GAIT ANALYSIS IN THE PIG ISCHEMIC STROKE MODEL TO DETERMINE EFFICACY 
OF INSC THERAPY ON MOTOR FUNCTION RECOVERY

by

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(Under the Direction of Franklin D. West)

ABSTRACT

Most stroke survivors suffer from severe motor disabilities, including impaired manual 
dexterity and gait in the paretic limb. A novel stem cell therapy, induced pluripotent stem cell-
derived neural stem cells (iNSCs), has been shown to differentiate into neural cells and integrate 
into the stroked brain to reduce infarct size and improve motor recovery in rodents. However, 
there is a need to test iNSC therapy in a gyrencephalic brain more similar in composition to that 
of humans, such as the pig brain. In this study, we used computer-based quantitative gait analysis 
to determine the efficacy of hiNSC transplantation in a pig MCAO ischemic stroke model. Pigs 
underwent spontaneous recovery within 12 weeks following stroke and saline injection. hiNSC-
treated pigs showed less deficits in temporal gait parameters, including swing and stance time 
and limb support phase times, compared to vehicle-only pigs after 1 week. Together, our 
findings show that intraparenchymal injection has a minimal effect on motor function and that 
hiNSC therapy is a promising rehabilitative strategy for improved gait recovery after stroke.

INDEX WORDS: Ischemic Stroke, Induced Neural Stem Cells, Pig Stroke Model, Stem Cell 
Therapy, Gait Analysis, Motor Function
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DEDICATION

I would like to dedicate this work to my parents, who risked everything to come to America for the promise of a better future and endured challenges I could never imagine facing with such courage and resilience. Thank you for the privileges and opportunities you have given me through all your sacrifices and unconditional love despite my shortcomings. To my dad, with his unrivaled work ethic that has earned him success in both his career and family life, who taught his daughters the value of education and hard work and always challenged us to push ourselves. To my mom with her generous and loving heart, who always put our happiness before her personal gain and taught me what being truly selfless means. I am incredibly lucky to have you both in my life.
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CHAPTER 1

THE EFFECT OF STEM CELL-BASED THERAPY ON MOTOR FUNCTION IN ISCHEMIC STROKE

Stroke is currently among the leading causes of mortality and long-term disability worldwide and is especially prevalent in developing countries [1-3]. An estimated 795,000 Americans suffer from a new or recurrent stroke each year with varying debilitating impairments in mobility, speech, memory, and learning [4]. As the leading cause of long-term disability in the United States, stroke has huge socioeconomic implications on victims and their families with a costly $34 billion spent annually in the nation on long-term care [4]. Ischemic stroke accounts for more than 80% of all strokes and results from an embolic or thrombotic occlusion in a cerebral blood vessel, subsequently interrupting blood flow in the brain [4-6]. Ischemic injury often leaves stroke patients with severe disabilities, which commonly manifest in motor function impairments affecting muscle control or movement of the contralateral face, arm, and leg [7]. Even with intensive rehabilitation, most patients suffer from persisting motor function deficits after stroke, including impaired manual dexterity, walking ability, and activities of daily living (ADL) [8, 9].

Despite the high incidence of stroke and subsequent motor dysfunction, very few advances in treatment have been made over the last 20 years [10, 11]. Most therapies focus on limiting the extent of injury, targeting the more viable ischemic penumbra between the infarct core and normally perfused tissue [10]. Pharmacological and mechanical approaches of stroke therapy have been explored with limited success by either promoting recanalization of occluded
blood vessels to restore blood flow to cerebral tissue or increasing neuroprotection to reduce the cytotoxic and inflammatory cascade that occurs following a cerebral ischemic event. Both therapeutic approaches act to prevent further cell death, subsequently leading to an increase in motor function recovery [10, 11]. These strategies have only yielded a handful of viable stroke treatments; currently, the only Food and Drug Administration (FDA)-approved thrombolytic stroke therapies include tissue plasminogen activator (tPA) and, more recently, select mechanical thrombolysis and stenting techniques [10-15]. These methods are fairly effective in improving clinical outcome after stroke but have many limitations. With its restrictive 4.5-hour time window for treatment (although limited success is still observed up to 6 hours) and increased risk of intracranial hemorrhage, tPA is administered to only ~5% of ischemic stroke patients of which more than half remain disabled after 3 months [10, 12, 13, 15]. Alternatively, the Mechanical Embolus Removal in Cerebral Ischemia (MERCI) system can be used up to 8 hours after stroke, especially useful in patients ineligible for tPA treatment, but only restores blood flow in about 48% of patients [15, 16]. Of those patients with successful recanalization, only 46% have good clinical outcome after 90 days ranging from slight disability but with no assistance required to complete recovery (modified Rankin score of 2 or less) [16, 17]. The Stent-Assisted Recanalization in Acute Ischemic Stroke (SARIS) trial, the first FDA-approved stenting trial for stroke, has a shorter 6-hour time window than the MERCI system. Despite its 100% recanalization rates, stenting yields high risks associated with intracranial stent placement with only moderate clinical outcome in 60% of patients (mRS score of 3 or less), where patients still require some help but are able to walk without assistance [14, 15]. To date, over 1,000 neuroprotective therapies have been investigated in preclinical trials for their potential to modulate secondary injury responses after ischemic stroke, including free radical production,
apoptosis, and inflammation; however, of the 200 neuroprotective agents tested in clinical trials, none have successfully passed despite strong preclinical data [11, 18]. Both thrombolytic and neuroprotective approaches to stroke therapy only act to prevent further injury for acute stroke but have little to no regenerative potential. Any previously damaged tissue due to ischemia cannot be salvaged. This major limitation of current stroke treatment methods has garnered a growing interest in stem cell therapy for its regenerative potential.

In an effort to improve the success of stroke therapies in clinical trials, the Stem Cells as an Emerging Paradigm in Stroke (STEPS) group, comprised of leaders from academia, industry, the FDA, and National Institute of Neurological Disorders (NINDS), was established under the Stroke Academic Industry Roundtable (STAIR) model. From the Consortium meetings (STEPS I, II, III), the group identified several criteria to improve stroke treatment development, including the need for a regenerative cell therapy that will not only protect cells from ischemic injury but also replace any lost or damaged tissue and the use of a more translatable large animal model [19-21]. These STEPS publications and overwhelming lack of progress in thrombolytic and neuroprotective stroke therapies have increased the interest in stem cell therapy as a promising and novel candidate for ischemic stroke treatment. Various cell types have been used in translational stroke therapy research, including embryonic stem cells (ESCs), adult stem cells, neural stem cells (NSCs), and induced pluripotent stem cells (iPSCs) [22, 23].

In this review, we will explore the potential application of stem cells for motor function recovery after ischemic stroke using a large animal model with a brain more similar in anatomy and physiology to that of humans. The aim of our research is to develop a stem cell-based therapy that will both replace lost brain tissue and secrete regenerative trophic factors to improve motor function recovery in a pig stroke model. Our research will help contribute to the
development of more effective clinical stroke therapies with the capability to improve motor function recovery and subsequently increase quality of life for stroke survivors and their families.

**GAIT ANALYSIS: APPLICATIONS FOR ISCHEMIC STROKE THERAPY**

More than 80% of stroke survivors experience gait impairments manifested most commonly as severely limited mobility [24-26]. While most stroke patients regain some walking ability, approximately 35% of patients do not regain walking function, and 25% of all survivors require physical assistance to walk [27]. Even after a stroke patient regains the ability to walk, lower limb impairments and abnormal gait patterns often persist, where the impaired lower limb is unable to balance, posture, and initiate and control movement normally due to muscle weakness [28-30]. Gait recovery is often considered synonymous with stroke recovery, as the goal of most stroke patients is to regain normal walking function [31, 32]. In response, there has been an extensive focus on the measurement of gait impairments in order to identify effective therapies for gait recovery after stroke [33-36]. Quantitative gait analysis is frequently used in clinical applications in order to identify and measure gait impairments, determine appropriate treatments, and evaluate the outcome and efficacy of therapeutic interventions after stroke [37, 38]. However, gait analysis may prove useful in elucidating effective stroke therapies for functional recovery in preclinical animal models, such as stem cell-based treatments. Both thrombolytic and stem cell-based stroke treatments focus on mediating damage in cerebral tissue with the hope of subsequent improvement or reversal of functional and behavioral deficits. The exact role of the central nervous system (CNS) in both normal and post-stroke gait is not truly understood since gait is a fairly complex interaction between the CNS and peripheral musculoskeletal systems. Therefore, it is important to understand the mechanisms involved in motor function recovery and
biomechanical abnormalities following stroke in order to develop more effective interventions to restore gait and determine reliable parameters to measure post-gait disturbances using quantitative gait analysis [39].

1.1 Motor Recovery after Stroke

Stroke Lesion Location and Size

Ischemic or hemorrhagic injury to the motor cortex, premotor cortex, motor tracts, or associated pathways in the cerebrum or cerebellum can cause motor impairment and disability in stroke patients [7]. The extent of motor impairment and subsequent recovery after stroke is variable among individual patients, where some motor functions recover rapidly and others remain as permanent deficits [40, 41]. Results of structural imaging studies have correlated motor recovery and subsequent long-term effects of stroke with the size and location of the initial stroke lesion [42-49]. Clinical evidence suggests that injury to the corticospinal tract is associated with higher motor impairment independent of lesion size [50, 51]. Lesions of the insula and surrounding opercular cortex have also been reported to result in severe motor deficits and low functional outcome in speech and language, hand-and-eye motor movement, space and body perception, and cardiovascular regulation [50, 52-55]. Higher modified Rankin scale (mRS) scores are associated with lesions in the corona radiata and internal capsule, suggesting more severe post-stroke disabilities such as limb weakness [50]. Another study has further confirmed that patients with internal capsule injury are also likely to have poor motor outcome [56]. Decrease in upper limb motor recovery is associated with lesions located in areas of motor-related cortical regions, corona radiata, and internal capsule [43, 48].

Though multiple studies have reported variable findings regarding effects of lesion size and lesion location on motor impairment and recovery, most confirm that stroke lesion size and
location are important predictors of motor and functional outcome. The ability to predict and understand motor recovery patterns from lesion characteristics is crucial for the development of more effective rehabilitation interventions to restore motor deficits, including gait and walking function.

**Brain Response to Ischemic Injury**

Motor weakness is frequently reported in almost all patients who suffer from middle cerebral artery infarcts, which often span the motor cortex and corticospinal tract [57]. Lesions in the motor cortex and corticospinal tract often disrupt motor signals transmitted via the corticospinal tract to the spinal cord motor neurons, resulting in hemiparesis [28-30]. Fibers in the corticospinal tract originate mainly from the primary motor cortex (M1), but other motor related brain regions, such as dorsolateral premotor cortex, supplementary motor area, and cingulate motor areas, that form the secondary motor cortex have direct corticospinal connections to motor neurons in the spinal cord [58]. In response to brain injury affecting the primary motor output system and resulting in the reduction of motor signals from M1, these secondary motor regions may compensate by providing outputs to the corticospinal tract [59]. However, these projections are not perfect substitutions as they are less efficient at exciting spinal cord motor neurons [60]. This adaptive response suggests that the brain has the capability to change structure and function in response to ischemic injury, which may explain why stroke patients often experience dramatic recovery in motor function within the first month without any rehabilitative interventions [61]. This potential mechanism of spontaneous motor recovery is referred to as neuroplasticity.

Mechanisms of plasticity following stroke have been extensively studied in many animal models. Rehabilitative training after stroke resulted in functional reorganization in the adjacent
undamaged motor cortex in non-human primates [62]. In a rodent focal ischemic stroke model, remapping of sensory circuits and development of prolonged sensory responses were observed in both the peri-infarct zone and distant sites [63]. Molecular and cellular substrates of plasticity may play a role in the remodeling that occurs after stroke. Developmental proteins normally absent in the adult brain are expressed hours after ischemic brain injury, usually at a time of limited cellular metabolic resources, and remain increased for weeks or months [64]. These proteins include NeuroD, Nestin, MAP-2, GAP43, synaptophysin, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), p53, and Cyclin D and are involved in neuronal growth, apoptosis, angiogenesis, and cellular differentiation [65-71]. Structural changes, evidenced by increased dendritic branching, synaptogenesis, axonal sprouting, and neural connection rewiring, have also been observed in perilesional and distant cortex after stroke [72, 73]. These results suggest that not only can cortex reorganization occur after stroke in the adult mammalian cortex but also rehabilitative training can promote this functional reorganization. This is particularly important as the manipulation of these processes for rehabilitative approaches might increase motor recovery potential in stroke patients.

**Time Window for Rehabilitative Therapy**

In most animal models, heightened plasticity and subsequent recovery in the brain usually occurs for a month after stroke [74]. Identifying an optimal rehabilitation period within this time of increased neuronal plasticity is critical in maximizing motor function recovery. Generally, earlier initiation of rehabilitation therapy yields better functional and motor outcome. In animal studies, rehabilitative training within the first week of stroke is most effective in improving motor function and enhancing neuroanatomical changes in both the ispi- and
contralateral hemispheres [75]. In a rat MCAO stroke model, rehabilitation initiated at 5 days after stroke significantly increased functional outcome and dendritic branching in the contralateral side of the lesion but not with later rehabilitation at 14 or 30 days post-stroke [76]. Another rodent study compared forelimb-reaching skills after stroke with training initiated at 4 or 25 days post-stroke; reaching skills were significantly enhanced in the early trained group [77]. While these results clearly indicate that early training improves motor outcome, initiating therapy too early may also lead to decreased recovery. Increased lesion size and cell death rate have been observed after early excessive limb training immediately after stroke due to NMDA-mediated excitotoxicity in an already hyperexcitable peri-infarct area [78]. In animal studies more closely modeling early intervention in humans, rehabilitation at 1-3 days post-stroke increased cell death but improved motor function long-term [79]. After considering findings from multiple animal studies, it seems as though the optimal time to initiate rehabilitative therapy with the greatest chance of motor recovery is five days after stroke [74].

1.2 Stroke Rehabilitation

Most stroke rehabilitation approaches focus on motor learning in order to promote new synapse formation, alter existing synapses, and increase neurochemical production and in turn, facilitate motor function recovery after stroke [80]. One common approach, task-oriented training, focuses on the repetitive practice of motor performance to improve functional abilities [81]. Task-specific training has been shown to induce long-lasting motor learning and associated cortical reorganization and assist the natural pattern of functional recovery [81]. In addition to task specificity, enriched environments, which provide greater opportunity for physical therapy and motivation, are also crucial for stroke rehabilitation [82]. Therefore, successful stroke rehabilitation programs should combine these two approaches and utilize repetitive and intensive
task-specific movement training in an enriched environment to promote neural plasticity and motor recovery [81]. The use of motor learning-based stroke rehabilitation strategies has been evaluated in many studies with beneficial effects.

**Constraint-Induced Movement Therapy (CIMT)**

Because stroke patients mostly use their nonparetic limb to perform daily activities, this disproportionate use of the nonparetic limb leads to learned nonuse in the paretic limb, which severely limits motor function recovery [83]. Constraint-induced movement therapy (CIMT) is used to overcome this problem of learned nonuse in the paretic limb by restraining the nonparetic arm into a sling or glove and thus forcing the paretic arm to perform repetitive activities [84]. Studies have shown evidence of structural and physiological changes following CIMT, including increased neural plasticity and altered neural network activity [85, 86]. Most patients undergoing CIMT within 3-9 months of stroke exhibit strength improvement in the paretic upper limb for up to 2 years [87]. These findings indicate that CIMT is very effective in reducing upper limb disability, especially in those with chronic stroke.

**Body Weight-Supported Treadmill Training (BWSTT)**

Most stroke patients experience abnormal control of the lower paretic limb, resulting in asymmetric gait [88]. Body weight-supported treadmill training (BWSTT) involves patients walking on a treadmill with their body weight partially supported to improve gait and walking function by enabling repetitive practice of complex gait cycles [89]. By allowing patients to practice nearly normal gait patterns and avoid developing compensatory walking habits, such as hip hiking and circumduction, BWSTT results in improved swing time asymmetry, stride length, and walking speed [31, 90, 91]. Results from animal studies have found that BWSTT increases activity in bilateral primary sensorimotor cortices, cingulate motor areas, caudate nuclei, and
thalamus of the affected hemisphere and alters central pattern generator activation, suggesting that BWSTT may work to induce neural plasticity in humans as well [92, 93]. As repetitive task-specific method of rehabilitation, BWSTT is particularly successful in improving gait deficits.

**Robotic-assisted Training**

Robotic training involves the patient trying to move with the robot assisting or resisting the movements during repetitive practice in order to provide intensive, reproducible, and task-specific movement therapy [94]. Most commercial robotic systems are mainly for upper limb training, including the Assisted Rehabilitation and Measurement (ARM) guide, Mirror Image Movement Enabler (MIME), MIT Manus, and Hand Wrist Assistive Rehabilitation Device (HWARD); however, a few robotic gait training devices, such as Lokomat and Robotic-Assisted Gait Training (RAGT) device, exist [94, 95]. The use of HWARD robotic-assisted training in the upper limbs results in cortical reorganization and associated motor recovery [94, 95]. Robotic therapy is also particularly efficient in facilitating muscle control in the paretic arm and has long-term effects from several months to several years [94]. Although robotic-assisted training has proved to be beneficial in upper limb motor recovery, it remains unclear whether this type of rehabilitation is more effective than conventional therapy due to its limited use in clinical studies [96].

1.3 Human Biomechanics of Gait

**Normal Gait**

Normal human walking is considered a smooth progression through the gait cycle. The gait cycle is comprised of two phases: stance phase and swing phase. Each phase is then subdivided into different stages, as follows:

1. **Stance phase**
1. Initial contact: heel strike, hip flexed through loading response, lengthening contraction of the ankle dorsiflexors in early stance phase

b. Loading response: foot flat, lengthening contraction of the ankle plantar flexors throughout most of stance phase

c. Mid stance: neutral hip, body supported by one leg

d. Terminal stance: hip extended

e. Preswing: toe off, controlled forward shift of the center of the body mass by propulsive power, mainly of the ankle plantar flexors

2. Swing phase

a. Initial swing

b. Mid swing

c. Terminal swing: controlled fall of the body into next stance phase [97]

Various spatial and temporal parameters have been defined for biomechanical gait analysis. Common spatial parameters measured include step length (the linear distance between two successive points of contact of the right and left limbs) and stride length (the linear distance between two successive points of contact of the same foot). Temporal parameters include stance time (the amount of time during the gait cycle where the foot is in contact with the ground); swing time (the amount of time during the gait cycle where the foot is off the ground and in the air); gait speed; and stride duration (the amount of time to complete a single gait cycle). Normal gait is generally symmetric both spatially and temporally, meaning that these measured spatial and temporal variables would be equal in both left and right limbs in a normal patient [98, 99].

Abnormal Stroke Gait

Stroke often results in unilateral gait deficits, resulting in asymmetric gait due to severe
muscle weakness in the contralateral limb. Limb-specific differences in kinematic and kinetic measures of gait and in overall spatiotemporal parameters serve as quantitative gait analysis confirming asymmetry [100, 101]. Studies examining gait patterns in stroke patients typically find that swing phase duration is prolonged and single-limb support time is shortened in the paretic limb as compared to the nonparetic limb [100-104]. Overall, the proportion of time spent in stance vs. swing is significantly different after stroke; compared with healthy subjects walking at the same speed, stroke patients spend a longer proportion of time in stance phase on both the paretic and nonparetic sides [103, 105]. Double-limb support duration is also increased compared to normal, while gait speed and stride length is generally decreased [103]. In stroke patients, the symmetric step length observed during normal gait is missing; instead, some patients exhibit longer step length on the paretic limb [106, 107], while others have longer step length on the nonparetic limb [105]. These asymmetries observed in patients are very significant, as they lead to inefficient energy expenditure, falls, abnormal joint loading, joint damage, and pain [108, 109].

1.4 Quantitative Gait Analysis in Animal Models

Gait Analysis in Rodents

To date, several methods of analyzing gait changes have been well established in rodent models. Behavioral tests are often used to assess dynamic gait changes in spontaneous open field locomotion or fine coordination skills as animals walk either on a narrow beam [110], rope [111], grid [112], horizontal ladder [113], ladder rung [113], rotating rod [110], or over a runway [114]. Footprint analysis using ink [115], X-ray [116], cinematography [117], or scanner [118] can determine static changes in gait. While these tests detect distinct gait impairments due to neurological diseases, such as stroke, they are not sensitive enough to reveal subtle motor
changes. However, using quantitative gait analysis can provide a more comprehensive and sensitive evaluation of motor function impairment by evaluating a variety of biomechanical gait parameters in rodent models of neurological disorders.

Several rodent studies using gait analysis have found significant deficits in multiple gait parameters synonymous to impairments observed in human patients [119-123]. Video-based gait analysis typically uses high-speed cameras to film rodents as they walk across a glass plate [124] or on a treadmill [125]. However, many studies have used automated gait analysis systems, such as CatWalk, successfully in rodent models of neurological disorders, including stroke [120, 124, 126-132]. The huge advantage to using automated gait analysis systems is their ability to rapidly detect and quantify several gait parameters, including spatiotemporal parameters for individual limbs, position, pressure, and surface area of each limb, and interlimb coordination [124]. In human stroke patients, GAITRite is a similar automated system used to reliably detect changes in gait post-stroke, such as decreased walking speed, cadence (steps/sec), and step length [133, 134].

Several groups have investigated motor impairments in rodent models of stroke using automated quantitative gait analysis methods. Most studies found significant impairments in gait parameters after stroke but with varying results possibly due to the different methods of stroke induction. Overall, results in rodent studies revealed that hindlimbs were typically the most affected in different gait parameters after stroke compared to forelimbs [120, 126, 127]. In a mouse transient MCAO model, maximum contact area, stride length, and swing speed were significantly decreased in the contralateral hindlimb [111]. Gait analysis in a rat model of cerebral ischemia found a significant decrease in cadence and increase in base of support [112]. Another transient MCAO model resulted in short-term deficits in intensity, stride length, and
placement time of impaired forelimb. Decreased hindlimb swing speed and placement time were more long lasting [115]. Similarly, another study found that reduced intensity, print area, and maximum width area of contralateral hindlimbs were severely affected one day after stroke [116]. Intensity and maximum area of affected forepaw was decreased four days after the induction of a cortical lesion stroke. Increased double limb support time in ipsilateral paws and impaired interlimb coordination were also observed with these gait impairments persisting for up to 5 weeks after stroke [117]. These findings in rodent models show that quantitative gait analysis can reliably detect subtle changes in gait after stroke and reflect gait impairments often observed in humans, including decreased speed, cadence, and stride length.

**Gait Analysis in Pigs**

Quantitative gait analysis in rodent stroke models has yielded promising results for its use to determine the efficacy of therapies in improving gait and motor function. However, rodents have many limitations as a translatable model with significantly different brain anatomy and physiology from that of humans. With brains similar in anatomy and physiology to humans, pigs may serve as a more predictive model of stroke therapy [135, 136]. In fact, pig models of ischemic stroke have resulted in functional deficits similar to those observed in human stroke patients [137-140].

Behavioral and functional assessments in pigs have not been as extensively developed as in rodent models. This poses as a significant hurdle to overcome for researchers interested in evaluating gait impairments following stroke. However, prior studies have used biomechanical gait analysis in pigs to determine the effects of pen flooring conditions on gait using force plates and video-based approaches [141, 142]. Video data was used to analyze several gait parameters, including walking speed, stride length, swing and stance time, and limb support phases. Normal
pig gait on dry, clean floors was symmetric with alternating two- and three-limb support phase pattern comparable to that of dogs, sheep, dairy cows, and horses [141, 142]. Pigs altered their gait in soiled floor conditions by reducing walking speed, shortening stride length, prolonging stance time, and employing more three-limb support phases [141, 142]. These findings have established basic characteristics of normal and impaired pig gait, providing a baseline for future quantitative gait analysis studies in pigs.

Understanding the differences between normal and abnormal pig gait is crucial to interpreting gait changes in pig models of neurological disorders, such as stroke. Quantitative gait analysis has been used previously in a porcine stroke model with promising results [138]. Normal pig gait was symmetric in swing time, stance time, step length, step velocity, and maximum step height. MCAO stroke resulted in significant gait asymmetries, most notably in hindlimb maximum hoof height and swing and stance time [138]. After stroke, pigs displayed an asymmetric gait with increased stance time and reduced swing time in the contralateral limb. Gait abnormalities in human stroke patients are the opposite with longer swing times and shorter stance times in the paretic limb. Direct comparisons between bipedals and quadrupeds cannot be made since their gait is inherently different. However, these changes in pig gait after stroke reflect that of the pigs walking on soiled flooring in the previously mentioned study with prolonged stance time and shortened swing time. In stroked pigs, these compensations in gait may help distribute weight of the pig on the affected side by increasing ground contact.

Results of these studies indicate that quantitative gait analysis has the capability to detect subtle motor function changes in pigs. These changes in gait can be compared to those exhibited in human stroke patients, confirming that the pig can be used to predict the efficacy of novel stroke therapies on motor function recovery.
CHAPTER 2

STEM CELL TYPES FOR ISCHEMIC STROKE THERAPY

Stem cell-based therapy for ischemic stroke has resulted in promising functional outcomes in preclinical animal studies. Different cell types have been used to improve function and recovery after stroke, including embryonic stem cells, adult stem cells, neural stem cells, and induced pluripotent stem cells. Each cell type both has its advantages and limitations with varying levels of efficacy and safety in experimental stroke studies, prompting further investigation to determine the best choice for ischemic stroke treatment.

EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are cells derived from the inner cell mass of the preimplantation blastocyst with the ability to self-renew and differentiate into nearly any somatic cell type of the three germ layers [143]. Their unlimited capability to proliferate and differentiate into many clinically relevant cell types in vitro make ESCs an attractive candidate for cell transplantation studies [144, 145]. However, major limitations to undifferentiated ESC transplantation into an ischemic stroke animal model include the spontaneous production of teratomas and even highly malignant teratocarcinomas [146, 147]. Under specific culturing conditions, ESCs can be induced to a neural fate for potential use as a regenerative treatment for neural disorders such as stroke [148-153]. This is critical as differentiating ESCs prior to transplantation in vivo may circumvent the malignant transformation of ESCs. A few studies have transplanted ESC-derived neural cells into an ischemic injury model with moderate success. After mouse ESC-derived neural cell transplantation into rat cortex with severe focal ischemia,
ESC-derived neural cells were found to have integrated into the host tissue and improved functional recovery; in this study, recovery was considered a significant decrease in neurological severity score, which evaluates sensorimotor behavior, coordination, balance, and motor function [154]. In rats with focal ischemia induced by middle cerebral artery occlusion (MCAO), intrastriatal transplantation of mouse ESC-derived neuron-like cells improved dopaminergic function and behavioral recovery [155]. Subsequently, several studies have utilized ESC-derived neural stem/progenitor cells (NSPCs) in animal models of ischemic stroke [156-161]. Most results found that ESC-derived neural stem/progenitor cells migrated and engrafted to the area of ischemic infarct [156-161]; differentiated into either mature neurons [156, 158, 161] or a neuronal phenotype [157, 159, 160] and glial cells [158, 159]; mostly survived within the infarct core for up to 12 weeks in one study [156-161]; improved functional recovery [156-161]; and formed synaptic networks with host neuronal circuits [156, 159]. One study in a rodent MCAO model found that most human NSPCs did not survive after transplantation, but those that did survive differentiated to neuronal phenotypes, suggesting that the presence of these cells contributed to some functional improvement [160]. Despite pre-differentiation, transplanted human ESC-derived neural cells still formed teratomas after transplantation [162]. Prolonged differentiation of ESCs or different culturing conditions in vitro may reduce the risk of tumorigenesis [163]. Overall, ESC-derived neural cell transplantation resulted in some functional recovery in a battery of sensorimotor tests and improved asymmetric motor behavior, making ESCs a promising cell choice for motor function recovery.

**ADULT STEM CELLS**

Most stroke therapy studies have focused on adult stem cells (ASCs) due to their low immunogenicity and easy method of isolation, leading to a large number of clinical trials using...
ASCs to treat stroke [20, 164]. Adult stem cells include both hematopoietic and mesenchymal stem cell populations, but most stroke therapy studies focus on the use of mesenchymal stem cells (MSCs) [20]. MSCs have the capacity for self-renewal and differentiation into several mesenchymal-specific lineages without the controversy associated with ESCs [165]. While MSCs are traditionally found in the bone marrow, they have been successfully isolated from various tissues, including circulating blood, UCB, menstrual blood, placenta, heart, adipose tissue, skeletal muscle, pancreas, and dental pulp [166]. With the ability to differentiate into neural cells in vitro and secrete cytokines and trophic factors, which may lead to increased neurogenesis, angiogenesis, and attenuation of inflammation, MSCs are a viable cell candidate for motor function improvement after stroke [167-170].

The effects of MSCs in stroke animal models and patients have been extensively investigated with moderate success. Long-term (~5 years) intravenous transplantation of autologous MSCs did not cause harm and may improve recovery in stroke patients; the study measured recovery with the modified Rankin score and found that the proportion of patients with a mRS score of 0-3 (full recovery to mild disability) increased in the MSC-treated group [164]. In another study, administration of autologous MSCs in 12 patients with chronic stroke resulted in significant neurological improvement in NIH Stroke Scale and modified Rankin scores [171]. However, many preclinical studies have shown that, while transplanted MSCs express neuronal or glial markers, survival of grafted and differentiated cells was very low [172-175]. These results strongly suggest that the beneficial effects of MSCs on ischemic brain injury may be due to neuroprotective signaling factors, which play critical roles in promoting angiogenesis, synaptogenesis, and endogenous neurogenesis [172, 176, 177]. Studies have confirmed that MSCs reduce tissue death by secreting these trophic factors, which stimulate low levels of
regeneration and reduce the immune and inflammatory responses that typically follow ischemic injury [178, 179]. After MSC transplantation in ischemic stroke models, anti-apoptotic, cell survival, proliferation, and angiogenic factors mediate the cytotoxic ischemic cascade and lead to improved recovery [180]. Given the current data, ASCs are proven to be safe and beneficial for use as a potential stroke therapy resulting in moderate functional improvement. It seems that ASCs mostly promote endogenous repair and regeneration as opposed to acting as a means of neuronal cell replacement, which may also increase brain plasticity and further augment spontaneous motor recovery.

**NEURAL STEM CELLS**

Neural stem cells (NSCs) are the most obvious choice for transplantation in central nervous system disease models, including ischemic stroke, as they have the ability to self-renew, proliferate, and differentiate into multiple neural lineages, such as neurons, astrocytes, and oligodendrocytes [181, 182]. Exogenous NSCs can be derived from several sources, including ESCs [183], iPSCs [184], MSCs [185], and embryonic NSCs [186]. Growth factors, such as EGF, FGF, and LIF, and retinoic acid are often used to induce NSC proliferation and differentiation *in vitro*, respectively, making this class of cells an attractive candidate for ischemic stroke therapy [181]. Additionally, NSCs express little to no MHC molecules, avoiding the potential problem of immunorejection after transplantation [187]. Overall, animal studies using ESC-derived NSCs demonstrated a greater ability to proliferate and differentiate into neurons after transplantation and had a higher rate of survival in ischemic tissue, lending to greater functional recovery and reduction in lesion size than in models using adult NSCs [157]. In most studies, NSCs were found to differentiate into neural and glial phenotypes, suggesting that NSC transplantation improved ischemic injury through cell replacement. NSCs also had the
ability to form functional synapses and integrate into host neuronal circuitry [188]. NSC transplantation has been shown to increase functional recovery, particularly measured in sensorimotor tests and motor function recovery scales, such as the modified Rankin scale [189, 190]. These findings indicate that NSCs may be used for ischemic stroke therapy to improve motor function.

**INDUCED PLURIPOTENT STEM CELLS**

Induced pluripotent stem cells (iPSCs) represent a novel class of stem cell, where mature somatic cells can be reprogrammed into pluripotent cells capable of differentiating into any cell type in the body through the overexpression of defined factors (Oct3/4, Sox2, c-Myc, and Klf4) [191]. iPSCs are very similar to embryonic stem cells (ESCs) in morphology, immunoreactivity, global gene expression, epigenetics, and developmental plasticity but without the controversy and ethical concerns [192-194]. iPSCs offer an ideal approach for a stem cell-based stroke therapy as patients would be treated with pluripotent cells derived from their own somatic cells, which would subsequently avoid immune rejection. Recent studies showed that there was no evidence of immune rejection to iPSC transplantation, further bolstering the idea that autologous iPSCs can be safely transplanted into human stroke patients [195, 196]. However, very much like ESCs, transplantation of undifferentiated iPSCs into rodent stroke models led to the formation of tumors [197, 198]. The use of induced pluripotent stem cell-derived neural stem cells (iNSCs) circumvents any safety concerns regarding tumorigenesis; new reprogramming technologies allow somatic cells to be directly reprogrammed into NSCs and neurons without a pluripotent stem cell intermediate [199-201]. Promising data from rodent stroke models have shown that iNSC transplantations led to decreased infarct sizes and functional recovery with improvements in sensorimotor tests, such as grasping and beam walking [202-205].
Transplanted iNSCs successfully migrated to the site of injury and differentiated into neurons, astrocytes, and oligodendrocytes [202, 203], formed synapses, and functionally integrated into host neural circuitry [203]. However, most beneficial effects of iNSCs seem to be independent of iNSC survival, suggesting that iNSCs act by another means outside of cell replacement [202, 203]. Although the exact process is not thoroughly understood, iNSCs may serve a dual purpose by replacing lost cells and secreting regenerative paracrine factors that enhance endogenous tissue regeneration (e.g. VEGF, BDNF, GDNF). Proposed mechanisms include the exogenous iNSCs secreting the trophic factors themselves or stimulating endogenous protective pathways to decrease inflammation and promote neural regeneration, angiogenesis, and plasticity [202, 203]. This dual mechanism of iNSCs makes them a very promising candidate for stroke therapy to increase motor function recovery by both potentially replacing lost neural tissue and promoting neural plasticity to further drive spontaneous motor recovery.
CHAPTER 3
LARGE ANIMAL MODELS OF ISCHEMIC STROKE

For the past 30 years, there has been an extensive focus on neuroprotective strategies for ischemic stroke treatment. However, these efforts have yet to yield any approved clinical neuroprotective therapies [8, 14]. In an effort to bridge this significant disparity, the Stem Cells as an Emerging Paradigm in Stroke (STEPS) group have addressed concerns with the current lack of clinical translatability of animal stroke models and published several criteria to ensure the success of future stroke therapies in clinical trials. These published guidelines (STEPS I, II) included the recommendation of large animal model use. While rodent models of ischemic stroke are well established, the use of large animal models with comparable brain size and structure to that of humans may be crucial in translating stroke therapies to human patients [20].

Lissencephalic rodent brains lack the convolutions (gyri and sulci) characteristic of more complex species, such as humans, and comprise of mainly gray matter [206]. Abnormalities in gyrification in humans have been directly correlated to neurological diseases, suggesting that ischemic injury might affect the brain differently in the presence of gyrrification [207, 208]. The rodent brain also significantly differs in gray and white composition from the human brain. White matter is mostly composed of astrocytes, oligodendrocytes, and myelinated and unmyelinated axons, while gray matter consists of neuron cell bodies, dendrites, and synapses. These distinctions between white and gray matter composition lead to different responses to ischemic injury. The cellular composition of gray matter increases its vulnerability to ischemic damage due to the more rapid oxygen and glucose consumption occurring in this compartment of
the brain, leading to higher levels of damage and secondary injury relative to white matter. Ischemic injury in white matter can lead to the loss of axon myelination, ultimately resulting in impaired cognitive, motor, and sensory function [209]. Since white and gray matter react differently to ischemic injury, rodent brains with a small proportion of white matter may not accurately model an ischemic stroke in humans who have a significantly larger white to gray matter ratio [210-213]. In fact, most strokes in humans occur in white matter. While a vast majority of stroke studies utilize rodent models, it is clear that there are significant differences between the rodent brain and human brain. However, many large animal models of ischemic stroke with many similarities in brain size, anatomy, and physiology to that of humans currently exist, including dogs, cats, pigs, sheep, and non-human primates. Use of these large animal models for stroke therapies may increase translatability to clinical application.

**CANINE MODEL OF ISCHEMIC STROKE**

Although the true incidence of ischemic stroke in canines is unknown, strokes are identified more frequently in dogs than before due to the increased availability of magnetic resonance imaging (MRI) in veterinary medicine [214, 215]. Subsequently, canine models of ischemic stroke can be clinically relevant in the veterinary field in terms of diagnosis and potential therapeutic routes. With a gyrencephalic brain similar in composition to a human brain, dogs also serve as a more translatable model of ischemic stroke for humans than rodents [216, 217]. Several studies were able to establish highly reproducible and consistent canine ischemic stroke models in terms of lesion size and behavioral deficits [218-221]. These stroke-related deficits are similar to those observed in humans, varying from ipsilateral circling, head turning, contralateral blindness, contralateral ataxia, to proprioception deficits [217]. Additionally, dogs can be easily obtained and are relatively cheap in terms of cost and care, especially compared to
non-human primates. However, the cerebral arterial supply in dogs is extremely different from that in humans and non-human primates and can easily compensate for any cerebral artery occlusion, which may affect the translatability of a canine stroke model depending on the targeted area of occlusion [214]. The canine model of stroke may also garner significant controversy and ethical concerns over the use of a domesticated animal.

**FELINE MODEL OF ISCHEMIC STROKE**

Cats have gyrencephalic brains and are easily obtained and cheap, making them an attractive candidate for a translatable stroke model [222, 223]. However, their vascular anatomy prevents an endovascular approach for occlusion and forces a transorbital surgical approach, which increases the risk of complications post-surgery such as swelling and edema [224]. Although their brains are similar in anatomy and composition, feline brains are also fairly small compared to other large animals, like pigs or sheep. For stem cell studies, size plays an important role in determining number of cells to transplant. Very few stroke studies use cats, which might be due to the particular surgical route used for stroke induction and ethical issues.

**OVINE MODEL OF ISCHEMIC STROKE**

The use of sheep as an animal model for stroke provides major advantages as a tool to develop and evaluate novel stroke therapies for clinical translation. Sheep have a gyrencephalic brain, dense white matter tracts, strong fibrous dura mater and tentorium cerebelli, and similar neurovascular anatomy to that of humans (save for the presence of a complex of arteries called the rete mirabile) [225]. Sheep also exhibit similarities to non-human primates in hematologic parameters and blood grouping [226]. Reliable middle cerebral artery occlusion models have been established in sheep with fairly reproducible results in terms of lesion size and neurological impact, low mortality, and potential for longitudinal study uses [225, 226]. Clinical assessment
of motor function after MCAO revealed ipsilateral circling and delayed hemistanding and hopping reactions [226]. Sheep not only have larger brains than dogs and cats, which may prove useful for cell-based therapies in terms of cell transplantation numbers and ease of evaluation, but also avoid any public dissent or controversy. More recently, autologous bone marrow cell transplantation in a sheep model was found to reduce lesion extension, lymphocytic infiltration, and axonal degeneration [227]. A disadvantage of the ovine model is that sheep reach the age of maturation at 12 to 18 months, severely limiting the flexibility of this model [226]. The presence of the rete mirabile, a complex and dense network of blood vessels, in the skull also poses as a complication for surgical stroke induction as endovascular or embolic approaches cannot be used due to blood vessel collateralization within the rete mirabile and the inability to pass an embolus or catheter beyond it [226].

**PORCINE MODEL OF ISCHEMIC STROKE**

The pig brain greatly resembles the human brain in both anatomy and physiology, making it an excellent animal model of choice for ischemic injury [136]. Pig brains have a similar gyral pattern and distribution of gray and white matter to that of humans [135, 136]. Since cerebral white matter is mostly affected in stroke patients, it is crucial that models of translation have similar brain composition for the development of stroke therapies [210]. Because both human and pig brains comprise of more than 60% white matter, cellular responses to ischemic injury and subsequent recovery should be very similar [135]. The pig brain is only 7.5 times smaller than the human brain, making it more comparable in size than the rodent brain and very similar to non-human primate brains [137]. The similarities in size provide pigs an advantage for stem cell-based therapies since size greatly affects the number of cells for transplantation, ability of cell engraftment, sites of injection, and ease of evaluation. Robust pig
models of ischemic stroke have been established with consistent ischemic injury and functional deficits similar to human stroke patients [137-140]. Following stroke, pigs demonstrated unilateral deficits, including contralateral menace, ipsilateral circling, and proprioception and motor impairment. As in human stroke patients, pigs exhibited difficulty walking and weakness in the paretic limbs [137, 138]. Weakness in contralateral limbs manifest itself as prolonged stance time and shortened swing time in pigs, which is the opposite of human gait post-stroke; however, the inherent differences in gait patterns between quadrupeds and bipeds need to be considered in comparing motor deficits [138]. However, difficulty of housing and caring for such large animals is a significant disadvantage to the pig model. Like the sheep model, pigs also possess a rete mirabile in the skull, limiting stroke surgical approaches [137]. Overall, these findings prove that the use of a pig model for ischemic stroke has many advantages, particularly for evaluating changes in motor function after therapy.

**NON-HUMAN PRIMATE MODEL OF ISCHEMIC STROKE**

While variation in brain and vascular anatomy and physiology exist among non-human primate (NHP) species, most NHP brains are very similar to that of humans [206, 228]. Gyrencephalic primate species are generally large and possess brains with deep gray and white matter tracts, white and gray matter composition, and cerebral vascular anatomy closely resembling human brains [206, 228, 229]. Thresholds for ischemia and infarction are very similar between humans and monkeys, making NHP an ideal candidate for translational stroke studies [230]. Due to the variation among NHP species, considerations must be made in choosing a NHP that would most closely model ischemic stroke in humans. The baboon (*Papio anubis*) has been the most commonly used species for middle cerebral artery occlusion (MCAO) surgeries [231-236]. However, permanent MCAO stroke models in baboons resulted in severe
edema requiring intensive care and high risks of mortality [232]. Additionally, baboon cerebral vasculature has rich collateralization, making it significantly different from that of humans [234-236]. Alternatively, macaque monkeys, whose cortical and vascular anatomies are very similar to humans, have been used in permanent and transient MCAO models with success in modeling acute stroke injury [237-240]. Overall, stroke models of primates are quite varied in surgical approaches, though most involve occlusion of the MCA by some means, and have yielded very variable and inconsistent infarcts [240-242]. However, methods can be developed to very closely mimic ischemic stroke in human patients and also target specific areas of injury in the brain, such as the cortex [237]. Studies have shown that human NSCs can be safely transplanted in a monkey following MCAO and survive and differentiate but did not evaluate functional or motor changes [243]. However, there are considerable ethical issues surrounding primate research with an emphasis on potential physical and emotional discomfort and pain resulting from experimental stroke [244, 245]. The need for specialized housing and care, expertise, and research costs also pose as considerable challenges for primate models.

These large animal models previously discussed have their advantages and limitations as models of ischemic stroke. While non-human primates and humans are more closely related, studies using NHP need to be controlled and are costly in the amount of care and expertise needed. Dogs and cats are cheap and readily available but are considerably smaller in size than humans. Sheep and pigs pose as the more intermediate options and circumvent any ethical concerns associated with other animals. Previous pig studies have shown that changes in pig gait can be detected accurately with quantitative gait analysis and are comparable to gait deficits observed in humans after stroke, making the pig an excellent candidate for evaluating stem cell efficacy on motor function recovery.
CHAPTER 4
GAIT ANALYSIS IN PIG ISCHEMIC STROKE MODEL TO DETERMINE EFFICACY OF INSC THERAPY ON MOTOR FUNCTION RECOVERY

INTRODUCTION

Ischemic stroke is currently one of the leading causes of death and long-term disability worldwide [1-4]. More than 80% of stroke survivors suffer from severe motor function impairments, which significantly impacts mobility and gait [24-27]. Persisting gait abnormalities and lower limb weakness often leave stroke patients permanently disabled, requiring long-term care and adding to the profound health and socioeconomic burden on victims and their families [4, 28-30]. Since the main goal of most stroke patients is to regain normal walking function, gait improvement acts as an important parameter for stroke prognosis and treatment. Currently, the only FDA-approved therapies for ischemic stroke include tissue plasminogen activator (tPA) and a few mechanical thrombolysis devices with only a small minority of stroke patients able to receive them [12, 15-17]. Of the patients who do receive treatment, almost half remain disabled.

Efforts to develop new effective pharmacological treatments have failed. However, these treatments aim to only prevent further injury from ischemic stroke but cannot regenerate lost tissue. Interest in stem cell-based therapies has significantly increased due to their potential to not only act as a neuroprotectant, but to replace damaged tissue and neural networks and restore lost cognitive, sensory, and motor function. In particular, induced pluripotent stem cell (iPSC) derived neural stem cells (iNSCs) represent a novel class of autologous neural stem cells for
stroke therapy. Mature somatic cells can be reprogrammed into pluripotent cells and then differentiated into specific neuronal subtypes [191]. Rodent studies have found that human iNSCs can successfully differentiate into neurons, astrocytes, and oligodendrocytes and integrate into host neural circuitry, leading to reduced infarct and functional recovery [202-205]. Additionally, iNSCs were shown to secrete regenerative trophic factors, which promote endogenous neural regeneration, angiogenesis, and plasticity [202, 203]. These findings suggest that iNSCs are a promising avenue to explore for potential ischemic stroke treatment.

Though iNSC transplantation studies in rodents have yielded exciting results, inherent differences in brain anatomy and physiology of rodents and humans have garnered concern over therapeutic translatability of the rodent model. Due to the lissencephalic architecture and small proportion of white matter in the rodent brain, stem cell-based therapies need to be tested in a large animal model with brain size and architecture closer to humans [19, 20]. The pig brain closely resembles the human brain in anatomy, physiology, and size [136]. Robust pig models of ischemic stroke have been established with consistent ischemic injury and functional deficits similar to human stroke patients, making the pig a more predictive model for iNSC transplantation studies [137-140].

Quantitative gait analysis is commonly used in clinical applications to characterize motor control deficits in order to determine and evaluate appropriate therapy approaches, but use in preclinical animal model studies is essential to assessing the efficacy and safety of stem cell therapies for motor function recovery [134-137]. Gait analysis in rodent models has revealed significant deficits in multiple gait parameters following ischemic injury similar to gait changes observed in human patients including decreased cadence, walking velocity, and stride length [119-123]. More recently, gait analysis was performed in a porcine model to establish a baseline
for detecting abnormalities in pig gait after MCAO [138]. These studies have demonstrated that gait analysis is a valuable and highly sensitive tool for evaluating the potential of iNSC treatment to significantly improve functional recovery in a preclinical pig model.

In this study, we used a quantitative computational gait analysis system to investigate long-term effects of stroke and intraparenchymal saline injection in a porcine brain on gait parameters. We then transplanted induced pluripotent stem cell derived neural stem cells (iNSCs) into a pig MCAO stroke model and performed gait analysis to determine the effects of human iNSC (hiNSC) treatment on pig motor function for up to one week. We showed that stroked pigs exhibit gait deficits immediately after stroke and injection but almost fully recover at the end of 12 weeks. We also find that hiNSC transplantation may promote faster gait recovery.

MATERIALS AND METHODS

All work in this study was performed in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines.

Gait Study

Three adult male Landrace pigs were familiarized and trained to walk through a semi-circular track prior to data collection. Gait data was collected at multiple time points throughout the longitudinal 12-week study. Pigs were video recorded on three different dates one week prior to middle cerebral artery occlusion (MCAO) surgery. After stroke surgery, pigs were filmed at 1, 3, and 5 days post-stroke. At 5 days post-stroke, pigs received intraparenchymal injections of sterile
PBS. Pigs were then video recorded at 1 and 3 days post-injection and 1, 2, 4, 6, 9, and 12 weeks post-injection.

After assessing long-term safety of intraparenchymal injection, eight adult male Landrace pigs were trained to navigate the gait track used from the prior 12-week study. Data was collected at three time points prior to stroke induction. All pigs then underwent MCAO surgery. At 1 day post-stroke, pigs received either hiNSC or vehicle-only injection for treatment and control groups, respectively. Pigs were then recorded at 3 and 7 days post-stroke.

Stride duration, swing time, stance time, individual limb two-limb support, individual limb three-limb support, total two-limb support, total three-limb support, and breakover were analyzed for changes in gait after stroke and injection for all pigs.

**Gait Analysis**

**Video Collection**

Gait analysis videos were collected at multiple time points throughout the 12-week and 1-week studies. One week prior to data collection, adult male Landrace pigs were trained to navigate through a semicircular track (4 m in diameter) into a straight recording chute (0.6 m in width with a 2.4 m prerecording distance, 2.4 m recording frame, and 1.7 m postrecording distance). Pigs were encouraged to walk through the track clockwise by a handler walking behind them with a noisemaker. Two synchronized high-speed GigEye Ethernet cameras (IDS Imaging Development Systems, Obersulm, Germany) were positioned 3 m from either side of the recording chute at a height of 24 cm to capture (70 frames per second) the left and right profile of the pigs as they walked through the 2.4 m recording frame. Pigs were timed with electrical
timers (Farmtek, Wylie, TX) for each recording until 5 repetitions were collected. The mean was calculated for 5 repetitions to eliminate any repetition falling outside of 10% on either side of the mean to ensure gait consistency. Pigs were recorded until 5 useable repetitions were achieved.

*Video Analysis*

Gait videos were recorded utilizing the program, EquineTec (Monroe, GA). Forelimbs and hindlimbs of the pigs were analyzed with the video analysis software, Kinovea, for the following temporal gait parameters: swing time (frame where hoof first leaves the ground to frame where hoof first touches the ground); stance time (frame where hoof first touches the ground to frame where hoof first leaves the ground); stride duration (frame where hoof first leaves to ground to frame where hoof leaves the ground again); individual limb two-limb support (amount of time animal spends on two limbs, from frame where hoof first touches the ground to frame where hoof first leaves the ground); individual limb three-limb support (amount of time animal spends on three limbs, from frame where hoof first touches the ground to frame where hoof first leaves the ground); total two-limb support (amount of time animal spends on two limbs, from frame where hoof first touches the ground to frame where hoof touches the ground again); total three-limb support (amount of time animal spends on three limbs, from frame where hoof first touches the ground to frame where hoof touches the ground again); and breakover (frame where heel leaves the ground to frame where toe leaves the ground).

*Middle Cerebral Artery Occlusion Surgery for Ischemic Stroke Induction*

A right-sided middle cerebral artery occlusion (MCAO) surgery was utilized to induce an ischemic stroke in the pigs, as established in previous studies [137-138]. Briefly, a fronto-temporal craniectomy with orbital rim osteotomy was performed to expose the MCA distal to the
Circle of Willis. Following an incision in the dura mater, the MCA was permanently occluded with bipolar electrocautery forceps, leading to blood flow loss in areas of the frontal, parietal, and temporal lobes of the brain.

**Intraparenchymal Transplantation**

**Saline Injection: 12-week Study**

Pigs (n = 3) received intraparenchymal injection of sterile PBS at 5 days after MCAO. Injection was performed with a specialized large animal stereotaxic frame and a mounted Quintessential stereotaxic injector system (David Kopf Instruments). A craniotome or air drill was utilized to ensure chosen injection sites were accessible. Injection sites were determined using MRI data at 1 day post-stroke to identify and target the penumbra region, which may allow for greater survival and engraftment of cells in future cell transplantation studies. Because of the large area of the penumbra region, sterile saline (33.3 μL) was injected into four different sites: two white matter and two gray matter areas.

**Cell Transplantation: 1-week Study**

Intraparenchymal transplantation of DiR-labeled human iNSCs (GlobalStem, Gaithersburg, MD) or sterile PBS was performed stereotaxically (as described previously for saline injections) at 24 hours after MCAO in treatment (n = 4) and control (n = 4) pigs, respectively. On the day of transplantation, 5x10^6 hiNSCs were resuspended in 33.3 μL of sterile PBS at a concentration of 150,000 cells/μL per injection. For each pig, two injections were made in a region of gray matter and a region of white matter, 5 mm apart.
Statistical Analysis

Data was analyzed using PROC GLM with SAS Version 9.3 (Cary, NC) using time, treatment and side of the animal (e.g., left and right) as variables. Treatment * time, treatment * side, time * side, and treatment * time * side interactions were run to determine significance at each time point. Stride duration was calculated by adding swing and stance time. Swing and stance time was measured as the percentage of time spent in swing phase in one stride cycle calculated by dividing swing time by stride duration. Limb support phases were measured as the percentage of time spent in two-limb support in one stride cycle. Data was adjusted to eliminate any individual variation between pigs to allow for direct comparisons. Adjustments were presented as a percent of change from pre-stroke values at each time point. For example, to determine the percentage of change of swing time at 1 day post-stroke from pre-stroke, the calculations would be as follows:

\[
\frac{\text{Mean swing time (1d Post)} - \text{mean swing time (Pre)}}{\text{Mean swing time (Pre)}} \times 100
\]

For all comparisons, significance was reported at \( P \leq 0.05 \).

RESULTS

Stroked pigs show significant gait impairments early after MCAO and transplantation

Three pigs underwent MCAO surgery for ischemic stroke induction. Five days after stroke, pigs received intraparenchymal saline injection. Significant gait deficits were observed in all parameters immediately after stroke and transplantation. The average of left and right limbs for all parameters was used for all comparisons. Due to the variability in gait among the pigs,
analysis was also presented as a percentage of change from pre-stroke gait for more accurate comparisons. Stride duration increased by more than 50% at 1 day post-stroke compared to pre-stroke stride duration in forelimbs and hindlimbs (Fig. 1A, B; Fig. 2A, B). Significantly longer stride duration was still observed at 3 and 5 days post-stroke for front and hind limbs (Fig. 1A, B). Immediately after intraparenchymal saline injection, front and hind stride duration was nearly doubled the pre-stroke time and longer than at 1 day after MCAO (Fig. 1A, B; Fig. 2A, B). This increase from 5 days post-stroke prior to injection and 1 day post-injection was significant (Fig. 2A, B). Pigs exhibited significantly longer stride duration up to 6 weeks post-injection; at 9 and 12 weeks post-injection, stride duration returned to pre-stroke time (Fig. 2A, B).

Stance and swing times were calculated as a percentage of swing time in one stride cycle and presented as a percentage of change from pre-stroke swing time. Prior to stroke, pigs spent almost an equal amount of time alternating between swing and stance phase in one stride cycle for both front and hindlimbs (Fig. 1C, D). After stroke, a significant decrease in the percent of time spent in swing phase in a stride cycle was observed in both forelimbs and hindlimbs, indicating that pigs prolonged their stance time (Fig. 2C, D). At 1 day post-stroke, swing time in the stride cycle decreased by more than 30% compared to pre-stroke. Immediately after injection, swing time in the stride cycle decreased by more than 40% from pre-stroke time in front and hindlimbs (Fig. 2C, D). Hindlimb swing time returned to pre-stroke time by 2 weeks post-injection, earlier than forelimb swing time (Fig. 2C, D). In both forelimbs and hindlimbs, swing time returned to normal at 9 and 12 weeks post-injection with pigs spending more time in swing phase than at pre-stroke (Fig. 2C, D).

At 1 day post-stroke, breakover time increased by nearly 100% and more than 100% in forelimbs and hindlimbs respectively, compared to pre-stroke times (Fig. 2E, F). Front breakover
time was only significantly increased at 1 day post-stroke and 1 and 3 days post-injection and returned to pre-stroke time by 1 week post-injection (Fig. 1E, 2E). Hind breakover time was increased at 1, 3, and 5 days post-stroke and 1 and 3 days post-injection, returning to normal by 1 week post-injection (Fig. 1F, 2F).

Pre-stroke, pigs spent more than 50% of their stride cycle in individual limb two-limb support phase in forelimbs and hindlimbs (Fig. 1G, H). Immediately after MCAO surgery, two-limb support of the stride cycle decreases by more than 50% compared to pre-stroke time (Fig. 2G, 2H). After injection, two-limb support time of the stride cycle decreased by more than 70% compared to pre-stroke in both front and hindlimbs (Fig. 2G, H). By 4 weeks post-injection, individual two-limb support time of the stride cycle returned to normal in both forelimbs and hindlimbs (Fig. 2G, H).

These results indicate that MCAO surgery and transplantation led to immediate gait impairments for all parameters. The intraparenchymal injection method had a minimal, short-term effect on motor function. By 9 weeks post-injection, pig gait returned to pre-stroke baseline for all parameters. This data shows that while stroke induces motor function deficits, pigs experienced a large amount of spontaneous recovery with no detectable differences in gait parameters observed by the end of 12 weeks.

**hiNSC transplantation led to faster recovery in gait parameters one week after stroke**

Ischemic stroke was induced in eight pigs by MCAO surgery. At 1 day post-stroke, pigs received hiNSC (n = 4) or vehicle-only injection (n = 4) into the stroke-damaged brain. In both control and hiNSC-treated groups, front stride duration time was not significantly different from pre-stroke stride duration after stroke and transplantation (Fig. 3A). No significant differences in
average front stride duration were observed between both groups at all time points (Fig. 3A). Overall, hiNSC-treated pigs display longer front stride duration times than control pigs. However, when data is adjusted to account for individual variation between pig groups, control pigs show a significant 40% increase in front stride duration from pre-stroke time at 3 days post-stroke (Fig. 4A). Front stride duration in hiNSC-treated pigs increased by about 20% after stroke, but this increase was not significantly different from pre-stroke time (Fig. 4A).

Significant differences were observed in hind stride duration for both control and hiNSC-treated pigs after stroke, where hiNSC-treated pigs had a longer stride duration time than control pigs at 3 days post-stroke (Fig. 3B). hiNSC-treated pigs exhibited a significant increase in hind stride duration compared to pre-stroke up to one week after MCAO, while control pigs returned to almost normal pre-stroke time at 7 days post-stroke (Fig. 3B). Significant changes in hind stride duration were also observed in adjusted data. Although hiNSC-treated pigs had longer average hind stride duration time than control pigs, hiNSC-treated pigs had less of an increase in stride duration after stroke compared to pre-stroke stride duration at 3 days post-stroke. At 3 days post-stroke, stride duration was significantly increased by more than 40% and by more than 20% compared to pre-stroke time in control and hiNSC-treated pigs respectively (Fig. 4B).

No significant differences were observed in front and hind swing time for both control and iNSC-treated pigs after stroke (Fig. 3C, D; Fig. 4C, D). Overall, hiNSC-treated pigs had a greater decrease in swing time at 3 and 7 days post-stroke compared to control pigs (Fig. 4C, D).

Pre-stroke, both pig groups spent most of their stride cycle in two-limb support phase in forelimbs and hindlimbs (Fig. 3E, F, Fig. 4E, F). At 3 days post-stroke, control and hiNSC-treated pigs spent significantly less amount of time in two-limb support for front and hindlimbs compared to pre-stroke (Fig. 3E, F). At the end of one week, front and hind two-limb support
time returned to normal in control pigs, while average front and hind two-limb support times for hiNSC pigs were still significantly decreased (Fig. 3E, F). Significant differences were also observed in forelimbs at 7 days post-stroke and hindlimbs at 3 and 7 days post-stroke between two groups with hiNSC-treated pigs exhibiting significantly decreased two-limb support time (Fig. 3E, F). However, control pigs had a significant 30% decrease in front and hind two-limb support time at 3 days post-stroke (Fig. 4E, F). Overall, hiNSC-treated pigs showed a smaller amount of change in two-limb support time from before stroke compared to control pigs (Fig. 4E, F). Together, these results show that hiNSC transplantation prevented significant changes in two-limb support time and stride duration in stroked pigs relative to vehicle-only control pigs.

Pig gait remains symmetric after MCAO and transplantation

Left and right sides of forelimbs and hindlimbs were analyzed and compared at each time point for swing and stance time, limb support phase time, and breakover time symmetry in all pigs. Prior to stroke, no statistical differences were observed between the left and right limbs, indicating that normal pig gait is symmetric. Swing and stance time, limb support phase time, and breakover time remained symmetric in left and right limbs at all time points following stroke and transplantation. These results reveal that stroke surgery and transplantation did not impact gait symmetry in all parameters measured (see Appendix).

While more gait impairments were detected in hiNSC-treated pigs than the control pig group when comparing unadjusted data, accounting for individual variation in gait revealed that iNSC-treated pigs had less impairments in most gait parameters relative to control pigs. Overall, this data suggests that iNSC transplantation prevented significant changes in gait parameters.
DISCUSSION

In this study, we demonstrated that the intraparenchymal injection method has minimal, short-term effects on motor function and that intraparenchymal hiNSC transplantation can lead to faster motor function recovery in temporal gait parameters in a pig ischemic stroke model. Quantitative gait analysis showed an overall increase in stride duration, decrease in swing time, increase in stance time, decrease in two-limb support time, increase in three-limb support time, and increase in breakover time in all limbs after MCAO surgery. Generally, gait changes observed at 1 day post-injection were worse than at 1 day post-stroke; however, it is important to note that intraparenchymal injection is a fairly invasive method of cell delivery and requires reopening of the surgical cite after only five days post-surgery. In our longitudinal study, pigs exhibited gait impairments early after stroke and injection but began undergoing significant spontaneous recovery by 6 weeks and nearing full recovery by the end of 12 weeks. Human stroke patients often experience dramatic motor function recovery within the first month without any rehabilitative interventions [61]. Several animal models have shown that this spontaneous recovery may be due to significant structural changes and cortex remodeling following cerebral injury in the motor cortex of the mammalian brain; in other words, the brain is plastic [62-64]. Developmental proteins that are typically absent in the adult brain modulate this plastic response in the brain after ischemic injury and remain expressed for weeks or months following stroke [64]. Many of these proteins include trophic factors involved in neuronal growth, apoptosis, angiogenesis, and cellular differentiation [65-71]. Although cortex reorganization can occur naturally in response to stroke, task-oriented rehabilitative training can increase this functional reorganization. The spontaneous recovery observed in our study may indicate that pig brains also undergo this cortex remodeling following ischemic stroke as well.
Stroke often results in unilateral deficits leading to asymmetric gait in humans. Limb-specific differences observed in spatiotemporal parameters usually confirm asymmetric gait [100, 101]. Stroke patients typically exhibit prolonged swing phase and shortened single-limb support time in the paretic limb [100-104]. Our results show that our pigs exhibit the opposite gait pattern after stroke with a longer stance phase time and shortened swing phase time. These findings correlate with a prior gait analysis study in stroked pigs, where pigs displayed increased stance time and decreased swing time on the contralateral limb [138]. However, direct comparisons cannot be made between humans and pigs as quadruped and bipeds have inherently different gait patterns. In other gait studies observing the effect of soiled flooring on pig gait, pigs made compensatory adjustments in their gait by prolonging stance time and employing three-limb support. Similar gait patterns were observed in our study following stroke, where stroked pigs spent an increased amount of time in three-limb support. Similarly, stroke patients typically experience increased double-limb support duration [103]. In this study, we also observed breakover time in our pigs. Immediately following stroke, pigs had prolonged breakover time in all limbs. While breakover time is not a parameter typically measured in stroke studies, breakover time is often used in horses to characterize uneven or asymmetric feet, which often lead to the development of lameness [246]. In horses, prolonged breakover time is associated with uneven hooves, where hoof lands toe first, shortening gait and increasing shock absorption. Surprisingly, no differences were observed in left and right sides for all gait parameters in all pigs from this study, suggesting that stroke had no effect on gait symmetry. The previous study in pigs found significant asymmetries in spatiotemporal parameters, including reduced hind swing and decreased hind maximum hoof height [138]. Our surgical approach to stroke induction typically causes lesions in areas of the frontal, parietal, and temporal lobes of
the brain. Variability in lesion size and location may have contributed to the fairly symmetric gait and spontaneous recovery. While stroke affecting the frontal and parietal lobes in humans usually results in motor function deficits in humans, some studies have suggested that the pig motor cortex does not play a large role in motor control of limbs [246].

Our data revealed promising implications regarding hiNSC therapy in a pig stroke model. Although more gait impairments were observed in the hiNSC-treated pig group than in the control group, our gait analysis measurements did not account for individual variation between pigs. After making adjustments to compare the amount of change from pre-stroke gait baseline between groups, we found that hiNSC-treated pigs had faster motor function recovery compared to control pigs. Previous rodent studies have shown that iNSCs may serve a dual purpose by both replacing lost cells and secreting regenerative paracrine factors that promote endogenous tissue regeneration, angiogenesis, and plasticity while reducing inflammation [202, 203]. The spontaneous recovery observed in the pigs without hiNSC treatment suggests that there is an increase in plasticity and subsequent cortex remodeling in the pig brain following ischemic stroke. hiNSC transplantation may have modulated this natural recovery and further increased gait recovery by secreting trophic factors to promote neuroplasticity. However, this data is not conclusive, as our study only used temporal gait parameters to determine the efficacy of hiNSC transplantation. Our findings need to be further correlated with infarct area and lesion sizes from MRI analysis. Additionally, a long-term study in a pig MCAO model is critical in order to distinguish between the role iNSCs might play in motor function recovery and endogenous healing.
CONCLUSION

Middle cerebral artery occlusion (MCAO) in pigs led to significant impairments in several temporal gait parameters, including increased stride duration, increased stance time, decreased swing time, increased three-limb support time, decreased two-limb support time, and increased breakover time in all limbs. However, pigs exhibited spontaneous recovery by the end of 12 weeks without hiNSC treatment. Pigs receiving hiNSC transplantation had faster motor function recovery than control pigs after one week. Results of this study will help bridge the translatability gap between preclinical models and clinical trials and bring researchers closer to developing successful cell-based therapies for stroke. However, further studies in pigs must be conducted in order to determine long-term effects of iNSC treatment on motor function recovery.

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Figure 1

A) Front Stride Duration

B) Hind Stride Duration

C) Front Swing Time

D) Hind Swing Time

E) Front Breakover

F) Hind Breakover

G) Front 2-Limb Support

H) Hind 2-Limb Support
**Figure 1: Stroke results in significant deficits in temporal gait parameters.** Front stride duration was significantly increased at 1, 3, and 5 days post-stroke, 1 and 3 days post-injection, and 1, 2, and 6 weeks post-injection compared to pre-stroke (A); hind stride duration was significantly increased at 1, 3, and 5 days post-stroke, 1 and 3 days post-injection, and 1 and 6 weeks post-injection compared to pre-stroke (B); front swing time was significantly decreased at 1, 3, and 5 days post-stroke, 1 and 3 days post-injection, and 1, 2, and 6 weeks post-injection compared to pre-stroke (C); hind swing time was significantly decreased at 1, 3, and 5 days post-stroke, 1 and 3 days post-injection, and 1 week post-injection compared to pre-stroke (D); front breakover time was significantly increased at 1 day post-stroke and 1 day post-injection compared to pre-stroke (E); hind breakover time was significantly increased at 1, 3, and 5 days post-stroke and 1 and 3 days post-injection compared to pre-stroke (F); front two-limb support was significantly decreased at 1, 3, and 5 days post-stroke, 1 and 3 days post-injection, and 1 and 2 weeks post-injection compared to pre-stroke (G). * indicates significant difference compared to pre-stroke at p<0.05.
Figure 2

A. Front Stride Duration
B. Hind Stride Duration
C. Front Swing Time
D. Hind Swing Time
E. Front Breakover
F. Hind Breakover
G. Front 2-Limb Support
H. Hind 2-Limb Support
Figure 2: Significant gait recovery is observed by 9 and 12 weeks post-stroke. Front stride duration recovered to pre-stroke time at 4, 9, and 12 weeks post-injection (A); hind stride duration recovered to pre-stroke time at 2, 4, 9, and 12 weeks post-injection (B); front swing time recovered to pre-stroke time at 4, 9, and 12 weeks post-injection (C); hind swing time recovered to pre-stroke time by 2 weeks post-injection (D); front breakover time recovered to pre-stroke time at 3 and 5 days post-stroke and 1, 2, 4, 6, 9, and 12 weeks post-injection (E); hind breakover time recovered to pre-stroke time by 1 week post-injection (F); front two-limb support time recovered to pre-stroke time by 4 weeks post-injection (G); hind two-limb support recovered to pre-stroke time by 4 weeks post-injection (H). * indicates significant difference compared to pre-stroke; # indicates significant difference compared to 5 days post-stroke at p<0.05.
Figure 3

A. Front Stride Duration

B. Hind Stride Duration

C. Front Swing Time

D. Hind Swing Time

E. Front 2-Limb Support

F. Hind 2-Limb Support
Figure 3: hiNSC-treated pigs exhibit significantly more gait deficits relative to control pigs. No significant changes in front stride duration were observed after stroke (A); in control and hiNSC-treated pigs, hind stride duration significantly increased at 3 days post-stroke. hiNSC-treated pigs had a longer stride duration than control pigs (B); no significant changes in front swing time were observed after stroke (C); in control and hiNSC-treated pigs, hind swing time significantly decreased at 3 days post-stroke. hiNSC-treated pigs still had significantly decreased swing time at 7 days (D); in control and hiNSC-treated pigs, front two-limb support time was significantly decreased at 3 days. hiNSC-treated pigs had significantly decreased two-limb support time at 7 days (E); in control and hiNSC-treated pigs, hind two-limb support time was significantly decreased at 3 days. hiNSC-treated pigs had decreased two-limb support time at 7 days and was significantly different from control pigs at 3 and 7 days (F). * indicates significant difference compared to pre-stroke in control pigs; # indicates significant difference compared to pre-stroke in hiNSC-treated pigs; $ indicates significant difference between control and hiNSC-treated pigs at time point at p<0.05.
Figure 4

A. Front Stride Duration

B. Hind Stride Duration

C. Front Swing Time

D. Hind Swing Time

E. Front 2-Limb Support

F. Hind 2-Limb Support
Figure 4: hiNSC-treated pigs exhibit greater functional recovery in hindlimb gait parameters relative to control pigs. At 3 days post-stroke, control pigs had a significant increase in stride duration from pre-stroke time (A); at 3 days post-stroke, both control and hiNSC-treated pigs had significant increases in hind stride duration from pre-stroke time (B); no significant changes were observed for front swing time (C) and hind swing time (D); control pigs had a significant increase in front two-limb support at 3 days post-stroke (E); control pigs had a significant increase in hind two-limb support at 3 days post-stroke (F). * indicates significant difference compared to pre-stroke in control pigs; # indicates significant difference compared to pre-stroke in hiNSC-treated pigs; $ indicates significant difference between control and hiNSC-treated pigs at time point at p<0.05.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Most stroke victims experience persisting abnormalities in gait and walking function following ischemic injury. Despite intensive rehabilitative efforts, 35% of all stroke survivors never regain normal walking function. Our results demonstrated the potential of using an ischemic stroke pig model to test the efficacy of iNSC therapy in motor function recovery. We found that iNSC transplantation may have resulted in greater motor function recovery. However, further exploration of iNSC treatment in a pig ischemic stroke model is critical. From our 12-week study, we found that pigs exhibited motor function recovery without iNSC transplantation. Because our study only observed the effects of iNSC transplantation up to one week, we cannot eliminate any confounding variables, such as spontaneous recovery and healing, which may have contributed to the recovery observed in our iNSC-treated pigs. In order to address this issue, studies evaluating long-term effects of iNSCs in a pig stroke model will need to be conducted.

We also need to consider why our pigs seem to regain almost normal motor function by the end of 12 weeks without any treatment. Our surgical approach to stroke induction has been shown to cause lesions spanning the frontal, parietal, and temporal lobes of the brain. Due to the potential variation of infarct area and size, pigs may have received little to no damage to the motor cortex, resulting in spontaneous recovery. Studies have also suggested that the motor cortex mostly controls the snout in pigs, rather than limbs, and that the brainstem plays a crucial role in motor function instead [247]. While the pig brain greatly resembles that of a human brain in anatomy and physiology, specific methods need to be developed in order to more closely
mimic motor function deficits resulting from stroke in human patients. While computer-based quantitative gait analysis approaches have been proven to be sensitive tools for analyzing changes in gait after stroke, these methods can be tedious and result in inherent human variability in analysis. Automated quantitative systems, such as CatWalk, have been successfully used in rodent models of neurological disorders, including stroke [120, 124, 126-132]. Use of a similar automated system in our pig model would allow for quick and accurate measurements of several biomechanical gait parameters. Another limitation of our study lies in the gait parameters we chose to measure. For our study, we only analyzed temporal gait parameters. Clinical stroke studies not only measure spatiotemporal parameters but also utilize kinematics to assess joint angles and kinetic approaches of gait analysis. Additionally, our approach to quantitative gait analysis involved pigs walking at their own self-chosen gait, which may act as a confounding variable in our results. Although we excluded trials with inconsistent gait, using a treadmill to ensure consistent speeds in each trial and among pigs can ensure reliable and accurate gait analysis.

With the huge incidence of stroke and very few effective treatments available, it is crucial to develop more translatable animal models of stroke to test more efficient therapies. Large animal models, such as the pig, can help bring stem cell-based therapies from preclinical studies to clinical applications by increasing motor and functional recovery after stroke and subsequently improving the quality of life of stroke patients and their families.
REFERENCES


A. Gait Symmetry in 12-Week Pigs

A. Front Stride Duration

B. Hind Stride Duration

C. Front Swing Time

D. Hind Swing Time

E. Front 2-Limb Support

F. Hind 2-Limb Support

G. Front Breakover

H. Hind Breakover
B. Gait Symmetry in Control Pigs

A. Front Stride Duration

B. Hind Stride Duration

C. Front Swing Time

D. Hind Swing Time

E. Front 2-Limb Support

F. Hind 2-Limb Support
C. Gait Symmetry in iNSC-Treated Pigs

Front Stride Duration

Hind Stride Duration

Front Swing Time

Hind Swing Time

Front 2-Limb Support

Hind 2-Limb Support