulation. Stimulation sites were spaced 2 mm apart (anterior to posterior) in rows that were separated from the next by 2 mm (ventral to dorsal). The intensity of the evoked response was graded on a scale of 1 to 5. Stimulation sites with the lowest threshold and greatest recorded intensity were drawn on the photograph to create a map of the dominant hand area (Figure 3A). The electrode was then applied gently to the anterior bank of the central sulcus to determine the extent of the hand representation in the sulcus, before adding this to the map as well.

Following the generation of the electrophysiologically-determined cortical map, a small incision was made in the pia at the dorsal limit of the representation. Using a small glass micropipette, the pia and the penetrating arterioles were bluntly separated from the underlying cortex, depriving the cortex of blood supply and producing an ischemic lesion of the gray matter that degenerated to the underlying white matter (Figure 3B). The lesion was also continued down to the fundus on the rostral bank of the central sulcus adjacent to the surface representation.

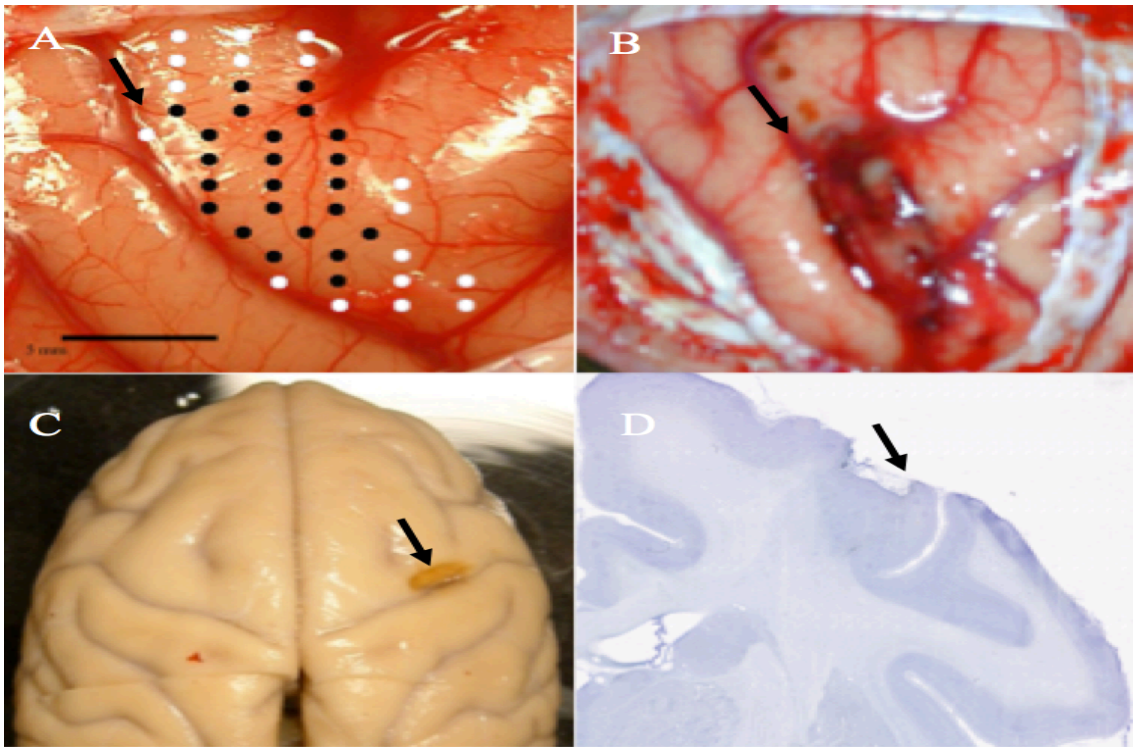

Figure 3: Mapping Surgery and post-euthanasia lesion. (A) Cortical map was determined electrophysiologically. Stimulation sites with the lowest threshold and greatest recorded intensity were drawn as dots on the photograph to create a map of the dominant hand area. (B) A lesion was created based on the stimulation sites that were determined electrophysiologically. (C) The lesion was created in the right precentral gyrus as seen in this photo. (D) Degeneration of the cortex by destruction of penetrating pial blood vessels can be seen be seen under a microscope.

All bleeding was stopped after the lesion was made. The dura was then closed, the bone flap was sutured back in place using small burr holes, and the muscle, fascia and skin were closed in layers. Following surgery, monkeys were moved to an incubator until they began to wake up and were then returned to their home cage and monitored for several hours. Immediately following surgery and for about a week, according to need, monkeys were monitored closely and given antibiotics and analgesics to minimize pain and prevent any signs of infection or complications.

Technical considerations. The approach for creating the ischemic lesion was taken for two reasons. First, there was no existing effective and controlled method for occluding a vessel and producing a reproducible stroke in the monkey cortex that would only yield damage confined to defined area (locus and size). Second, while useful for many endpoints, previous studies that had created strokes by occluding the middle cerebral artery or its branches produced severe neurological impairments, disabilities that impair the monkey's ability to feed and groom, as well as highly variable motor and sensory deficits. The present model produced a focal neurological impairment affecting only the dominant hand, and while the impairment was severe enough to be detected by our motor tasks and required significant recovery, the animal was able to retain effective mobility of the other arm and both legs to feed, groom and move around their cage in a healthy manner.

With regard to mechanism of damage, regions of gray matter are supplied by arterioles that penetrate vertically into the cortex from the overlying pia mater. Once the territory of M1 controlling the hand was localized electrophysiologically by surface stimulation, the pia was nicked at the dorsal margin of the mapped area and a small (0.50 mm) blunt glass suction tube was used to dissect the penetrating vessels bluntly. Suction through the tube was used only to clear extravisated blood and fluids used to flush and keep the field clean. Thermal cautery, otherwise known as electrocautery, was not needed and was specifically not used to avoid damage to underlying white matter or vessels in adjacent areas. Care was taken to avoid poking into gray matter and the diameter of the pipette was chosen so that it could not pull in blood vessels themselves. Hence, this was not an "aspiration" lesion where gray or white matter was aspirated during surgery but instead damaged underlying cortex, depriving it of blood flow and producing local ischemia and limited lesion (Fig 3C & D). Overall, this model was unique, providing solid endpoints comparable to human stroke recovery and allowing for more focused observation without the effects of side-effects and too many other motor and sensory deficits.

Post-operative cage-side assessments of upper extremity function. Beginning on the day of surgery and continuing for two weeks after the operation, the degree of motor impairment in the upper extremity (UE) was observed and recorded for each monkey.

Table 1: NHP Upper Extremity Motor Dysfunction Scale. *(adapted from Zhang et al. 2000 and the NIH Stroke Scale).* The degree of motor impairment in the upper extremity was assessed using a 3-, 4-, 5- scale of impairment (0 indicating no impairment) for post-operative days 0-14.

1	Observed tone in upper limbs 0 1 2	 Normal Decreased muscle tone (flaccid, arm hangs limply at side) Increase muscle tone (arm is held in flexed position)
2	Tremor in upper limbs 0 1 2 3	 Absent Occasionally present Present much of the time Continuously present
3	Fine motor function of hands 0 1 2 3 4	 Normal use Mild impairment in ability to retrieve food Moderate impairment in ability to retrieve food Severe impairment in ability to retrieve food Unable or refuses to retrieve food
4	Functional strength of hand 0 1 2 3 4	 Normal – no signs of weakness Mild weakness in UE when reaching or grasping Moderate weakness in UE when reaching or grasping Severe weakness in UE when reaching or grasping Unable or refuses to reach or grasp
5	Flexion of digits 0 1 2 3 4	 Normal flexion in digits Mild impairment in flexion of digits Moderate impairment in flexion of digits Severe impairment in flexion of digits No flexion observed
6	Movements of forearm/wrist 0 1 2 3 4	 Normal and full movement of wrist Mild impairment/weakness/slowness of wrist Moderate to severe impairment/weakness/slowness of wrist Severe impairment/weakness/slowness of wrist Unable to move UE at wrist joint
7	Movements of arm/shoulder 0 1 2 3 4	 Normal and full movement of arm and shoulder Mild impairment/weakness/slowness of arm and shoulder Moderate impairment/weakness/slowness of arm/shoulder Severe impairment/weakness/slowness of arm and shoulder Unable to move UE at arm and/or shoulder joints

An adapted NHP Upper Extremity Motor Dysfunction Scale (Zhang et al. 2000 and the NIH Stroke Scale) was used to assess tone, tremor, fine motor function in the hand, strength of the hand, digit flexion, and movement of the forearm, wrist, arm, and shoulder (Moore et al. 2013). Scores were rated on a 3-, 4-, or 5-point scale of impairment with 0 indicating no impairment and the highest score (3, 4, or 5) indicating total lack of motor function (Table 1). Fine motor function and functional strength in the dominant hand were given the most attention by the observer for the purposes of this study.

Post-operative Inosine Administration. Immediately following surgery, monkeys began receiving their individual daily dose of inosine (500mg) that was determined pre-operatively. The inosine dose or placebo was added to a yogurt cup for each animal. The tester verified that the monkeys ingested the same dose by having them complete the entire cup of yogurt each day directly after post-op testing. Blood chemistries were analyzed 2 weeks after beginning administration and then again prior to euthanasia.

Post-operative Testing. Post-operative HDT and DCT testing began 14 days after surgery and continued for a total of 60 days or 12 weeks. Similar to the pre-operative testing, post-operative testing was performed 5 days a week with 32 trials of the HDT (20 trials to impaired hand and 12 trials to intact hand) on Monday, Wednesday and Friday and 16 trials (8 trials per hand) of the DCT on Tuesday and Thursday. Performance on each testing day was videotaped for future assessment of grasp mechanics and

comparison of grasp patterns to allow ratings of motor function using a NHP grasp assessment scale.

RESULTS

Pre-operative HDT results. To confirm that there was no significant difference in initial performance on the HDT between our control and treatment groups, as well as the effectiveness of our pseudo-random balancing method, the mean time (in seconds) to retrieve a food reward with the dominant hand was determined for each monkey for the last 5 days of pre-op testing. Separate two-tailed Student's t-tests were used to compare our independent samples, the mean performances of the control and treated groups on the two different-sized wells. According to the data analysis, there was no significant difference in retrieval time between the control and treated monkeys on either the large ($t(6)=0.288$, $p=0.783$) or small well ($t(6)=0.897$, $p=0.404$).

Pre-operative DCT results. To confirm that there was no significant difference in initial performance on the DCT between our control and treatment groups and, again, the effectiveness of our the pseudo-random balancing method, the mean time (in seconds) to retrieve a food reward with the dominant hand was determined for each monkey for the last 5 days of testing. Separate two-tailed Student's t-tests were used to compare the mean performances for the control and treated groups on the dominant well. According to the data analysis, there was no significant difference between the control ($\overline{x}=1.08$) and treated ($\overline{x}=0.85$) monkeys in retrieval time on the DCT ($t(6)=-1.461$, $p=0.194$).

Post-operative NHP Upper Extremity Motor Dysfuction Assessment Score analysis. The median cage-side ratings (Table 1) were found for each animal in terms of dominant hand fine motor function and functional strength on the first three (1-3) and last

three (12-14) post-operative days. Lower scores indicated a higher level of function. Both the control and treated groups showed higher levels of fine motor function on days 12-14 (control: $\tilde{x}$=3.00; treated: $\tilde{x}$=3.25) than days 1-3 (control: $\tilde{x}$=4.00; treated: $\tilde{x}$=3.50) (Figure 4). Both the control and treated groups also showed higher levels of functional strength in their dominant hand on days 12-14 (control: $\tilde{x}$=3.00; treated: $\tilde{x}$=1.00) than days 1-3 (control: $\tilde{x}$=4.00; treated: $\tilde{x}$=3.50) (Figure 4).

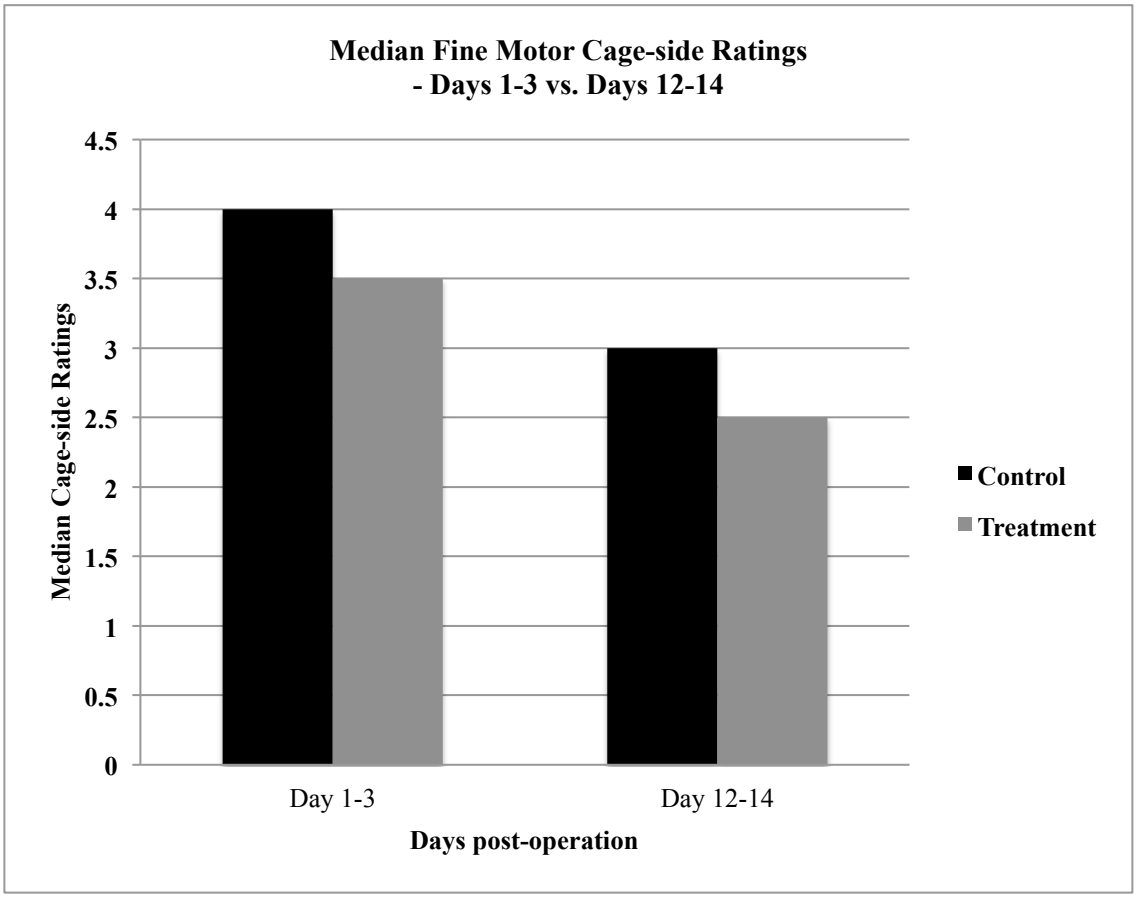

Figure 4: NHP Median Cage-side Ratings for fine-motor function in control and treatment groups on post-op days 1-3 vs. days 12-14. Both control and treated groups showed lower median cage-side ratings (higher level of function) for days 12-14 compared to days 1-3 after surgery. However, both groups demonstrated the same degree of recovery in terms of the assessment scale and, therefore, displayed no significant difference.

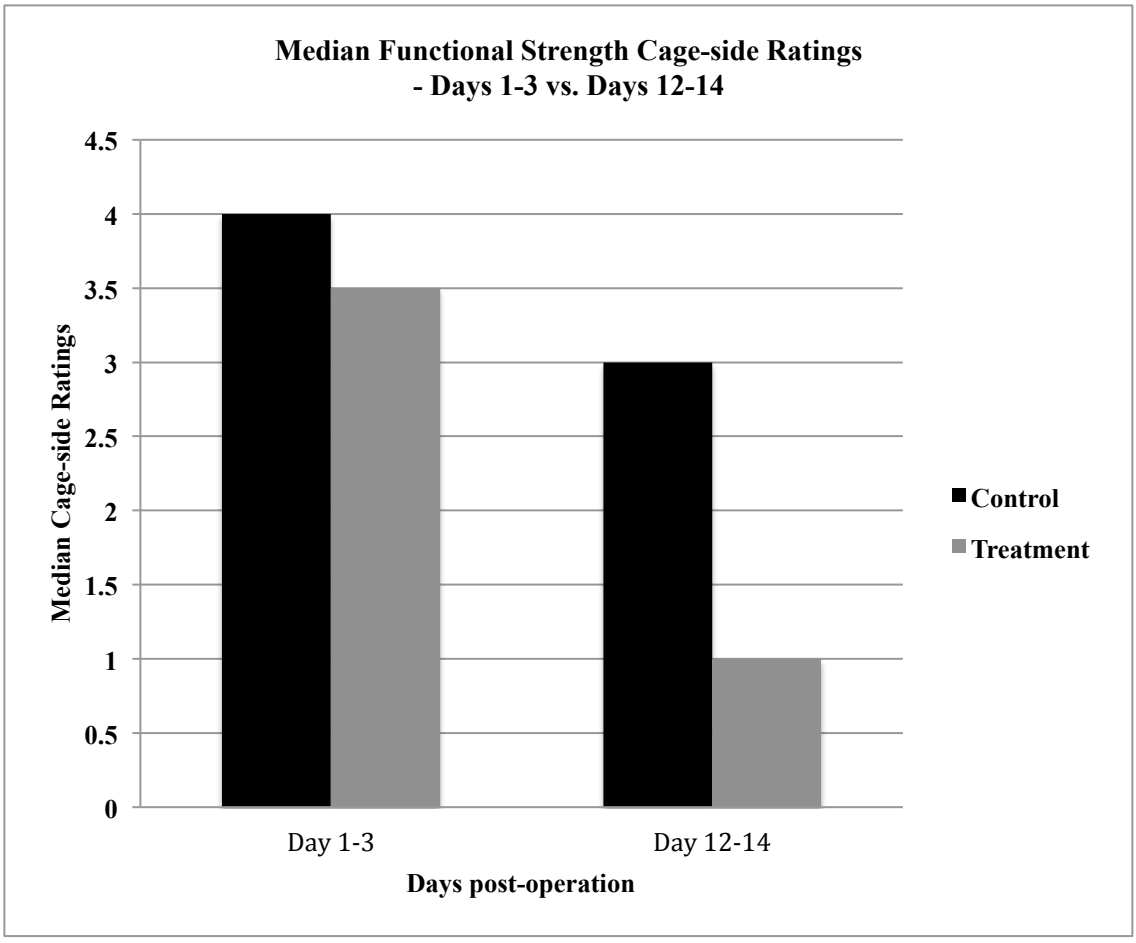

Figure 5: NHP Median Cage-side Ratings for functional hand strength in control and treatment groups on post-op days 1-3 vs. days 12-14. Analysis of the medians for the control and treated groups demonstrated a trend for the treated group towards greater recovery in functional strength for days 12-14. Median scores for animals treated with inosine were significantly lower than those of the control animals 2 weeks after having their surgically-induced lesion.

Non-parametric Mann-Whitney U tests were used to compare the difference in median scores on measures of fine motor function and functional strength of hand on days 1-3 and days 12-14 between the control and treatment groups. Median scores for fine-motor function were compared between days 1-3 ($p=0.127$) and days 12-14

(p=0.369). Analysis showed no significant difference in fine-motor recovery between the control and treatment groups at either time point (Figure 5). Median scores for functional strength were compared for days 1-3 (p=0.127) and days 12-14 (p=0.072). Analysis showed no significant difference but the p-value for days 12-14 suggested a trend towards greater functional strength recovery in the dominant hand for animals in the treated group than those in the control (Figure 5).

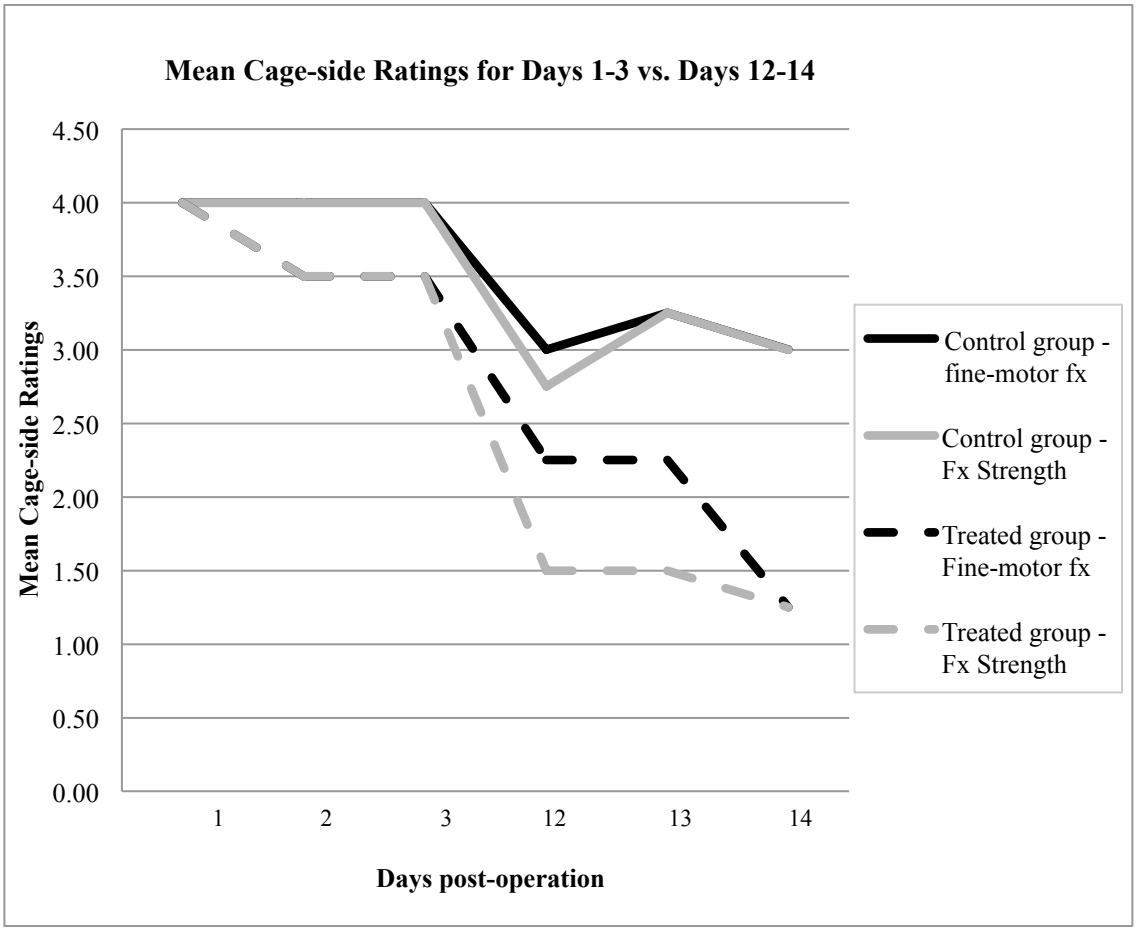

Figure 6: NHP Mean Cage-side Ratings for control and treatment groups post-op days 1-3 vs. days 12-14. The treated group demonstrated greater functional recovery than the control group in both fine motor function and functional strength from days 1-3 to days 12-14, demonstrating a higher rate of recovery over time.

The mean cage-side ratings were also calculated for each animal's fine motor function and functional strength of hand on the first three (1-3) and last three (12-14) post-operative days for additional observation of the trend and as a supplementary comparison between the control and treatment groups. Both the control and treated groups showed higher levels of fine motor function on days 12-14 (control: $\bar{x}$=2.75; treated: $\bar{x}$=1.92) than days 1-3 (control: $\bar{x}$=3.75; treated: $\bar{x}$=4.00). Both the control and treated groups also showed higher levels of hand strength on days 12-14 (control: $\bar{x}$=2.67; treated: $\bar{x}$=1.92) than days 1-3 (control: $\bar{x}$=3.75; treated: $\bar{x}$=4.00). The treated group demonstrated the greatest amount of change over time in both cage-side assessment measures (Figure 6)

Post-operative HDT results. The mean times (in seconds) of the control and treated groups to retrieve a food reward with the dominant hand on the HDT for the last 5 days of post-op testing were compared using a two-tailed Student's t-test. The analysis revealed no significant difference between the groups for the large ($t(6)=0.975$, $p>0.05$) and small ($t(6)=0.121$, $p>0.05$) wells. The mean number of days required to reach criterion (three consecutive days at or below pre-op levels) was also calculated using the mean times of both groups and used for further analysis. The mean number of days required to reach criterion was found to be statistically greater for the treatment group than the control group on both the large (control: $\bar{x}$=70.75; treated: $\bar{x}$=99.00) and small (control: $\bar{x}$=57.50; treated: $\bar{x}$=88.25) wells. Another group of two-tailed Student's t-tests

run comparing these numbers also revealed no significant difference between the control treatment animal groups for the large ($t(6)=0.179$, $p>0.05$) or small ($t(6)=0.273$, $p>0.05$) wells.

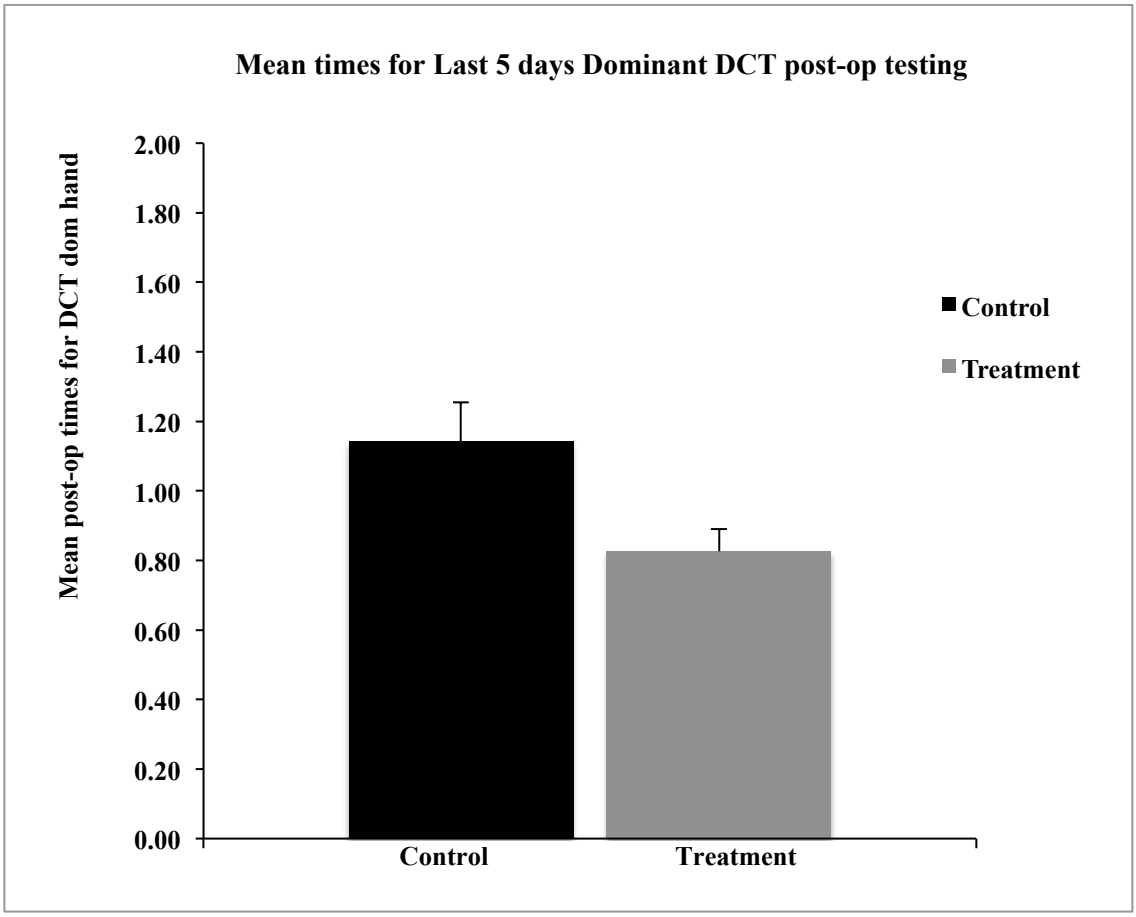

Figure 7: Mean times for last 5 days DCT post-op testing on dominant hand. Lower mean times were found for dominant hand retrieval of the food reward by the treated group during DCT testing but showed no significant difference from those recorded for the control group.

Post-operative DCT results. The mean times (in seconds) of the control and treated groups to retrieve a food reward with the dominant hand on the DCT for the 5 last days of post-op testing were compared using a two-tailed Student's t-test. Although, the

treated group showed lower mean times ($\bar{x}$=0.84) than the control group ($\bar{x}$=1.09) (Figure 7), the analysis revealed no significant difference between the group mean times for the dominant hand ($t(6)=1.02$, $p>0.05$). The mean number of days required to reach criterion (two consecutive days at or below pre-op levels) was also recorded and used for further analysis. The mean number of days taken for the treatment group was found to be greater than the control group for the dominant hand on the DCT (control, $\bar{x}$=75.75; treated, $\bar{x}$=97.75). Another group of two-tailed Student's t-tests were run comparing these numbers and, similar to the post-op HDT analysis, the data collected revealed no significant difference between the control and treatment performance on the DCT ($t(6)=0.275$, $p>0.05$).

DISCUSSION

Summary. There was one main finding in this study. Statistical analysis of cage-side assessment rating medians showed a trend in the treated group towards greater functional strength recovery of the dominant hand on days 12-14 when compared to the control group. Although there were no significant differences found in fine-motor function and functional hand strength recovery between the control and treatment groups, a trend was enough support for our hypothesis considering the small sample size and the subjectivity of the scale. There were also no significant group differences found in terms of mean times to retrieve the food reward with the dominant hand during pre-op and post-op HDT testing on either of the two differently-sized wells, or the mean times recorded during pre-op and post-op DCT testing. This is thought to have occurred as a result of behavioral variability or differences in grasp mechanics between animals on the DCT test, in particular.

Pre-operative HDT analysis. The results of the statistical analysis carried out on HDT data from the last 5 days of pre-op testing were as expected, indicating no baseline differences in dominant hand function and motor performance prior to the animals having their surgically-induced stroke. These results confirmed the effectiveness of the pre-operative pseudo-random balancing of the animals into control and treatment groups and that all animals began training at approximately the same level of dexterity and motor performance.

Pre-operative DCT analysis. Analysis of pre-op data was done with the sole purpose of confirming that our control and treatment animal groups did not demonstrate initial differences in performance so that recovery of function in these groups could later be compared to assess the efficacy of the inosine treatment after the surgically induced stroke. The results of the statistical analysis carried out on pre-op DCT data indicated no baseline differences in dominant hand function and motor performance prior to the animals having their surgically-induced stroke. These results confirmed the effectiveness of the pre-operative pseudo-random balancing of the animals into control and treatment groups and that all animals began training at approximately the same performance level.

Although the DCT was designed to be the more difficult of the two motor tasks, animals did not test on this task as expected. While the HDT required that the animals use precisely two digits to extract or "pick" the food reward from the wells, the DCT task did not have the same ability to force the animals to use the same exactitude of movement. Some animals were able to "scoop" or use their entire fist to pull the food reward sideways from the vertical boards of the apparatus, adding to the variability the motor performance on this test between animals and between testing days. Therefore, it is important to note that the mean times recorded may not have been reliable representations of the animals' abilities at baseline level.

Post-operative HDT analysis. The results of the statistical analysis carried out on post-op HDT data from the last 5 days of testing did not support our hypothesis that

animals treated with inosine after receiving a surgically induced stroke would show greater functional recovery than animals in the control group. It is possible that the mean retrieval times recorded for the animals during the last 5 days did not provide the best representation of their actual long-term functional recovery. The mean number of days to reach criterion was also found to be greater for the treated animals than the controls. According to the hypothesis, treated animals would show greater recovery by reaching criterion, or pre-op levels, in a shorter amount of time. Not only was this result unexpected but also further statistical analysis indicated no obvious inosine effect.

Behavioral data is subject to a great amount of fluctuation and variability. Testing was carried out in a quiet room to minimize sounds that may affect the animal and the collection of data. However, some animals were more susceptible to the slightest of noises and prone to losing focus while retrieving a food reward during trials. Although much was done to maintain regularity and routine with these animals, mean retrieval times may have changed due to distractions and outliers while carrying out the post-op HDT testing.

Post-operative DCT analysis. Analysis of post-op DCT data from the last 5 days of testing demonstrated no significant difference between treatment groups. The results did not support our hypothesis that animals treated with inosine after receiving a surgically induced stroke would show greater functional recovery than animals in the control group. Again, it is possible that the mean retrieval times recorded for the animals

during the last 5 days did not provide the best representation of their actual long-term functional recovery over the entirety of testing. Data was also affected by behavioral variability as with HDT testing.

The mean number of days to reach criterion calculated based on the mean times of both groups was also found to be greater for treated animals than the controls. According to the hypothesis, treated animals would show greater recovery by reaching criterion, or pre-op levels, in a shorter amount of time. These numbers were unexpected and further statistical analysis indicated no obvious inosine effect. The variability of grasp and DCT performance seen during pre-op testing also played a role during post-op testing and it is possible that, although inosine may have had a potential effect, it could simply not be observed through the DCT and our method of motor function testing.

Post-operative NHP Upper Extremity Motor Dysfunction Assessment Scale Analysis. Analysis carried out on cage-side ratings (Table 1) of animal fine motor function and hand functional strength during post-operative days 1-3 and 12-14 provided support for our hypothesis that inosine may facilitate functional motor recovery post-stroke. Individual medians and means calculated for both control and treated groups showed that all animals demonstrate recovery in fine motor function and functional strength over time. The treated group seemed to show a greater rate of recovery compared to the control group (Figure 5 & Figure 6), but the significance of this observed difference had still yet to be determined.

Mann-Whitney U tests were used to analyze the difference between the median scores of the control and treatment groups on measures of fine motor function and strength of hand from days 1-3 and days 12-14. Statistical analysis showed no significant difference in recovery of fine-motor function between the control and treated groups. However, non-parametric analysis of functional strength medians of both groups demonstrated a trend towards greater recovery in inosine-treated animals two weeks after surgery than control animals that were not given the inosine treatment.

Although the trend was not found to be statistically significant, the value produced during analysis was still important to the study considering our very small sample size and the subjectivity of the cage-side ratings collected. Scores were based on the observer's judgment and the defined ordinal values of the NHP Upper Extremity Motor Dysfunction Scale. To limit the amount of variability during data collection, one human observer was used for the entirety of the two-week cage-side rating period. However, half-point changes could not be recorded and the ratings assigned were most often dependent on the observer's estimation. With only four animals per treatment group and this amount of subjectivity, the significance difference calculated between groups for functional hand strength on days 12-14 provides enough evidence of an effect.

Deductions and future analysis. Times in seconds that were recorded for food retrieval in the apparatus during testing may not have been the most reliable measure of recovery considering the variability between animals and testing days. However, it is

possible that stronger support for our hypothesis could come from added assessment of hand and grasp pattern, using a NHP grasp assessment scale (See Moore et al. 2013) to analyze video recordings that were taken of each trial and all 60 post-op testing days for each monkey. Future analysis of these recordings may provide more significant and striking data on the motor improvement of the animals with time and with or without inosine treatment. Inosine may not work quickly enough to affect retrieval times but may affect grasp and finger movement in a way that cannot be captured by photocells and a tester's quick observation.

MRIs given, photographs taken during surgery, and tissue sections collected following euthanasia may also be used for future studies of the effects of inosine on anatomical reorganization and reduction in lesion size. Lesion size before and after post-op testing may provide an interesting cofounder for analysis of the effects of inosine treatment. Quantification of axons originating in the intact hemisphere and crossing into the denervated region could also potentially show the effects of inosine on axon growth and brain repair. Inosine-treated neurons would be expected to show greater sprouting. Axonal reorganization, however, is often difficult to describe convincingly due to the issue of distinguishing new connections from those that were not damaged in the first place. Again, this growth has been correlated to improved performance on behavioral tasks (Chen et al. 2002).

Although analysis of post-op mean times and mean days to criterion for both the HDT and DCT did not produce the expected results based on our hypothesis, a trend was observed in the cage-side ratings of hand strength for the treated group providing critical support for further research into the potential use of inosine as a long-term stroke treatment. Considering the subjectivity and small sample size of this study, a trend is enough evidence that inosine may still have a role in brain repair.

LIST OF JOURNAL ABBREVIATIONS

Acta Neuropathol	Acta Neuropathologica
Arch Neurol	Archives of Neurology
Arch Phys Med Rehabil	Archives of Physical Medicine and Rehabilitation
Brain Res	Brain Research
Biochim Biophys Acta	Biochimica et Biophysica Acta
Cell Mol Life Sci	Cellular and Molecular Life Sciences
CNS Spectr	CNS Spectrums
Eur J Neurosci	European Journal of Neuroscience
Free Radic Biol Med	Free Radical Biology and Medicine
Int J Dev Neurosci	International Journal of Developmental Neuroscience
JAMA Neurology	Journal of the American Medical Association - Neurology
J Alt and Comp Med	Journal of Alternative and Complementary Medicine
J Biol Chem	Journal of Biological Chemistry
J Cereb Blood Flow Metab	Journal of Cerebral Blood Flow & Metabolism
J Clin Invest	Journal of Clinical Investigation
J Neuro	Journal of Neuroscience
J Neuroinflammation	Journal of Neuroinflammation
J Neurosci Nurs	Journal of Neuroscience Nursing
J Neurophysiol	Journal of Neurophysiology
J Physiol	Journal of Physiology

J Pineal Res	Journal of Pineal Research
J Rehabil Res Dev	Journal of Rehabilitation Research and Development
Nat Vital Stat Rep	National Vital Statistics Reports
Neurobiol Aging	Neurobiology of Aging
Neurobiol Dis	Neurobiology of Disease
Phys Med Rehabil Clin N Am	Physical Medicine & Rehabilitation Clinics of North America
PNAS	Proceedings of the National Academy of Sciences
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences
Rev Neurol	Revue Neurologique
Rom J Morphol Embryol	Romanian Journal of Morphology and Embryology
Somatosens Mot Res	Somatosensory and Motor Research
Stem Cells Cloning	Stem Cells and Cloning: Advances and Applications
Trends Neurosci	Trends in Neurosciences

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