Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Juanmarco Gutierrez Date

Preclinical Validation of Multilevel Intraspinal Stem Cell Therapy for Amyotrophic Lateral Sclerosis (ALS)

By

Juanmarco Gutierrez
Master of Science

Clinical Research

_____
Nicholas M. Boulis, M.D.
Advisor

_____
Henry M. Blumberg, M.D.
Committee Member

_____
Mitchel Klein, Ph.D.
Committee Member

_____
John E. McGowan, M.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Preclinical Validation of Multilevel Intraspinal Stem Cell Therapy for Amyotrophic Lateral Sclerosis (ALS)

By

Juanmarco Gutierrez
M.D., Universidad Anahuac, Mexico, 2010

Advisor: Nicholas M. Boulis, M.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Clinical Research
2014

Abstract

Preclinical Validation of Multilevel Intraspinal Stem Cell Therapy for Amyotrophic Lateral Sclerosis (ALS)
By Juanmarco Gutierrez

Background: Amyotrophic Lateral Sclerosis (ALS) is a fatal and relentlessly progressive neurodegenerative disease with a median survival after symptom onset that ranges from 2 to 5 years. The only approved treatment, riluzole, prolongs this survival by a matter of months. Cell therapies for ALS attempt to restore motor function through replacement of neuronal and non-neuronal cells. Multiple clinical trials using this approach are now underway in many countries around the world. The current study tested the spinal cord's tolerance to increasing volumes and numbers of injections in Gottingen minipigs.

Methods: Twenty-five female minipigs received human neural progenitor cell injections using a stereotactic platform device developed by the Emory group. Cell transplantation in groups 1 to 5 (n = 5 pigs each) was undertaken with the intent of assessing the safety of an injection volume escalation (10, 25, and 50 microL) and an injection number escalation (20, 30 and 40 injections). Sensory and motor function, as well as general morbidity was assessed for 21 days. Full necropsy was performed; spinal cords were analyzed for graft survival and microscopic tissue damage.

Results: No mortality or permanent surgical complications were observed within the 21-day study period. All animals returned to preoperative baseline within 14 days, showing complete motor function recovery. The histological analysis of the tissue showed that there was no significant decrease in neuronal density between groups and the engraftment percentage ranged from 11-31% depending on the injection paradigm. However, significant tissue damage was identified when injecting high volumes into the spinal cord (> 25 microL).

Conclusion: This series supports the functional safety of various injection volumes and numbers in the spinal cord, and gives critical insight to important safety thresholds. The results from this study are relevant to all translational programs delivering cell therapeutics to the spinal cord.

Preclinical Validation of Multilevel Intraspinal Stem Cell Therapy for Amyotrophic Lateral Sclerosis (ALS)

By

Juanmarco Gutierrez
M.D., Universidad Anahuac, Mexico, 2010

Advisor: Nicholas M. Boulis, M.D.

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Clinical Research
2014

Acknowledgements

This work is dedicated to both my parents and siblings who have given me unconditional support throughout this long, and tough yet joyful journey that I embarked many years ago.

Thanks to Ceci for being a special company throughout the last 2 years of this journey and for always being there to support me, this work is dedicated to her as well.

Thanks to my mentors Nicholas M. Boulis, Carlos Del Rio and Thais Buchman for guiding me and supporting me throughout the process.

Thanks to my thesis advisors Mitch Klein and John McGowan for giving me useful feedback and comments throughout the darkest times of this process.

Thanks Eleanor Barber for reading this thesis work a thousand times and always giving me great comments.

Thanks Jason Lamanna, Natalia Grin, Jonathan Riley, Rebecca Shapiro and Lindsey Urquia for being the best teammates throughout this research study and for supporting me.

Thanks to all the Boulis Lab members for always being open to help me with my work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a fatal and relentlessly progressive neurodegenerative disease that involves death of upper motor neurons (UMNs) and lower motor neurons (LMNs). Death occurs a couple of years after diagnosis, usually due to the progressive motor weakness that inevitably leads to respiratory failure. The only Food and Drug Administration (FDA) approved treatment for this condition, riluzole, increases the survival only by a matter of months (1). Thus, the development of effective treatment options becomes a clear and urgent need.

Stem cell transplantation represents a promising approach for the treatment of ALS, but many issues have to be addressed in order to successfully translate this therapy into a clinical setting. Although, multiple clinical trials are currently underway using this approach to treat ALS, there are still many gaps in our knowledge (2). These gaps include: understanding the maximum volume and number of stem cell injections tolerated by the spinal cord, understanding the immune response to cell transplantation in the spinal cord, optimizing immunosuppression treatment to minimize transplant rejection, and developing cell tracking methods that could enable clinicians to assess therapeutic efficacy and clinical outcomes in vivo. This thesis work will test the hypothesis that there is a threshold (toxic dose) for morbidity in terms of increasing volume and number of stem cell injections in the spinal cord of Gottingen minipigs. The results from the present work will be relevant to all of the translational programs attempting to deliver cell therapies to the spinal cord.

BACKGROUND

ALS also known as Lou Gehrig's disease, was first described by Jean-Marie Charcot in 1874 and it is the most common motor neuron (MN) disease (3). ALS has a reported incidence of between 1.5 and 2.6 per 100,000 person/years among Caucasian populations in Europe and North America. The prevalence in men is slightly higher when compared to women, recent population based studies in Europe report a male to female ratio ranging from 1.2-1.5:1 respectively. The individual's risk of ALS increases after the age of 40 years, peaking in the late sixties or early seventies, followed by a rapid decline (4). The disease is characterized by a progressive degeneration of the MNs that supply voluntary muscles, including UMNs in the cerebral cortex and LMNs in the spinal cord. This degeneration translates clinically into progressive motor weakness that leads to paralysis and ultimately to death, usually from respiratory failure (5).

The diagnosis of ALS, which depends on progressive UMN and LMN findings by history and examination, is accurate 95% of the time when made by an experienced clinician. Exclusion of differential diagnoses is made using imaging techniques and/or neurophysiology studies. The revised El Escorial diagnostic criteria help clinicians standardize the diagnosis (Table 1). Patients are classified according to the number of affected body regions: bulbar, cervical, thoracic and/or lumbosacral (4, 6). The Awaji algorithm may further improve the diagnostic sensitivity in patients with bulbar onset in whom limb motor deficits can be subtle (7). ALS constitutes a disease with highly variable clinical features and poor ability to predict prognosis, median survival after

diagnosis ranges form 2 to 5 years (5, 8). Riluzole, the only approved pharmacological treatment for ALS, has shown limited efficacy, prolonging the median survival of patients by only 2 to 3 months (9).

ALS is considered to be sporadic in most cases (90-95%). Mutations in four genes (C9ORF72, SOD1, TARDBP, and FUS/TLS) account for over 50% of familiar ALS (fALS) cases (10, 11). Some of these genes are known to alter the onset, severity or progression of the disease (12). fALS is predominantly hereditary and almost always through an autosomal-dominant inheritance pattern; X-linked or autosomal-recessive inheritance patterns are rare (13). Despite the available hypotheses about the etiology of this disease, over the last few years the question of whether ALS is a single disease with variable phenotypic expression or different diseases with heterogeneous causes has represented a matter of extensive debate (14). In some diseases the interaction between genetic background and environmental exposures contribute to disease susceptibility. It is not completely clear if an individual's risk of ALS increases with age, however it is the only identified non-genetic risk factor (15).

Even with the discovery of these genetic causes of fALS, the pathogenesis of the disease is not fully understood. Accumulation of disease causing mutant proteins and the neuroinflammatory reaction caused by activated glial cells are two common characteristics of many neurodegenerative diseases, such as ALS, Alzheimer's and Parkinson's. In ALS, dysfunction in RNA processing (e.g. expanded hexanucleotide repeat in the C9ORF72) and protein homeostasis (e.g. TDP-43 aggregates) are some of

the latest emerging themes (16). The current thinking is that disturbed natural protein homeostasis directly induces cellular stress that gives rise to axonal retraction, which leads to cell death by interfering with essential intracellular functions. Additionally, activated glial cells have been proven to contribute to MN death (Figure 1) (17). For more details of the underlying intracellular mechanisms of disease please refer to the latest comprehensive review published by Robberecht and Philips (13). The discovery of these underlying mechanisms of pathogenesis and the lack of an effective therapy for ALS provides a unique atmosphere for the discovery of new pharmacological and non-pharmacological therapeutics.

The development of cell therapies as treatment alternatives in conditions such as cancer and stroke has served as the scientific basis for the development of novel therapeutic strategies that could potentially be effective for various human neurological diseases (18, 19). Stem cells are defined by their capability to differentiate into several cells types, as well as the ability to maintain a self-renewing population (20). The various types of stem cells differ mainly in their intrinsic differentiation capabilities and source of origin. Pluripotent stem cells (pSC), such as embryonic stem cells (eSC), have the ability of differentiating into cells from all the three germ layers. In contrast, multipotent stem cells (mSC), such as neural progenitors (NPs) or adult stem cells, are inherently limited to differentiate into cells from the lineages from which they were derived. In the past decade or so, neurons and other glial cells (e.g. astrocytes and oligodendrocytes) have been successfully generated from various types of stem cells (21, 22).

Most diseases or injuries affecting the spinal cord have a poor prognosis, not only because of the nature of the disease itself but also because of the challenge that exists in developing and delivering new therapeutics to the spinal cord. When developing a cell therapy for ALS, it is of utmost importance to consider the underlying mechanisms of disease, as well as the delicate anatomy and physiology of the spinal cord (23). Due to the complexity of the underlying mechanisms of neurodegeneration in ALS, the development of a stem cell therapy should be aimed to preserve or restore lost motor function, and attenuate toxicity in the spinal cord. Stem cells and derived cells can be used to replace the lost of neurons, MNs and glial cells, such as astrocytes and oligodendrocytes. Furthermore, these cells can be engineered to secrete cytokines and growth factors that promote neuroprotection and promote cell regeneration (24). Recent studies using embryonic stem cells for motor neuron replacement have been able to successfully restore motor function in rodent models of ALS and spinal cord injury (25-28). However, it is impractical to attempt to translate it into the human because grafted neurons must form functional synapses and have the ability to direct axons throughout long distances to re-innervate muscles in order to retain muscular function. As mentioned previously, astrocytes and microglia contribute significantly to the pathogeneses of ALS. Glial cell replacement therapies attempt to enrich the spinal cord microenvironment by providing trophic support to diseased neuronal and non-neuronal cells, and by facilitating the reuptake of substances that are toxic. Therefore, this approach constitutes a promising alternative that could alleviate the dysfunction at the cellular level. Accumulated data from many preclinical studies support the use of this cell replacement therapy as an effective alternative treatment for ALS (2, 27, 29-32).

Delivering stem cells to the right location in the spinal cord represents a complex challenge. Consequently, many different delivery methods such as intravascular, intrathecal and intraparenchymal have been extensively studied. Each one of these methods possesses unique advantages as well as drawbacks. Intravascular delivery of cellular therapies to the spinal cord has the primary advantage of being a minimally invasive method. However, this delivery route has proven to be challenging due to the fact that the cells must bypass the blood brain barrier in order to get into the central nervous system (CNS). Other difficulties include the concern for a cell type dependent tumorigenesis and high probability of inducing a hypercoagulable state (33). Some small preclinical studies have attempted to deliver cell-based therapeutics to the site of interest using this method; unfortunately they were not highly effective (34-36). Intrathecal delivery to the spinal cord is achieved by accessing the subarachnoid space, usually using a standard technique for percutaneous access (e.g. lumbar puncture). The two main advantages of this method include the minimal invasiveness of the procedure and delivery of the cells adjacent to the spinal cord. The disadvantages include the risk of local or generalized infection secondary to the procedure (e.g. meningitis or encephalitis), activation of the immune system and the risk of localized or disseminated tumorigenesis. Preclinical and clinical studies using this approach to deliver cell therapies for spinal cord injury reported minimal engraftment of the transplanted cells at the injury site (37-40). Additionally, the few studies using intrathecal delivery of cell therapeutics for ALS yielded similar results (27, 41). Intraparenchymal delivery represents the most straightforward delivery method and it is achieved by directly injecting the spinal cord

using a cannula. The main advantage of this approach is that it can deliver cell grafts with anatomic specificity and offers the possibility of adjusting the dose at the target site. The disadvantages include the morbidity related to the surgical exposure of the spinal cord, damage to the spinal cord secondary to cannulation or manipulation, and tissue injury secondary to the immune rejection of the cell grafts (42, 43).

Many clinical trials using stem cell transplantation to treat ALS are currently underway in many countries around the world, including the USA, Spain, Italy, Israel and Turkey. Cell type, delivery method and immunosuppressive regimen are some of the main differences between these trials. Differences in these trials could be attributed to the lack of reliable scientific data to answer questions such as the maximum volume and number of injections tolerated by the spinal cord (44). This study will provide to the available body of literature critical insight towards understanding the tolerance of the spinal cord to intraparenchymal delivery of stem cells. Other issues such as characterizing the immune response to transplantation in the CNS, finding the optimal immunosuppressive therapy to reduce graft rejection and development of effective cell tracking methods are currently being studied by groups at Emory University but will not be addressed in the present thesis work.

METHODS

Study Design

Given that human death in ALS occurs secondary to respiratory failure and the loss of upper airway control, segmental therapy to the cervical spinal cord from levels 3-5 may prolong life by preserving phrenic motor neurons. In addition, neuroprotection in these segments may strengthen the proximal upper extremity. Treating the motor neurons for these three segments requires multiple injections. However, at some point, this advantage is balanced by the increasing risk of spinal cord injury. The present thesis work served to provide critical data on the safety of the cell transplantation technique developed by the Boulis Lab (42). It was critical to understand the time course of transient morbidity. Surgeons must have a threshold for determining when to re-explore these patients in search of reversible causes of unexpected morbidity such as epidural hematomas. Further anesthesia and surgical re-exploration will carry its own morbidity. Thus, these experiments help to refine the technique by investigating dangerous thresholds for number and volume of intraparenchymal injections. Finally, it helped to establish expectations to guide preoperative and postoperative care. Since cell graft survival was not the main goal of this thesis, a standard immunosuppressive regimen (Tacrolimus) was used after transplantation. The animal model in this study consisted of healthy minipigs, since it is difficult to evaluate surgical safety outcomes in debilitated animals.

Number escalation experiment

This experiment served to determine the maximum tolerated number of injections. Animals were divided into three groups (n = 5/group) and underwent an escalation of 20, 30, and 40 cervical injections distributed bilaterally using a rigid cannula, with a 10 μL cell suspension containing hNPCs (10^6 cells). The surgical technique is detailed in the Experimental Methods section. Based on previous observations of cell migration in rodent tissue, inter-graft distances of 2 mm were used. Sensory and motor behavioral assessments were performed as described in the Experimental Methods section. Immunosuppression consisted of monotherapy with Tacrolimus (0.025mg/kg, BID, IV). Animals were euthanized at 21 days after surgery and underwent transcardiac perfusion for histology (see details in the Experimental Methods section). The segments of spinal cord containing the injections were then harvested, post-fixed overnight, and transferred to a sucrose solution for 72 hours. Spinal cords were cut serially in 40-50 μm sections using a cryostat and mounted on slides. Tissue was stained with Cresyl Violet for motor neuron identification and with Human Nuclei Antibody (HuNu) for grafted cell identification. Histological morbidity was assessed using the microscopic predictors as described in the measurements section. Behavioral morbidity was assessed with the Tarlov score at postoperative day 14, to evaluate if the animals come back to normal motor function.

Volume escalation experiment

To identify optimal transplantation parameters, this experiment served to evaluate morbidity following variable graft volumes. Animals were divided into three groups (n = 5/group) and underwent 20 bilateral intraparenchymal injections using a rigid cannula with increasing volumes of hNPCs: 10, 25, and 50 microliters per injection. As previously described in the number escalation experiment, grafts were placed at 2 mm intervals and cell concentration remained the same. Immunosuppression consisted of monotherapy with Tacrolimus (0.025mg/kg, BID, IV). Sensory and motor behavioral assessments were performed as described in the Experimental Methods section. Animals were euthanized at 21 days after surgery and underwent transcardiac perfusion for histology (see details in the Experimental Methods section). The segments of spinal cord containing the injections were then harvested, post-fixed overnight, and transferred to a sucrose solution for 72 hours. Spinal cords were cut serially in 40-50 μm sections using a cryostat and mounted on slides. Tissue was stained with Cresyl Violet for motor neuron identification and with HuNu for grafted cell identification. Histological morbidity was assessed using the microscopic predictors as described in the measurements section. Behavioral morbidity was assessed with the Tarlov score at postoperative day 14, to evaluate if the animals come back to normal motor function.

Hypotheses

1. There is a threshold for detection of post-operative morbidity when increasing volume and number of stem cell injections in the spinal cord of Gottingen minipigs.

2. There is a threshold for detection of microscopic tissue damage when increasing volume or number of stem cell injections in the spinal cord of Gottingen minipigs.

Study Type

Treatment Trial.

Characteristics of the Study Population

To date, the Food and Drug Administration (FDA) has recognized the proof-of-principle data on therapeutic efficacy in rodent models, which are the most highly characterized. However, the use of large animals is considered critical for validating the combination of the surgical procedure and safety of the final product for human use, because of the recognition that the size, anatomy, and general vulnerability of the spine and spinal cord better models the human.

Similarity in size and morphology between the swine and human spines and cords renders the pig optimal for safety and distribution studies of grafting approaches and devices. Moreover, favorable cost compared to canine and non-human primate models, and fewer ethical concerns have increased the use of the pig model in large animal experimentation over the last few years (45, 46). The surgical process of exposing and manipulating the spinal cord as well as closing the wound in the pig is virtually indistinguishable from the human. Consequently, the pig is subject to the same

fundamental complications including spinal cord injury, epidural hematoma, abscess, and CSF leakage (47).

Inclusion Criteria

1. Pathogen free female Gottingen minipigs.
2. Age ranging from 6 to 9 months.
3. Weight ranging from 12 to 18 kilograms.

Exclusion Criteria

1. Pregnancy.
2. Congenital defects affecting the spine and/or spinal cord identified during surgery.
3. Intraoperative complications not related with the proposed surgical procedure.

Elimination Criteria

1. Permanent damage to the collected tissue caused during the process of perfusion, harvesting, sectioning or staining.

Experimental Methods

Description of the type of cells selected for transplantation

Over the last couple of years, Clive Svendsen's laboratory in Cedars-Sinai Medical Center, has provided mounting evidence supporting the use of human neural progenitors (hNPCs) harvested from fetal cortex as a means to prevent motor neuron death in ALS (48). These cells are isolated from human fetal brain tissue (collected from the NIH-funded fetal tissue bank, Seattle) and expanded in culture using human epidermal growth factor (hEGF) and leukemia inhibitory factor (LIF). They can grow for over 50 population doublings while retaining the potential to generate both neurons and astrocytes, but enter natural senescence between 50 and 70 population doublings (49). The Svendsen laboratory provided the hNPCs for use in this study.

Cell Preparation for Transplantation

Human fetal cortex-derived neural progenitor cells cultured as free-floating neurospheres were received (Svendsen Lab) between passage 25 and 35. The neurospheres were cultured in maintenance medium containing human EGF, LIF, and antimicrobial/antibacterial reagent. Prior to transplantation, the neurospheres were dissociated to single cells using Trypsin and DNAse. The cells were resuspended in transplantation medium at a concentration of 10,000 cell/μL and maintained on ice. Cell viability and concentration were calculated using a hemocytometer and trypan blue reagent.

Spinal Cord Stem Cell Delivery System

Direct CNS injection of therapeutics is a viable and potentially important option for the treatment of CNS disorders. Surgical penetration of the functional human spinal cord has an acceptable risk profile with a long history of application in a variety of neurosurgical disorders.

Spinal cord injections in humans have been performed using a free-hand syringe (50-53) or table-mounted pump devices (54-56). Free-hand intraspinal injection represents an ill-advised delivery strategy in humans for a variety of reasons: 1) It cannot reliably reproduce anatomic targeting of the specific structures (ventral horn in motor neuron disease, plaques in multiple sclerosis); 2) Movement of the unsteady needle can sheer white matter tracts; 3) The uncontrolled infusion rate has an increased potential for spinal cord mass effect from high pressure; 4) Finally, it provides an imprecise rate of infusion predisposing the injection to reflux up the catheter. Table-mounted devices are usually designed in combination with micromanipulators and microinjectors, offering better stability, better control of volume, speed of injection, and anatomical targeting through precise three-dimensional positioning. Despite these advantages, these systems allow for movement of the patient with respect to the injection needle both during ventilation in the prone position, and as a result of inadvertent jostling of the patient. As the chest expands during inhalation, the spine rises several millimeters relative to the bed, causing a similar displacement of the cord with respect to the injection cannula. This issue can be addressed by holding ventilation, but such an approach increases blood carbon dioxide and limits the duration of an injection. Each of these issues carries significant potential to promote suboptimal efficacy and the generation of significant neurologic morbidity. The

Boulis Lab has developed a spinal cord microinjection platform designed to facilitate safe and accurate administration of cellular and molecular therapeutics to humans (43). Despite this optimized technique, the appropriate arrangement of injections is dependent on an understanding of the tolerance of the spinal cord for multiple injections and volumes. Because this morbidity is a reaction to cord penetration and injection rather than graft cell type, these findings will be relevant to all of the translational programs currently in development.

Surgical Technique for Intrajugular Catheter Placement

The Emory University Institutional Animal Care and Use Committee approved all surgical procedures. Prior to any surgical procedure all animals were fasting for at least 12 hours. Animals were anesthetized with Ketamine (35 mg/kg, IM), Acepromazine (1.1 mg/kg, IM), Atropine (0.02 mg/kg, IM), and maintained for the duration of the procedure with Isoflurane (1.5-2.5%, Inhaled) mixed with oxygen. Before the spinal incision, the neck of the pig was prepped and draped. The external jugular was exposed surgically and cannulated with a central catheter, which was secured with a 3-0 silk tie. The proximal end of the internal jugular was ligated with a 3-0 silk tie. The catheter was then tunneled out of the neck skin dorsally and secured with 3-0 nylon stitches. The wound was irrigated and closed with a running 3-0 nylon stitch. The catheter was used to administer all IV medication for the duration of the experiments.

Surgical Technique for Transplantation

Pigs were placed in the prone position, with appropriate draping of the operative area. An approx. 10-15 cm incision was performed over the spine and a multi-level laminectomy between levels 3 to 7 was performed over the cervical spinal cord. Following laminectomy, the percutaneous posts were placed through 1 cm skin incisions above and below the primary incision. The upper posts were mounted to the occiput through small percutaneous incisions and the lower post were mounted to lamina below the primary incision. The microinjection platform was attached to the four posts, allowing the device to span the laminectomy. A 2 to 4 cm incision was made into the dura mater, allowing exposure of the spinal cord. The dura mater was then tacked away using 4-0 nurulon sutures. At this point, the microinjection device was placed and adjusted. Targeting to the area of interest within the spinal cord was achieved with the use of coordinate-based microinjection. The injections followed placement of the cannula 2 mm medial to the dorsal root entry and at a 4 mm depth. Immediately prior to this, a bolus of Methylprednisolone (125mg, IV) was given in an attempt to prevent spinal cord swelling. A custom infusion cannula of narrow diameter was used. For each injection, the appropriate volume of cell suspension was infused by a microprocessor-controlled syringe pump at the rate of 5 μL per minute. The needle was left in place for an additional 1 minute to prevent cell reflux up the cannula injection tract before extraction. Following needle removal, the stereotaxic apparatus was relocated to the next target site, separated by 2 mm as necessary to avoid visible blood vessels on the dorsal surface of the spinal cord. This process was repeated as proposed in each volume and number escalation experiments. Once all injections were made, the injection apparatus was removed and the

incisions were closed in four layers. The dura was closed using a 4-0 nurulon stitch, in a watertight fashion and the transplanted area was marked using 6-0 prolene stitches. A 0 vicryl suture was used for the deep muscular layer. The second layer, fascia, was also closed using 0 vicryl suture in a watertight fashion. The dermal layer was closed with 2-0 ethylon, with a running stitch. Following this, animals were taken off anesthesia and observed until full recovery. A fentanyl patch (75 μg) was placed for post-operative analgesia for 3 days (57).

Behavioral Assessment

Animals underwent a general neurological examination/observation before surgery for baseline assessment and following complete recovery from the procedure. Behavioral assessment of motor function was performed daily during the 7 first postoperative days and then once a week until euthanasia. Sensory evaluation took place in the form of a tactile stimulus to the perianal region. All four limbs were assessed. This stimulus is not noxious or painful but allows assessment of both sensation and motor function in response to limb retraction from a steadily applied force (withdrawal response to a mechanical stimulus). Gait and motor function was assessed according to the Tarlov scale. This scale provides objective criteria by which to evaluate ability to ambulate as a surrogate measure of motor function. The score is as follows: (0) no voluntary limb function; (1) only perceptible joint movement; (2) active movement but unable to stand; (3) to be able to stand but unable to walk; (4) complete normal hind-limb motor function (Table 2).

Euthanasia and Perfusion

At endpoints, animals were sedated with Ketamine (35 mgs/kg, IM), Acepromazine (0.8 mgs/kg, IM) and Euthasol (1 ml/10 lbs, IV). Following sedation, 10,000 USP Units/ml of Heparin Sodium were administered IV five minutes before euthanasia, while the heart was still beating. Transcardiac perfusion with a 0.9% NaCl solution followed by a 4% Paraformaldehyde solution was then performed to improve the quality of the tissue for immunohistochemistry (IHC). A peristaltic pump (Masterflex Console Drive pump (model 71-1420) was used for perfusions. Spinal cords were harvested as described above. Tissue was then frozen in optimal cutting temperature gel (OCT) and cryosectioned.

Histology

Frozen transverse spinal cord sections (40-50 μm thick) were cut. Free-floating sections were stained with Cresyl Violet for motor neurons and with HuNu for grafted cells, mounted on slides, and coverslipped. Images were captured bilaterally with a digital DS-Qi1 high sensitivity Cooled CCD camera using a Nikon E400 microscope supplied with a controlled motorized Z stage and a NIS-Elements imaging software (Nikon Instruments, Inc.).

Stereology Protocol

Stereology constitutes an interdisciplinary field that is largely concerned with the three-dimensional interpretation of planar sections of materials or tissues. It uses techniques for extracting quantitative information about a three-dimensional material from measurements made on two-dimensional planar sections of the material. A random, systematic sampling approach is used to provide potentially unbiased and quantitative data and is an important and efficient tool in many applications of microscopy. It may thus provide estimates of cell numbers, object size and shape with precision (58, 59). Briefly, the transplanted area in the spinal cord of every pig was sampled using unbiased random uniform sampling. All sections with grafted cells were considered for the sample, sections without grafted cells were discarded. One out of every six sections was included in the sample for analysis with a total distance between sections of 300 μm. A combination of the Cavalieri principle and the optical disector was applied to the neuron and grafted cell counting. The equipment used for the optical disector included a microscope (Leica DM2500) with a motorized x–y stage, an electronic microcator (Applied Scientific Instrumentation), which was used for measuring movements in the z direction, and the PC software Stereologer™ for cell counting. The optical disector frame provided inclusion and exclusion lines to prevent edge effects arising from sub-sampling. All neurons and grafted cells that came into focus within the disector height (15 μm) were counted, provided they did not touch any of the exclusion lines and fell in the inclusion lines. The sections were counted with a 60X oil-immersion objective (final magnification, 2000X). The person performing the analysis of the spinal cords was blinded to the experimental design throughout the process.

Measurements

Sources of data

Predictors

1. Injection volume (20 injections): 10 μL, 25 μL and 50 μL.
2. Injection number (10 μL): 20, 30 and 40 injections.

Outcomes: behavioral and histological.

Primary: all the primary outcomes were continuous variables.

1. Neuron number per mm^3: sum of the total number of neurons counted divided by the volume of reference in every spinal cord.
2. Number of damaged injections sites: sum of all the grafts found with damage in every spinal cord (damage is considered when >50% of the graft area is lost).
3. Motor function (Tarlov score) at day 14: motor function recorded at postoperative day 14 as described in the behavioral assessment section above.

Secondary: all the secondary outcomes were continuous variables

1. Engraftment percentage: remaining proportion of cells engrafted at the target site at a time point (21 days) after transplantation.

2. Number of identified grafts: sum of all the identified injection sites containing engrafted cells.
3. Average time back to baseline motor function (Tarlov score): number of days that took every animal to recover motor function completely after the surgical procedure.

Sample Size and Power

Sample size was originally calculated so that there would be the ability to detect a large effect size (50%), with an 80% power and a 0.05 significance level. Using SAS 9.1 software to perform this calculation, the output yielded an N of 7 per group. Due to the complex nature of the project and its financial constraints, an N of 5 per group was studied, which would give us a 67.3% power at the alpha=0.05 significance level.

Analytic Plan

Descriptive statistics: summary statistics will report mean, median, standard deviation, minimum and maximum values for each one of the outcomes stratified by predictors (injection volume or number).

Statistical inference: one-way analysis of variance (ANOVA) was used to test for statistically significant differences for each outcome between groups. Where necessary, Tukey's *post hoc* comparisons was used to interpret the ANOVA results. Results are presented in tables; box and whisker plots are used where necessary.

RESULTS

Twenty-five 12 to 18 kg female Gottingen minipigs received cervical intraspinal cell (hNPCs) injections at a concentration of 10,000 cell/μL using the stereotactic delivery system described above. Different injection volumes (10, 25 and 50 μL) and total number of injections (20, 30 and 40 bilateral) were used for the transplantation technique in each one of the 5 groups (n = 5 each), for more details refer to Figure 2. One animal from group 5 was eliminated for the tissue analysis because of extensive damage caused to the spinal cord during sectioning leaving this group with an N of 4.

Postoperative behavioral outcomes

Table 3 shows both pre- and postoperative neurological outcomes after transplantation of hNPCs for the volume escalation cohort (groups 1 to 3) holding the number of injections constant and for the number escalation cohort (groups 1, 4 and 5) holding the injection volume constant. Tables 5 and 6 show the descripting statistics of the outcomes. In all pigs assessed in groups 1 to 5, baseline motor function was regained by postoperative day 14 and maintained until postoperative day 21 when they were sacrificed. One animal in each of group 2 & 4 showed a slower trend towards recovery with respect to the rest of the animals. Despite increasing injection volumes and total number, no statistically significant difference (ANOVA ($F = 0.74$, $p = 0.50$)) was observed for the average time back to baseline among all groups (Tables 7 and 9). This indicates that neither increases

in microinjection volumes nor total number were associated with development of postoperative or permanent neurological sequelae.

Postoperative histological outcomes

Tables 5 and 6 show the descriptive statistics for number of neurons per mm^3 and number of damaged injection sites (primary outcomes) as well as number of identified injections and engraftment percentage (secondary outcomes).

Volume Escalation

The stereological analysis of the volume escalation cohort (Table 7 and Figure 4) shows that there was no statistically significant difference (ANOVA ($F = 2.81$, $p = 0.10$)) in neuronal density at increasing volumes from 10 to 50 μL across groups (1-3). The histological analysis (Table 7 and Figure 5) showed a statistically significant (ANOVA ($F = 8.94$, $p = 0.004$)) difference between increasing volumes and damaged injection sites across groups (1-3). The pair-wise comparison done using Tukey's *post-hoc* adjustment method (Table 8) showed a significant difference in damaged injection sites between groups 1 and 3 ($p = 0.03$). Furthermore, there was no significant difference in damaged injection sites between groups 1 and 2 ($p = 0.25$), as well as between groups 2 and 3 ($p = 0.06$). These results indicate that there was no significant loss in neuronal density when increasing the injection volume. However, there was a significant increase in tissue damage at higher volumes.

Additionally, the engraftment percentage remained between 11 - 17% depending on the injection volume (Table 5 and Figure 6) and group 2 (25 μL) showed the highest mean (12.8) for the total number of identified injection sites (Figure 7).

Number escalation

The stereological analysis of the number escalation cohort (Table 9 and Figure 8) shows that there was a statistically significant difference (ANOVA ($F = 7.19$, $p = 0.01$)) in neuronal density at increasing number of injections from 20 to 40 total across groups (1,4 and 5). The pair-wise comparison T-tests were done using Tukey's *post-hoc* adjustment method (Table 10) showed a significant difference in neuronal density between groups 1 and 4 ($p = 0.04$), as well as between groups 1 and 5 ($p = 0.01$). The histological analysis (Table 9 and Figure 9) showed that there was no statistically significant (ANOVA ($F = 2.56$, $p = 0.12$)) difference between increasing number of injections and damaged injection sites across groups (1,4 and 5). Moreover, there was a statistically significant difference (ANOVA ($F = <0.001$) between identified grafts (Table 9 and Figure 10) and increasing number of injections across groups. In the pair-wise comparison (Table 10) the difference was significant ($p = 0.02$) between groups 1 and 4, but not significant between groups 4 and 5 ($p = 0.05$). These results indicate that there is a significant increase in neuronal density and number of identified grafts when increasing the number of injections. Furthermore, there is no significant increase in tissue damage as the number of injection increases.

Additionally, the engraftment percentage remained between 17 - 31% depending on the injection number (Table 6 and Figure 11), with group 4 being the highest among all groups.

DISCUSSION AND CONCLUSIONS

Stem cell transplantation represents a promising approach for the treatment of various neurodegenerative diseases. Multiple clinical trials are currently underway using this approach to treat ALS, however the optimal injection parameters have not yet been defined (44). The appropriate strategy for injections is dependent on an understanding of the tolerance of the spinal cord for multiple injection parameters. The purpose of this thesis work was to test the tolerance of the cervical spinal cord to increasing volumes and numbers of intraspinal injections using a stereotactic delivery device. Understanding the safety thresholds for these parameters will enable current clinical studies to generate better efficacy data.

Safety: increasing intraspinal injection volumes and total numbers

The findings in this study proved that escalating the intraspinal injection volume up to 50 μL and the total injection number up to 40 results in no permanent neurological morbidity. All animals in the volume and number escalation groups returned to preoperative motor function within 14 days after the surgery was performed. Transient decline of motor function was seen in all animals during the first postoperative week, however this transient decline appears to be less severe (Figure 3) in group 1 (20 bilateral 10 μL injections) compared to all other groups. Previous work from the Boulis laboratory has looked at safety and accuracy of delivering cell therapies to the spinal cord. The early studies in large animals were focused mainly towards validating the stereotactic

delivery system, as well as proving feasibility and accuracy of delivering cells to the right location within the spinal cord. The elevated neurological morbidity (evaluated using the Tarlov score) seen during one of these studies prompted the search for further improvements to the delivery system and injection cannula (42, 60). In a later study, pigs received 5 to 10 total injections at different volumes (10, 25 and 50 μL) and at different infusion rates (1, 2.5 and 5 μL/min) (43). Another study looked at the long-term risks of multilevel intraspinal injections using similar injection numbers but lower volumes (47). In both series neither intraoperative mortality nor permanent neurological morbidity was observed. However, these studies did not include quantitative assessment of histological tissue damage as a measurement of safety. The primary histology outcomes were chosen to assess tissue damage at the cellular level that might be undermined by the limited sensitivity of the Tarlov scale (61, 62). In contrast, the secondary histological outcomes were selected to assess which injection paradigm yielded a greater cell survival.

Analysis of primary and secondary outcomes for the volume escalation groups indicate that at increasing volumes of injections there is no loss in neuronal density, but there is an increase in the number of damaged injection sites. Additionally, the 25 μL group showed a higher mean number of identified injection sites, while the 10 μL group showed a higher engraftment percentage (17%). Thus, suggesting that the optimal injection volume lies in between 10 and 25 μL at a cell concentration of 10,000 cells/μL. The analysis of the same primary and secondary outcomes for the number escalation groups indicates that at increasing number of injections there is an increase in neuronal density. Despite the higher accuracy achieved using the stereotactic delivery device, the possibility of the

stem cells being delivered at different depths and anatomical locations remains (43). Different anatomical locations in the spinal cord have different neuronal densities, thus this might explain the increased neuronal density at different injection sites (63). Another explanation might be that the transplanted cells began to differentiate into microglial or neuronal cell types, but this seems highly unlikely since animals were sacrificed 21 days after transplantation (22, 64, 65). In contrast with the findings in the volume escalation groups, no significant differences were detected in terms of the number of damaged injection sites across different injection numbers (20, 30 and 40). Group 5 showed the highest engraftment percentage (31%) among all groups, suggesting that an injection paradigm using a higher number of injections at lower volumes might increase cell survival. Additionally, as the number of total injections increases there is a significant increase in the number of identified grafts. This result was expected since it is the dependent variable in these number escalation groups.

The behavioral and histological outcomes analyzed in this work provide the means to define safety thresholds for intraspinal injection volume and number. Consequently, the optimal injection paradigm should use an injection volume between 10 and 25 μL at a fixed concentration of 10,000 cells/μL. Furthermore, the number of injections can be safely escalated up to a total of 40 when using a volume of 10 μL, however the best engraftment percentage (31%) is seen at 30 injections (Figure 12). These recommendations should be interpreted with caution when choosing an injection strategy for other applications or diseases. It is also important to take into count anatomical

location, underlying disease, patient's characteristics, cell type and concentration, delivery vehicle, and infusion rate among many others factors.

Clinical translation: from T1 to T2

The results from this study constitute strong scientific evidence for the safety and reliability of intraspinal microinjection in large animals across a wide range of injection volumes and numbers. Additionally, they provide critical insight towards answering the question of what is the optimal injection paradigm for cell therapies targeting the spinal cord across a variety of diseases. The chosen outcomes helped define important safety thresholds that can be used for the delivery of other types of therapeutics such as viral vectors or proteins (66-68). This work will help current clinical studies to better assess the safety of escalating their injection parameters in the spinal cord for future phases, and consequently enable them to generate adequate and comparable efficacy data (44, 69, 70). However, in order to unmask the full potential of cell therapies for neurodegenerative diseases many other issues need to be further studied. Cell line selection, understanding the immune response to transplant in the spinal cord and choosing the best immunosuppressive treatment to avoid graft rejection constitute some of the critical issues (2).

The selection of stem cells that are capable of differentiating into supportive cell types rather than replace existing cells in diseases like ALS is still a matter of debate. Current research in this field is attempting to unravel which cell type is more efficacious.

Additionally, engineering these cells to secrete various neurotropic factors has proved to increase their beneficial effects to the microenvironment of the diseased spinal cord (48, 65, 71). Spinal cord injury studies using cell-based therapies have provided some understanding of the inflammatory response in this condition (72). However, there is limited data on how the immune system responds to cell transplantation in the spinal cord. Increasing our understanding of the CNS immune mechanisms that are triggered by grafted cells might shed light on what needs to be done to optimize immunosuppression in transplanted patients in the future (73-75). However, these issues should be studied in a multifaceted and interdisciplinary manner that will enable a fast and successful clinical translation.

Limitations

Although an n of 5 per group did not achieve the intended statistical power, these experiments would be the largest large animal series using cell therapy in the spinal cord reported in the literature to date. Thus, the results of these experiments provide critical information about the tolerability of the spinal cord to increasing volume and number of stem cell injections. Other modifiable parameters like cell concentration, delivery vehicle and infusion rate might require further in depth safety studies in large animals. In retrospective, the use of a more sensitive motor function evaluation scale and every day assessments would have given us a better idea of the exact time that took some animals to come back to baseline motor function.

Future challenges

Despite the safety and reliability of the currently employed methods for direct intraspinal injection, future approaches using MR guidance for targeting and delivery could provide minimal invasiveness and also real time control of the delivery of cell therapeutics to the spinal cord. However in order for this to become a reality, developing efficient cell labeling methods that could provide real time tracking of these cells using imaging techniques is of the utmost importance.

This thesis work demonstrates the functional safety of escalating microinjection volume and number in the cervical spinal cord of Gottingen minipigs. Histological outcomes served as an additional measure of safety that allowed us to better determine safety thresholds future use. These findings are relevant to all translational programs currently attempting to deliver cellular therapeutics to the spinal cord. More importantly, this work provides critical information for the application of human embryonic stem cells in the treatment of ALS and other neurodegenerative diseases.

REFERENCES

1. Borasio GD, Miller RG. Clinical characteristics and management of ALS. *Seminars in neurology* 2001;21(2):155-66.

2. Boulis NM, Federici T, Glass JD, et al. Translational stem cell therapy for amyotrophic lateral sclerosis. *Nature reviews Neurology* 2011;8(3):172-6.

3. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *The New England journal of medicine* 2001;344(22):1688-700.

4. Logroscino G, Traynor BJ, Hardiman O, et al. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *Journal of neurology, neurosurgery, and psychiatry* 2008;79(1):6-11.

5. Gordon PH. Amyotrophic Lateral Sclerosis: An update for 2013 Clinical Features, Pathophysiology, Management and Therapeutic Trials. *Aging and disease* 2013;4(5):295-310.

6. Brooks BR, Miller RG, Swash M, et al. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases* 2000;1(5):293-9.

7. Carvalho MD, Swash M. Awaji diagnostic algorithm increases sensitivity of El Escorial criteria for ALS diagnosis. *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 2009;10(1):53-7.

8. Traxinger K, Kelly C, Johnson BA, et al. Prognosis and epidemiology of amyotrophic lateral sclerosis: Analysis of a clinic population, 1997-2011. *Neurology Clinical practice* 2013;3(4):313-20.

9. Musaro A. Understanding ALS: new therapeutic approaches. *The FEBS journal* 2013;280(17):4315-22.

10. Veyrat-Durebex C, Corcia P, Dangoumau A, et al. Advances in Cellular Models to Explore the Pathophysiology of Amyotrophic Lateral Sclerosis. *Molecular neurobiology* 2013.

11. Mok K, Laaksovirta H, Tienari PJ, et al. Homozygosity analysis in amyotrophic lateral sclerosis. *European journal of human genetics : EJHG* 2013.

12. Turner MR, Hardiman O, Benatar M, et al. Controversies and priorities in amyotrophic lateral sclerosis. *Lancet neurology* 2013;12(3):310-22.

13. Robberecht W, Philips T. The changing scene of amyotrophic lateral sclerosis. *Nature reviews Neuroscience* 2013;14(4):248-64.

14. Sabatelli M, Conte A, Zollino M. Clinical and genetic heterogeneity of amyotrophic lateral sclerosis. *Clinical genetics* 2013;83(5):408-16.

15. Al-Chalabi A, Hardiman O. The epidemiology of ALS: a conspiracy of genes, environment and time. *Nature reviews Neurology* 2013;9(11):617-28.

16. Ling SC, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron* 2013;79(3):416-38.

17. Philips T, Robberecht W. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet neurology* 2011;10(3):253-63.

18. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414(6859):105-11.

19. Bang OY, Lee JS, Lee PH, et al. Autologous mesenchymal stem cell transplantation in stroke patients. *Annals of neurology* 2005;57(6):874-82.

20. Kim SU, Lee HJ, Kim YB. Neural stem cell-based treatment for neurodegenerative diseases. *Neuropathology : official journal of the Japanese Society of Neuropathology* 2013;33(5):491-504.

21. Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 2008;132(4):661-80.

22. Vazin T, Freed WJ. Human embryonic stem cells: derivation, culture, and differentiation: a review. *Restorative neurology and neuroscience* 2010;28(4):589-603.

23. Donnelly EM, Lamanna J, Boulis NM. Stem cell therapy for the spinal cord. *Stem cell research & therapy* 2012;3(4):24.

24. Himes BT, Neuhuber B, Coleman C, et al. Recovery of function following grafting of human bone marrow-derived stromal cells into the injured spinal cord. *Neurorehabilitation and neural repair* 2006;20(2):278-96.

25. Bonner JF, Blesch A, Neuhuber B, et al. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. *Journal of neuroscience research* 2010;88(6):1182-92.

26. Bonner JF, Connors TM, Silverman WF, et al. Grafted neural progenitors integrate and restore synaptic connectivity across the injured spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011;31(12):4675-86.

27. Silani V, Calzarossa C, Cova L, et al. Stem cells in amyotrophic lateral sclerosis: motor neuron protection or replacement? *CNS & neurological disorders drug targets* 2010;9(3):314-24.

28. Xu L, Ryugo DK, Pongstaporn T, et al. Human neural stem cell grafts in the spinal cord of SOD1 transgenic rats: differentiation and structural integration into the segmental motor circuitry. *The Journal of comparative neurology* 2009;514(4):297-309.

29. Boucherie C, Schafer S, Lavand'homme P, et al. Chimerization of astroglial population in the lumbar spinal cord after mesenchymal stem cell transplantation prolongs survival in a rat model of amyotrophic lateral sclerosis. *Journal of neuroscience research* 2009;87(9):2034-46.

30. Clement AM, Nguyen MD, Roberts EA, et al. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 2003;302(5642):113-7.

31. Xu LY, Ryugo DK, Pongstaporn T, et al. Human Neural Stem Cell Grafts in the Spinal Cord of SOD1 Transgenic Rats: Differentiation and Structural Integration into the Segmental Motor Circuitry. *Journal of Comparative Neurology* 2009;514(4):297-309.

32. Yan J, Xu L, Welsh AM, et al. Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. *PLoS medicine* 2007;4(2):e39.

33. Furlani D, Ugurlucan M, Ong L, et al. Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc Res* 2009;77(3):370-6.

34. Akiyama Y, Radtke C, Honmou O, et al. Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* 2002;39(3):229-36.

35. Inoue M, Honmou O, Oka S, et al. Comparative analysis of remyelinating potential of focal and intravenous administration of autologous bone marrow cells into the rat demyelinated spinal cord. *Glia* 2003;44(2):111-8.

36. Ra JC, Shin IS, Kim SH, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem cells and development* 2011;20(8):1297-308.

37. Callera F, do Nascimento RX. Delivery of autologous bone marrow precursor cells into the spinal cord via lumbar puncture technique in patients with spinal cord injury: A preliminary safety study. *Exp Hematol* 2006;34(2):130-1.

38. Callera F, Melo CMTP. Magnetic resonance tracking of magnetically labeled autologous bone marrow CD34(+) cells transplanted into the spinal cord via lumbar puncture technique in patients with chronic spinal cord injury: CD34(+) cells' migration into the injured site. *Stem cells and development* 2007;16(3):461-6.

39. Liu J, Han D, Wang Z, et al. Clinical analysis of the treatment of spinal cord injury with umbilical cord mesenchymal stem cells. *Cytotherapy* 2013;15(2):185-91.

40. Mothe AJ, Bozkurt G, Catapano J, et al. Intrathecal transplantation of stem cells by lumbar puncture for thoracic spinal cord injury in the rat. *Spinal cord* 2011;49(9):967-73.

41. Prabhakar S, Marwaha N, Lal V, et al. Autologous bone marrow-derived stem cells in amyotrophic lateral sclerosis: a pilot study. *Neurology India* 2012;60(5):465-9.

42. Riley J, Butler J, Baker KB, et al. Targeted spinal cord therapeutics delivery: stabilized platform and microelectrode recording guidance validation. *Stereotactic and functional neurosurgery* 2008;86(2):67-74.

43. Riley J, Federici T, Park J, et al. Cervical spinal cord therapeutics delivery: preclinical safety validation of a stabilized microinjection platform. *Neurosurgery* 2009;65(4):754-61; discussion 61-2.

44. Lamanna JJ, Miller JH, Riley JP, et al. Cellular therapeutics delivery to the spinal cord: technical considerations for clinical application. *Therapeutic delivery* 2013;4(11):1397-410.

45. Vodicka P, Smetana K, Jr., Dvorankova B, et al. The miniature pig as an animal model in biomedical research. *Annals of the New York Academy of Sciences* 2005;1049:161-71.

46. Dehoux JP, Gianello P. The importance of large animal models in transplantation. *Frontiers in bioscience : a journal and virtual library* 2007;12:4864-80.

47. Raore B, Federici T, Taub J, et al. Cervical multilevel intraspinal stem cell therapy: assessment of surgical risks in Gottingen minipigs. *Spine* 2011;36(3):E164-71.

48. Gowing G, Svendsen CN. Stem cell transplantation for motor neuron disease: current approaches and future perspectives. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 2011;8(4):591-606.

49. Wright LS, Prowse KR, Wallace K, et al. Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. *Exp Cell Res* 2006;312(11):2107-20.

50. Chen L, Huang H, Zhang J, et al. Short-term outcome of olfactory ensheathing cells transplantation for treatment of amyotrophic lateral sclerosis. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2007;21(9):961-6.

51. Huang H, Chen L, Wang H, et al. Safety of fetal olfactory ensheathing cell transplantation in patients with chronic spinal cord injury. A 38-month follow-up with MRI. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2006;20(4):439-43.

52. Huang H, Chen L, Wang H, et al. Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury. *Chin Med J (Engl)* 2003;116(10):1488-91.

53. Knoller N, Auerbach G, Fulga V, et al. Clinical experience using incubated autologous macrophages as a treatment for complete spinal cord injury: phase I study results. *J Neurosurg Spine* 2005;3(3):173-81.

54. Feron F, Perry C, Cochrane J, et al. Autologous olfactory ensheathing cell transplantation in human spinal cord injury. *Brain* 2005;128(Pt 12):2951-60.

55. Mazzini L, Mareschi K, Ferrero I, et al. Stem cell treatment in Amyotrophic Lateral Sclerosis. *J Neurol Sci* 2008;265(1-2):78-83.

56. World's first clinical trial of human embryonic stem cell therapy cleared. *Regen Med* 2009;4(2):161.

57. Federici T, Hurtig CV, Burks KL, et al. Surgical technique for spinal cord delivery of therapies: demonstration of procedure in gottingen minipigs. *Journal of visualized experiments : JoVE* 2012(70):e4371.

58. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *Journal of microscopy* 1987;147(Pt 3):229-63.

59. Schmitz C, Hof PR. Design-based stereology in neuroscience. *Neuroscience* 2005;130(4):813-31.

60. Riley JP, Raore B, Taub JS, et al. Platform and cannula design improvements for spinal cord therapeutics delivery. *Neurosurgery* 2011;69(2 Suppl Operative):ons147-54; discussion ons55.

61. Muir GD, Webb AA. Mini-review: assessment of behavioural recovery following spinal cord injury in rats. *The European journal of neuroscience* 2000;12(9):3079-86.

62. Perot PL, Jr., Lee WA, Hsu CY, et al. Therapeutic model for experimental spinal cord injury in the rat: I. Mortality and motor deficit. *Central nervous system trauma : journal of the American Paralysis Association* 1987;4(3):149-59.

63. Aitken JT, Bridger JE. Neuron size and neuron population density in the lumbosacral region of the cat's spinal cord. *Journal of anatomy* 1961;95:38-53.

64. Popescu IR, Nicaise C, Liu S, et al. Neural progenitors derived from human induced pluripotent stem cells survive and differentiate upon transplantation into a rat model of amyotrophic lateral sclerosis. *Stem cells translational medicine* 2013;2(3):167-74.

65. Gowing G, Shelley B, Staggenborg K, et al. Glial cell line-derived neurotrophic factor-secreting human neural progenitors show long-term survival, maturation into

astrocytes, and no tumor formation following transplantation into the spinal cord of immunocompromised rats. *Neuroreport* 2013.

66. Keifer OP, Jr., O'Connor DM, Boulis NM. Gene and protein therapies utilizing VEGF for ALS. *Pharmacology & therapeutics* 2014;141(3):261-71.

67. Simonato M, Bennett J, Boulis NM, et al. Progress in gene therapy for neurological disorders. *Nature reviews Neurology* 2013;9(5):277-91.

68. O'Connor DM, Boulis NM. Cellular and molecular approaches to motor neuron therapy in amyotrophic lateral sclerosis and spinal muscular atrophy. *Neuroscience letters* 2012;527(2):78-84.

69. Glass JD, Boulis NM, Johe K, et al. Lumbar intraspinal injection of neural stem cells in patients with amyotrophic lateral sclerosis: results of a phase I trial in 12 patients. *Stem cells* 2012;30(6):1144-51.

70. Riley J, Glass J, Feldman EL, et al. Intraspinal stem cell transplantation in amyotrophic lateral sclerosis: a phase I trial, cervical microinjection, and final surgical safety outcomes. *Neurosurgery* 2014;74(1):77-87.

71. Lunn JS, Hefferan MP, Marsala M, et al. Stem cells: comprehensive treatments for amyotrophic lateral sclerosis in conjunction with growth factor delivery. *Growth factors* 2009;27(3):133-40.

72. Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, et al. A systematic review of cellular transplantation therapies for spinal cord injury. *Journal of neurotrauma* 2011;28(8):1611-82.

73. Yan J, Xu L, Welsh AM, et al. Combined immunosuppressive agents or CD4 antibodies prolong survival of human neural stem cell grafts and improve disease outcomes in amyotrophic lateral sclerosis transgenic mice. *Stem cells* 2006;24(8):1976-85.

74. Usvald D, Vodicka P, Hlucilova J, et al. Analysis of dosing regimen and reproducibility of intraspinal grafting of human spinal stem cells in immunosuppressed minipigs. *Cell transplantation* 2010;19(9):1103-22.

75. Hefferan MP, Johe K, Hazel T, et al. Optimization of immunosuppressive therapy for spinal grafting of human spinal stem cells in a rat model of ALS. *Cell transplantation* 2011;20(8):1153-61.

TABLES AND FIGURES

Table 1. Revised El Escorial criteria for the diagnosis of ALS.

The diagnosis of ALS requires:
A. the presence of: 1. Evidence of LMN degeneration by clinical, electrophysiological or neuropathologic examination, 2. evidence of UMN degeneration by clinical examination, and 3. progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination, **together with:** B. the absence of 1. electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and 2. neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

* LMN: lower motor neuron, UMN: upper motor neuron

Table 2. Four point Tarlov scoring scale to assess motor function in Gottingen minipigs during the postoperative period.

Score	Description
0	No voluntary limb function
1	Only perceptible joint movement
2	Active movement but unable to stand up
3	Able to stand up but unable to walk
4	Complete normal hind-limb motor function

Table 3. Tarlov scores to assess motor function during the post-operative period using different injection techniques as predictors.

Injection					Post-operative							
Volume	**Number**	**Subject**	**Baseline**	**Surgery**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 14**
10 μL	20	Pig 1	4	4	4	4	4	4	4	4	4	4
		Pig 2	4	3	4	4	4	4	4	4	4	4
		Pig 3	4	3	2	3	3	3	4	4	4	4
		Pig 4	4	2	4	4	4	4	4	4	4	4
		Pig 5	4	4	4	4	4	4	4	4	4	4
25 μL	20	Pig 1	4	2	4	4	4	4	4	4	4	4
		Pig 2	4	2	4	4	4	4	4	4	4	4
		Pig 3	4	2	3	4	4	4	4	4	4	4
		Pig 4	4	2	2	2	2	2	2	2	3	4
		Pig 5	4	2	2	4	4	4	4	4	4	4
50 μL	20	Pig 1	4	2	2	4	4	4	4	4	4	4
		Pig 2	4	2	4	4	4	4	4	4	4	4
		Pig 3	4	2	4	4	4	4	4	4	4	4
		Pig 4	4	2	3	4	4	4	4	4	4	4
		Pig 5	4	2	4	4	4	4	4	4	4	4
10 μL	30	Pig 1	4	2	4	4	4	4	4	4	4	4
		Pig 2	4	2	2	3	4	4	4	4	4	4
		Pig 3	4	2	4	4	4	4	4	4	4	4
		Pig 4	4	2	2	2	3	3	3	3	3	4
		Pig 5	4	3	4	4	4	4	4	4	4	4
10 μL	40	Pig 1	4	2	3	3	3	3	4	4	4	4
		Pig 2	4	1	1	3	3	4	4	4	4	4
		Pig 3	4	2	3	4	4	4	4	4	4	4
		Pig 4	4	2	3	4	4	4	4	4	4	4

Table 4. Primary and secondary outcome measures using different injection techniques as predictors.

Injection			Neuron number per mm^3	DIS*	Tarlov Score		Number of identified grafts	Engraftment Percentage
Volume	Number	Subject			Day14	Time back to baseline (days)		
10 µL	20	Pig 1	1,670	0	4	0	9	21.86
		Pig 2	1,641	0	4	1	3	2.60
		Pig 3	1,891	0	4	5	1	5.05
		Pig 4	1,653	0	4	1	14	15.27
		Pig 5	4,857	0	4	0	20	43.99
25 µL	20	Pig 1	1,851	0	4	1	15	13.32
		Pig 2	3,945	1	4	1	12	8.60
		Pig 3	1,709	0	4	2	9	12.25
		Pig 4	2,359	1	4	8	16	20.08
		Pig 5	1,438	2	4	2	12	4.43
50 µL	20	Pig 1	3,002	2	4	2	10	33.20
		Pig 2	1,805	3	4	1	13	4.95
		Pig 3	5,382	3	4	1	10	8.43
		Pig 4	4,523	1	4	2	12	3.94
		Pig 5	5,795	1	4	1	15	9.53
10 µL	30	Pig 1	3,049	1	4	1	19	31.77
		Pig 2	3,934	0	4	3	22	46.35
		Pig 3	4,563	1	4	1	23	49.62
		Pig 4	5,129	1	4	8	17	14.41
		Pig 5	4,215	0	4	1	25	13.20
10 µL	40	Pig 1	4,192	0	4	5	27	27.64
		Pig 2	4,380	0	4	4	38	18.38
		Pig 3	5,117	1	4	2	35	15.84
		Pig 4	5,206	0	4	2	27	10.95

* DIS = number of damaged injection sites

Table 5. Descriptive statistics of the primary and secondary outcome measures at increasing injection volumes.

Predictors								
Volume	**Number**	**Outcomes**	**n**	**Mean**	**Median**	**Standard deviation**	**Minimum**	**Maximum**
*10 μL	20	Neuron number per mm^3	5	2342.56	1669.70	1409.41	1641.44	4857.07
		Number of damaged injection sites	5	0	0	0	0	0
		Tarlov score day 14	5	4	4	0	4	4
		Time back to baseline Tarlov score (days)	5	1.4	1	2.07	0	5
		Number of identified grafts	5	9.4	9	7.83	1	20
		Engraftment percentage	5	17.75	15.27	16.60	2.60	43.99
25 μL	20	Neuron number per mm^3	5	2260.29	1850.91	999.43	1438.20	3944.96
		Number of damaged injection sites	5	0.8	1	0.84	0	2
		Tarlov score day 14	5	4	4	0	4	4
		Time back to baseline Tarlov score (days)	5	4	2	5.61	1	8
		Number of identified grafts	5	12.8	12	2.77	9	16
		Engraftment percentage	5	11.74	12.25	5.82	4.42	20.08
50 μL	20	Neuron number per mm^3	5	4101.14	4522.64	1670.73	1805.15	5794.51
		Number of damaged injection sites	5	2	2	1	1	3
		Tarlov score day 14	5	4	4	0	4	4
		Time back to baseline Tarlov score (days)	5	1.4	1	0.55	1	2
		Number of identified grafts	5	12	12	2.12	10	15
		Engraftment percentage	5	12.01	8.43	12.07	3.94	33.20

* Same group of animals was used for the injection volume and escalation experiment

Table 6. Descriptive statistics of the primary and secondary outcome measures at increasing injection numbers.

Predictors								
Volume	**Number**	**Outcomes**	**n**	**Mean**	**Median**	**Standard deviation**	**Minimum**	**Maximum**
*10 μL	20	Neuron number per mm^3	5	2342.56	1669.70	1409.41	1641.44	4857.07
		Number of damaged injection sites	5	0	0	0	0	0
		Tarlov score day 14	5	4	4	0	4	4
		Time back to baseline Tarlov score (days)	5	1.4	1	2.07	0	5
		Number of identified grafts	5	9.4	9	7.83	1	20
		Engraftment percentage	5	17.75	15.27	16.60	2.60	43.995
10 μL	30	Neuron number per mm^3	5	4177.86	4214.57	772.74	3048.61	5128.90
		Number of damaged injection sites	5	0.6	1	0.55	0	1
		Tarlov score day 14	5	4	4	0	4	4
		Time back to baseline Tarlov score (days)	5	4	1	5.66	1	8
		Number of identified grafts	5	21.2	22	3.19	17	25
		Engraftment percentage	5	31.07	31	17.14	13.20	49.62
10 μL	40	Neuron number per mm^3	4	4723.73	4748.33	512.87	4191.78	5206.46
		Number of damaged injection sites	4	0.25	0	0.5	0	1
		Tarlov score day 14	4	4	4	0	4	4
		Time back to baseline Tarlov score (days)	4	2.25	2	1.26	1	4
		Number of identified grafts	4	31.75	31	5.62	27	38
		Engraftment percentage	4	18.20	17.11	7.00	10.95	27.64

* Same group of animals was used for the injection volume and escalation experiment

Table 7. One-way analysis of variance (ANOVA) for primary and secondary outcomes using injection volume as dependent variable.

Independent variables (outcomes)	Degrees of freedom	Mean Square	F-value	p-value†	R^2
Neuron number per mm^3	2	5406750.79	2.81	0.099	0.32
Number of damaged injection sites	2	5.07	8.94	0.004*	0.60
Time back to baseline Tarlov score (days)	2	3.27	0.74	0.50	0.11
Number of identified grafts	2	15.8	0.64	0.54	0.10
Engraftment percentage	2	57.76	0.38	0.69	0.06

Note: dependent variable (injection volume: 10, 25 and 50 μL)
† α=0.05. Significant p-values are marked with an asterisk (*)

Table 8. Tukey's post-hoc pair-wise comparison for the statistically significant ANOVAs using injection volume as dependent variable.

Independent variable	Dependent variable	Groups	p-value†
Number of damaged injection sites	Injection volume	10 vs. 25 μL	0.25
		10 vs. 50 μL	0.03*
		25 vs. 50 μL	0.06

† α=0.05. Significant p-values are marked with an asterisk (*)

Table 9. One-way analysis of variance (ANOVA) for primary and secondary outcomes using injection number as dependent variable.

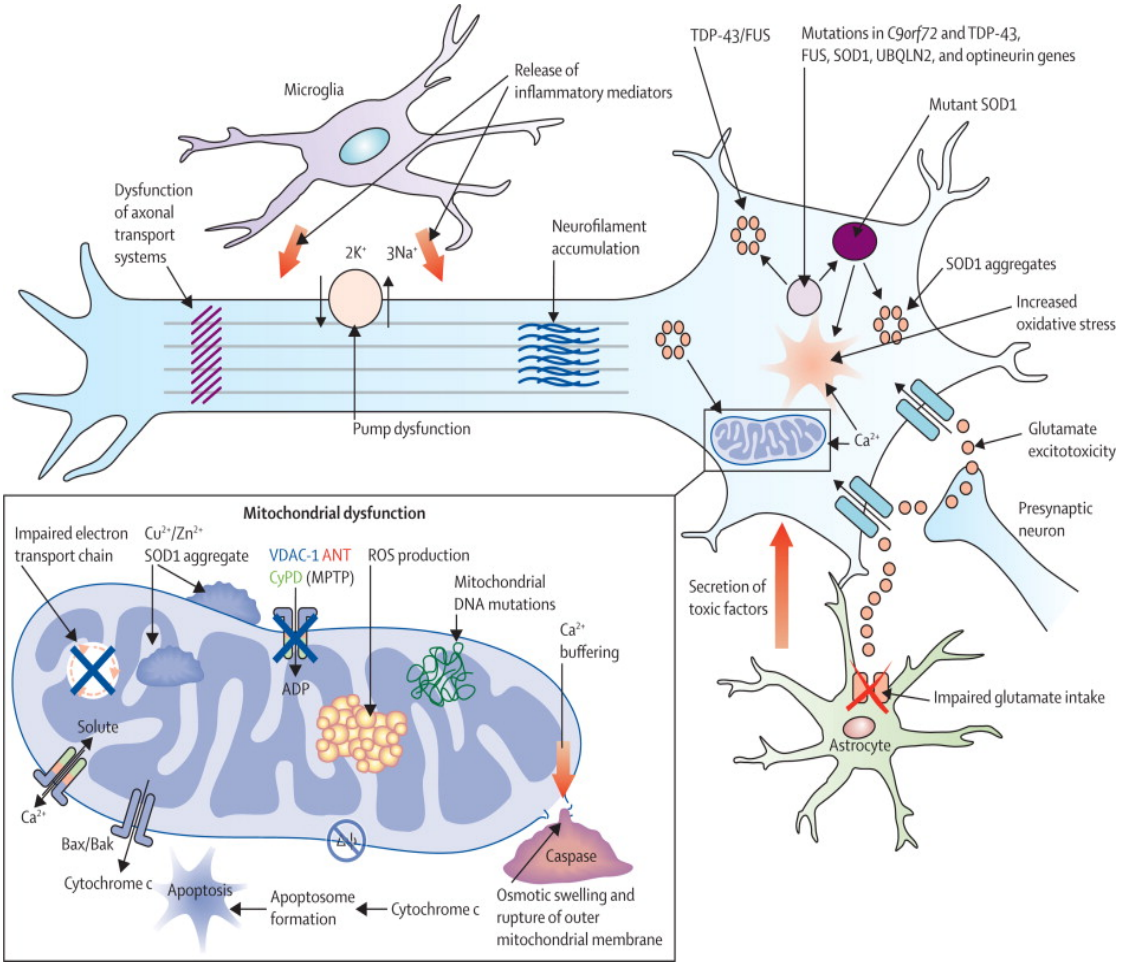
Independent variables (outcomes)	Degrees of freedom	Mean Square	F-value	p-value†	R^2
Neuron number per mm^3	2	7270261.12	7.19	0.01*	0.57
Number of damaged injection sites	2	0.45	2.56	0.12	0.32
Time back to baseline Tarlov score (days)	2	2.48	0.46	0.64	0.08
Number of identified grafts	2	560.625	16.20	0.0005*	0.75
Engraftment percentage	2	276.80	1.26	0.32	0.19

Note: dependent variable (injection number: 20, 30 and 40)
† α=0.05. Significant p-values are marked with an asterisk (*)

Table 10. Tukey's post-hoc pair-wise comparison for the statistically significant ANOVAs using injection number as dependent variable.

Independent variable	Dependent variable	Groups	p-value†
Neuron number per mm^3	Injection number	20 vs. 30	0.04*
		20 vs. 40	0.01*
		30 vs. 40	0.71
Number of identified grafts	Injection number	20 vs. 30	0.02*
		20 vs. 40	0.004*
		30 vs. 40	0.05

† α=0.05. Significant p-values are marked with an asterisk (*)



* Δ ψ =mitochondrial membrane potential.
** (Image source: Turner MR, Hardiman O, Benatar M, et al., 2013)

Figure 1. A schematic of the underlying mechanisms of neurodegeneration in ALS. Many of these mechanisms of cell death are shared by a variety of neurological disorders. Specific mechanisms involving recent genetic discoveries, such as the C9orf72 hexanucleotide repeat expansion, have not been fully elucidated. Neurodegeneration in ALS involves oxidative stress secondary to the generation of free radicals, cytoplasmatic aggregates of mutant proteins (SOD1, TDP-43), mitochondrial dysfunction and the disruption of axonal transport processes. The inhibition of VDAC1 conductance by SOD1 aggregates decreases mitochondrial energy production. Mitochondrial dysfunction is associated with increased production reactive oxygen species (ROS) that in combination with other trans-membrane processes induce cell death by apoptosis. Additionally, activation of neighbor microglia (e.g. astrocytes) results in secretion of proinflammatory substances that further enhance the cytotoxicity.

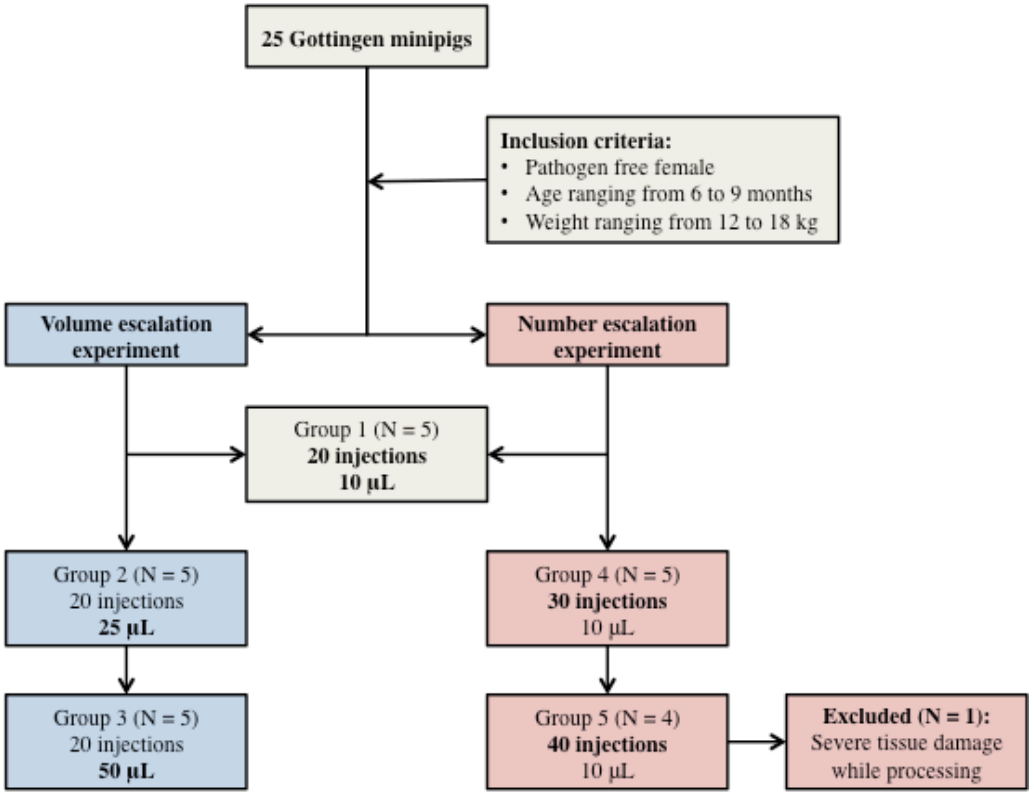


Figure 2. Schematic outlining the experimental design. Twenty-five Gottingen minipigs met the inclusion criteria to be included in the study. The animals were randomly assigned to one of the five groups. Group 1 was the reference group for both experiments and one animal from group 5 was excluded due to irreparable tissue damage during sectioning.

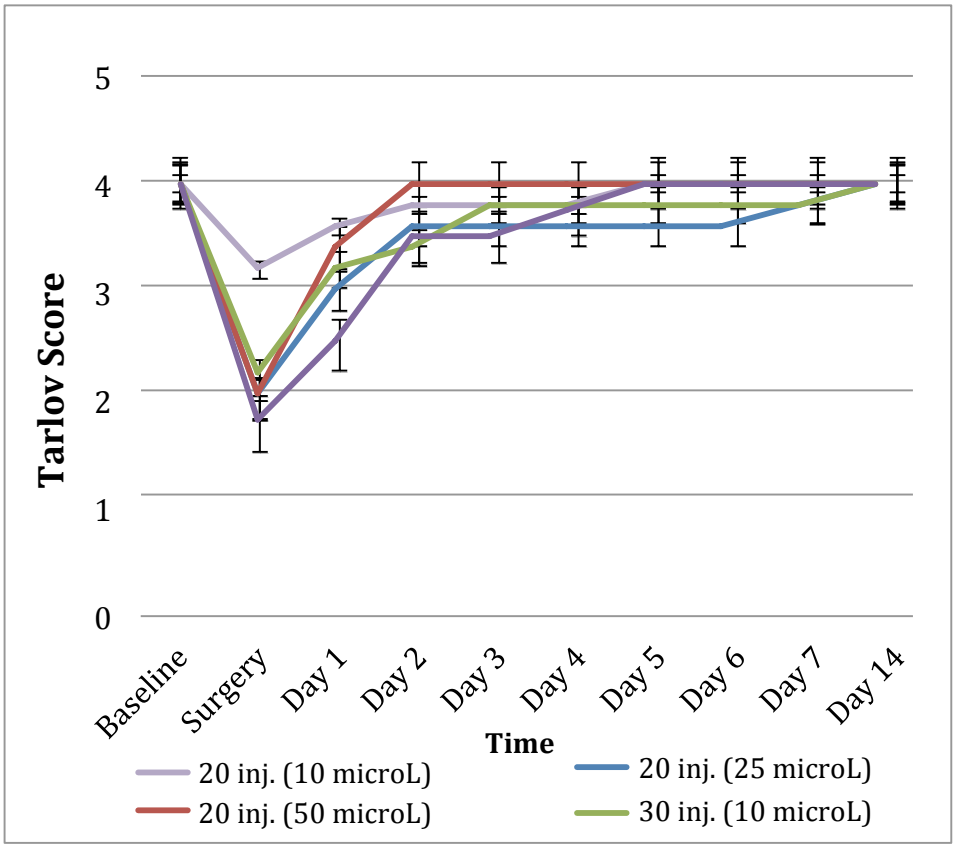


Figure 3. Postoperative motor function assessment of experimental groups using Tarlov score. This figure shows the postoperative motor function assessment (Tarlov score) for each one of the five study groups. All animals came back to baseline at postoperative day 14 regardless of the injection protocol. Additionally, one of the groups (20 inj. / 10 microL) appears to have better immediate postoperative morbidity when compared to the rest of the groups.

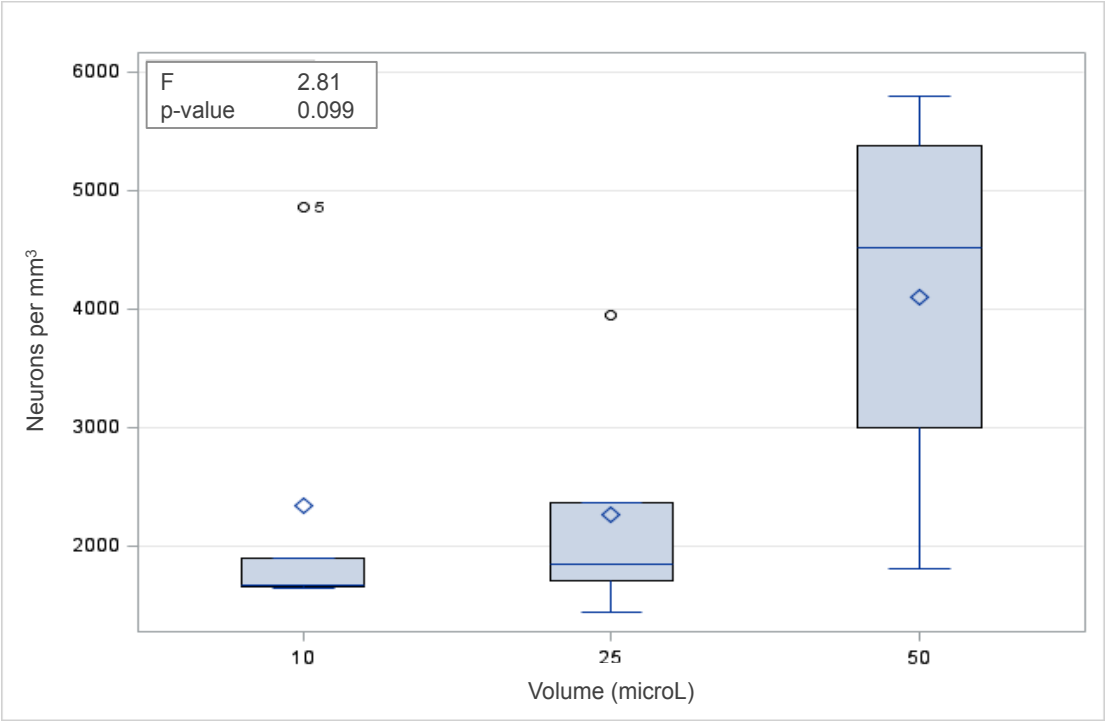


Figure 4. Distribution of number of neurons per mm^3 using injection volume as predictor. This figure indicates that as injection volume increases no loss in neuronal density was observed.

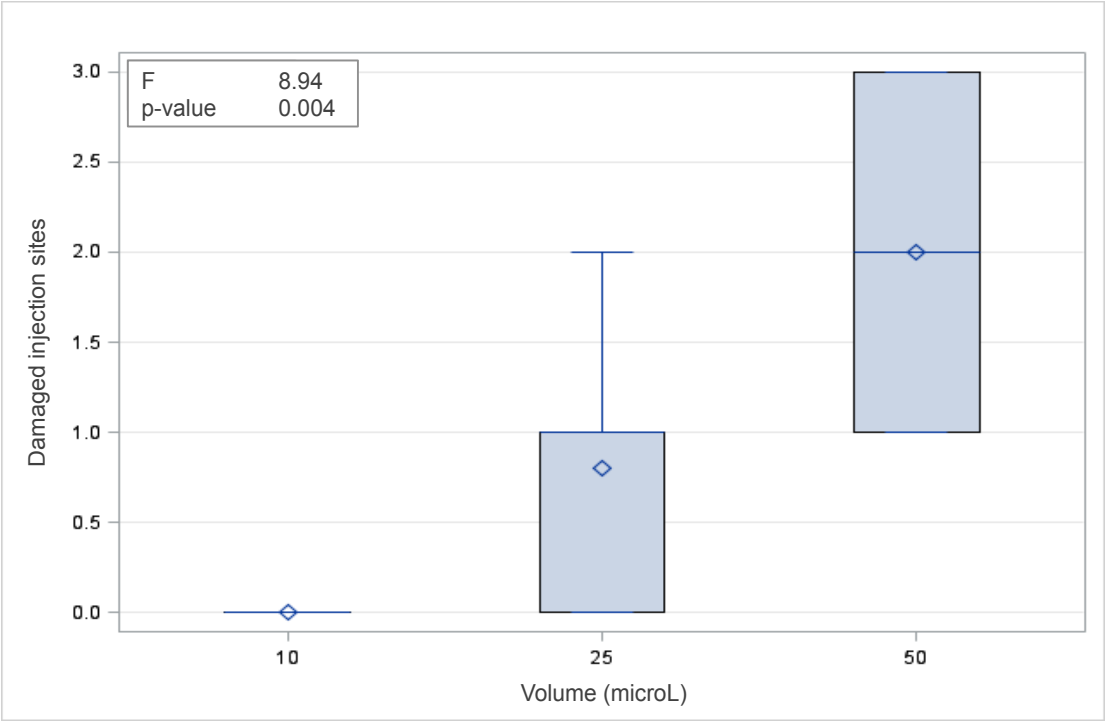


Figure 5. Distribution of number of damaged injection sites using injection volume as predictor. This figure shows that significant tissue damage was observed at higher injection volumes.

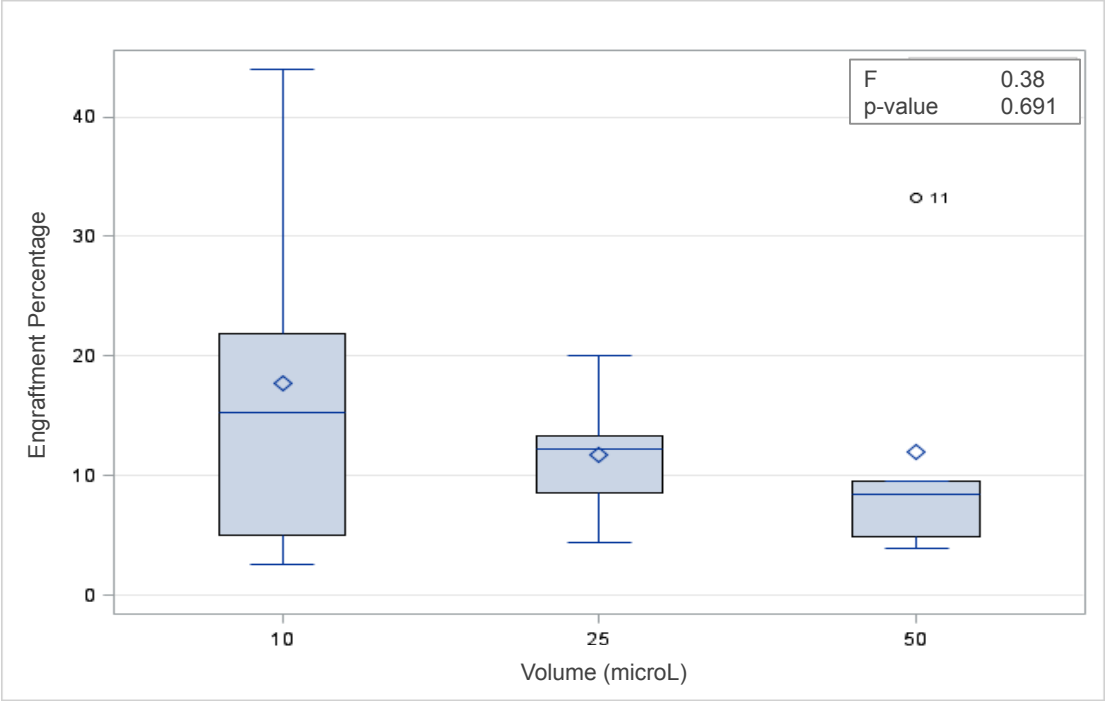


Figure 6. Distribution of engraftment percentage using injection volume as predictor. This figure shows the small variance in engraftment percentage across different injection volumes. However, this figure does not reflect the increased total cell survival seen at higher volumes.

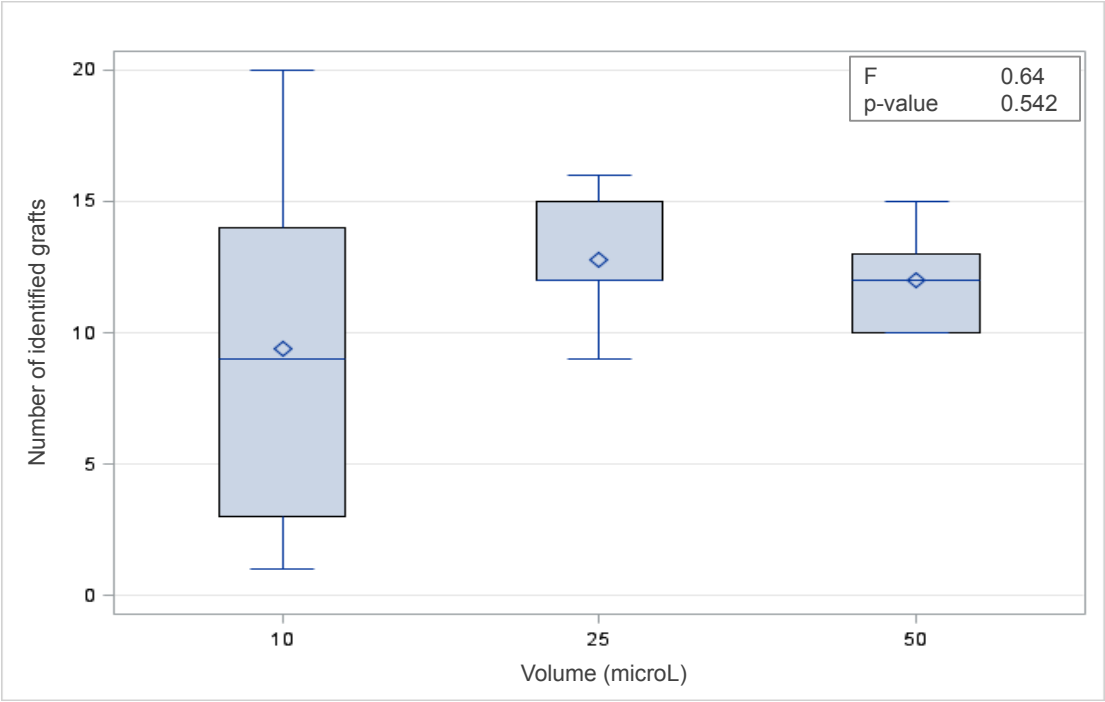


Figure 7. Distribution of number of identified grafts using injection volume as predictor. This figure shows that across different injection volumes the number of identified injections remained fairly constant

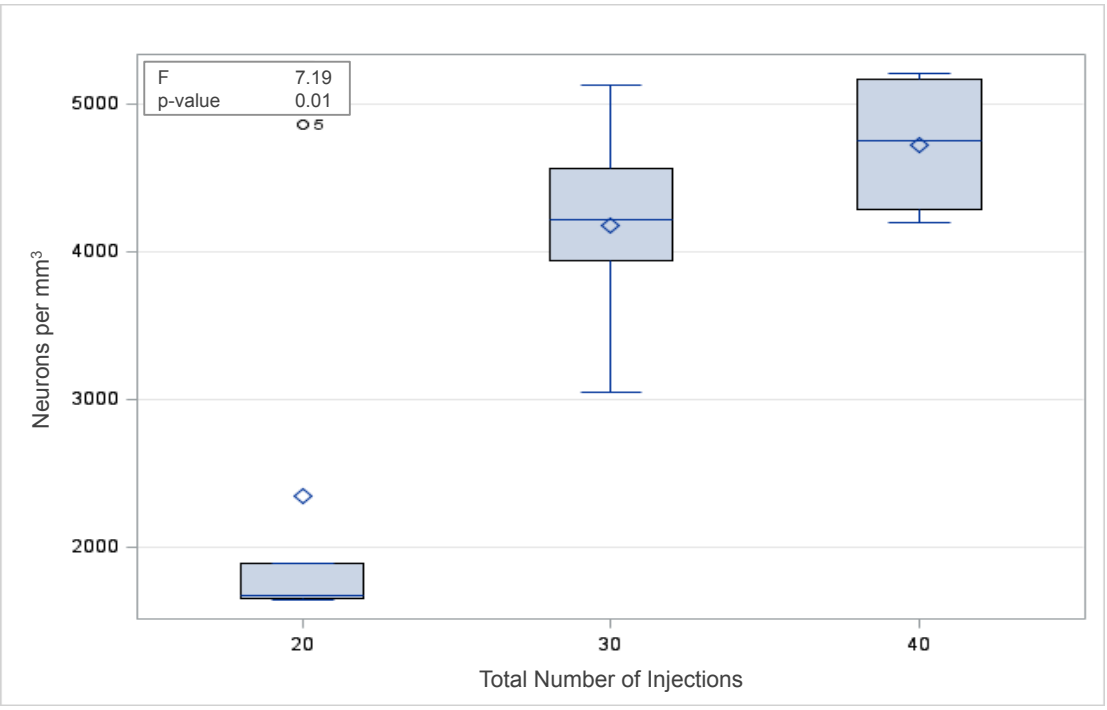


Figure 8. Distribution of neurons per mm^3 using injection number as predictor. This figure indicates that as the total number of injections was increased, a higher neuronal density was observed.

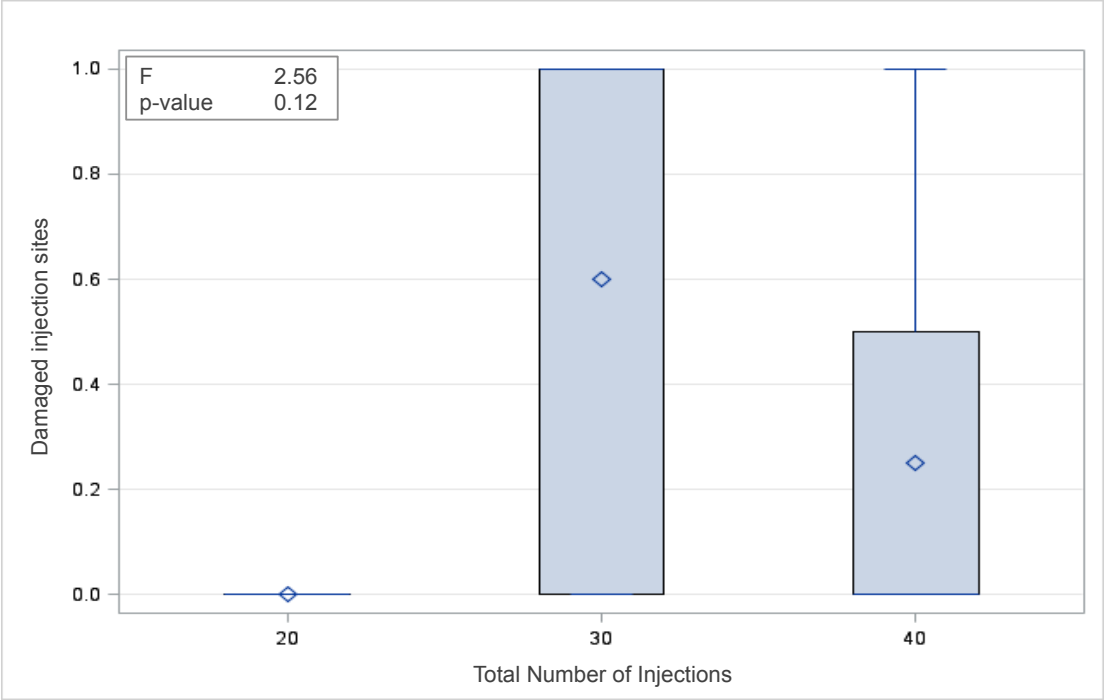


Figure 9. Distribution of damaged injection sites using injection number as predictor. This figure shows that as the total injection number was increased, no significant tissue damage was observed.

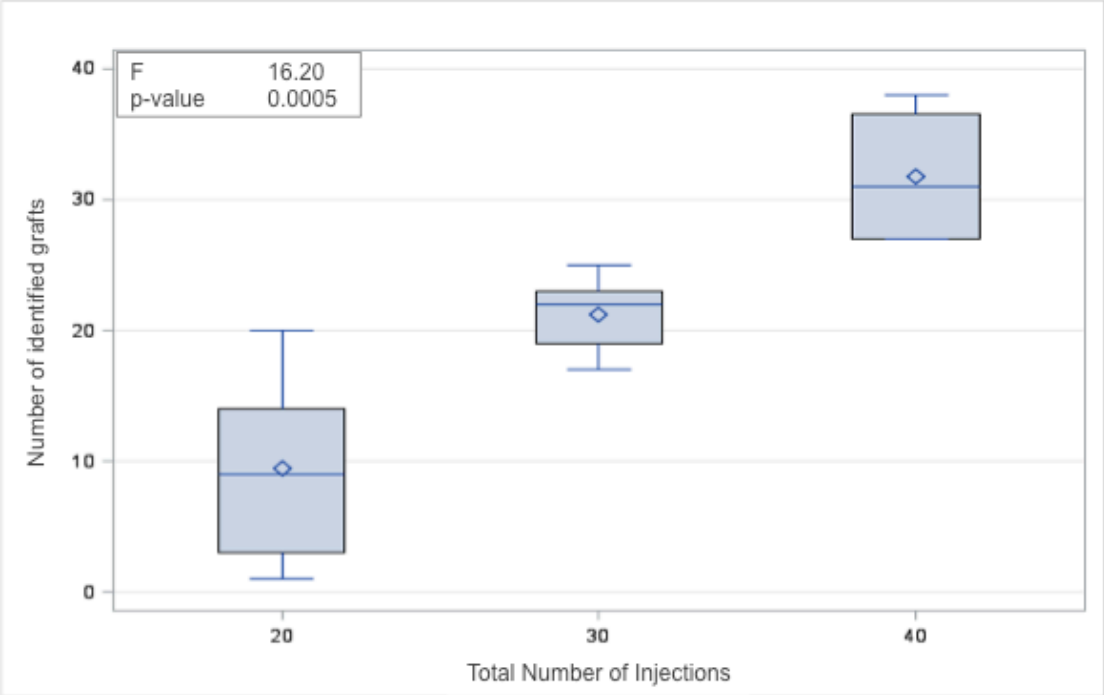


Figure 10. Distribution of number of identified grafts using injection number as predictor. This figures shows that the number of identified grafts significantly greater as the total number of injections was increased.

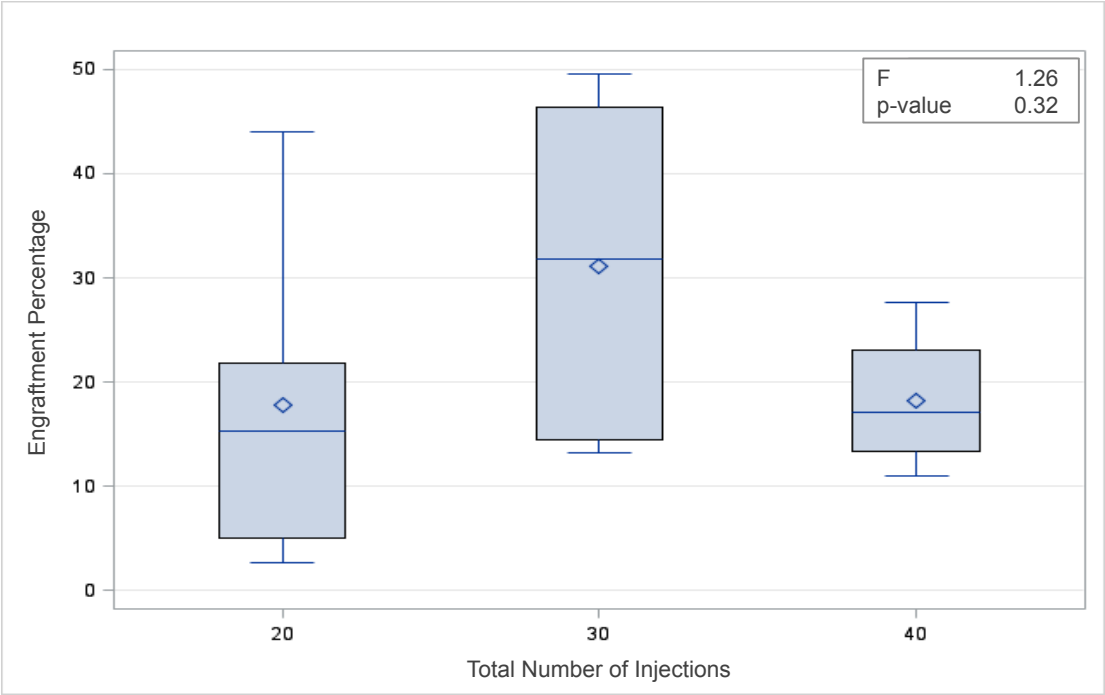


Figure 11. Distribution of engraftment percentage using injection number as predictor. This figure illustrates that the group receiving 30 (10 µL) injections had the best engraftment percentage (31%) when compared to the other groups.

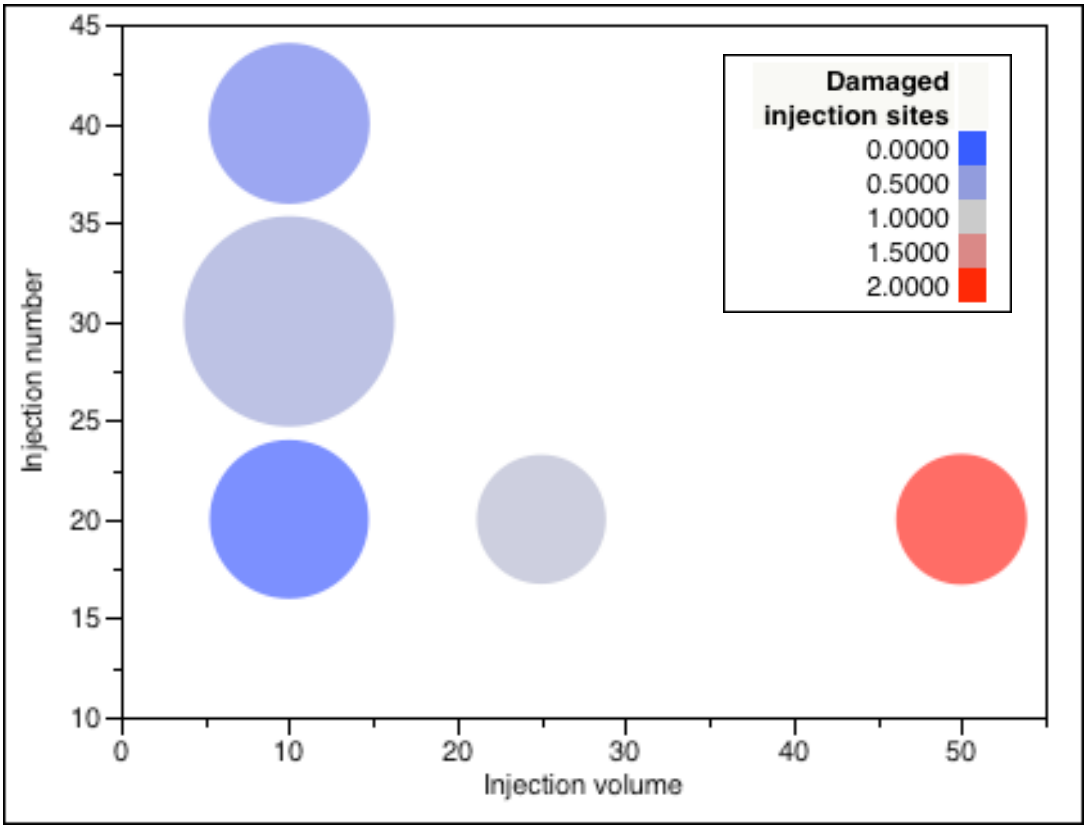


Figure 12. Comparison of injection number and volume sized by engraftment percentage and colored by the number of damaged injection sites. This graph shows that as injection volume increases the number of damaged injection sites increases and the engraftment percentage remains similar between groups (11-17%). Additionally, it shows that as number of injection increases tissue damage stays relatively the same but at 30 injections the best engraftment percentage is achieved (31%).