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ABSTRACT

THE *IN VIVO* RESPONSE TO PERIPHERAL NERVE CRYOPRESERVATION IN AN ANIMAL MODEL

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Adult male Sprague-Dawley rats were used as a model to study peripheral nerve freezing with the cryopreservation agent dimethyl sulfoxide (DMSO). Three experimental groups of ten rats were used. The first had a one centimeter segment of the sciatic nerve frozen with liquid nitrogen for 45 seconds. The second received a ten minute application of a ten percent solution of DMSO and then the nerve was frozen with liquid nitrogen. The third group was a control and had a sham operation. Muscle weights and functional and histological evaluations of nerve regeneration of the three groups were compared.

Results showed a complete functional loss post-operatively in both experimental groups by both quantitative gait analysis and three qualitative tests. Between week two and three, return of function began and was complete by week five in the freezing only group. In the freezing and DMSO group, a functional deficit remained until the conclusion of the experiment at ten weeks (-34.1% vs -4.2% for freezing only and -2.4% for controls). Tibialis anterior muscle weights at ten weeks averaged 100.9% of the non-operated side for controls, 88.2% for

freezing only, and 70.6% for freezing and DMSO. Axonal counts in the injured segment were equal to or greater than the proximal segment by the fifth week. At ten weeks, the freezing only group had 22% more axons in the injured segment than the proximal segment, and the freezing and DMSO group had 56% more.

The results show that freezing the rat sciatic nerve to -196° C after local pretreatment with 10% DMSO results in good reinnervation as measured by axonal counts, muscle weights, and functional testing. However, better results were seen with freezing only, with the limb being functionally indistinguishable from the contralateral side five weeks after freezing.

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Literature Review

I. Purpose of the Study

Freezing is useful in producing controlled tissue destruction. In the case of surgery for cancer, freezing produces tumor necrosis and avoids the problem of spillage of malignant cells (14). The freezing of peripheral nerve produces a prolonged functional deficit which typically resolves completely over a period of weeks (5,12,71). The purpose of this investigation is to utilize an experimental model of peripheral nerve freezing and the use of cryopreservative agents which is meaningful in terms of functional recovery. Many authors have reported the results of animal studies of nerve freezing in terms of histological examination or electrophysiological studies. These results have limited significance when extrapolated to humans (1). Others have looked at a single, qualitative functional evaluation in combination with histological or electrophysiological studies for the assessment of nerve function. In the human studies involving nerve freezing which have been performed, results are usually reported in terms of sensory return, and return of motor function has not been quantified. Therefore, we have explored the area of nerve freezing and use of a cryopreservative agent in the rat sciatic nerve model. Three groups of ten rats were

used: the first underwent nerve freezing alone, the second was pretreated locally with dimethyl sulfoxide before nerve freezing, and the third had a sham operation. Return of function was quantified by gait studies and three qualitative assessments of nerve function. The tibialis anterior muscle weight was measured and axonal counts were performed.

II. Clinical Aspects of Freezing Peripheral Nerve Tissue

A. Cryosurgery

The application of temperatures significantly below freezing produces cell death and tissue necrosis (14). As a surgical agent, cold produces anesthesia, coagulation, and controlled destruction (9). When used in surgery for cancer, freezing has the advantage of minimizing release of viable malignant cells into the circulation (14). For these reasons, several authors have explored the use of cryosurgery for cancer in both humans and animal models.

i. Human Studies

In 1963, Cooper (14) reported the use of extreme cold in the destruction of tissues during neurosurgical and general surgical procedures. He developed a liquid nitrogen cooled probe to induce both reversible physiological inhibition and permanent lesions in the

brain. The use of this apparatus in cryothalamectomy, en bloc resection of an astrocytoma, and freezing of a rectal carcinoma is described. Cooper concludes that cryosurgery is anesthetic, hemostatic, physiologically reversible at temperatures above freezing and is tolerated without insult to the organism.

Marcove and various co-authors have reported on the use of freezing in the treatment of many types of tumors (41-49). In 1964, they performed their first bone freeze in a patient with primary lung carcinoma and a painful metastasis to the humerus which was unreleived by two courses of x-ray therapy. Cryosurgery resulted in complete pain relief (41). Two methods of cold application are described. A double lumen probe cooled to -180° C and capable of producing a temperature of -20° C at a distance of one inch is used for at least three freeze-thaw cycles. A second method is employed after bone curettage where a funnel is sealed to the opening with gelfoam and the cavity is filled with liquid nitrogen.

In 1969, Marcove reported on a series of 50 cases of primary and metastatic bone tumors treated with cryosurgery (42,43). There were seven recurrences, and cryosurgery was repeated seven times. Six patients had preoperative fractures and four suffered postoperative fractures. Skin necrosis developed in six patients, two of which developed subsequent infections. Four patients developed nerve palsies, which resolved spontaneously. No mention is made

as to the time period required for resolution of the nerve palsy.

In 1972, Marcove (44) reported on the treatment of eight patients with bone metastases from renal cell carcinoma. Four had limited bone lesions and four had widespread metastasis. Seven of the patients were treated by direct application of liquid nitrogen as previously described, the other receiving bone freeze using the cryoprobe. Three of the four patients with limited bone lesions remained disease free, with follow-up to over seven years. In the group with widespread metastases, palliation was good in all cases and the longest survival was three years and four months after the onset of symptoms.

The treatment of low and medium grade chondrosarcoma with cyrosurgery is discussed in a 1977 paper by Marcove et al. (47). Eighteen patients (7 grade 1, 11 grade 2 on a scale of 1 to 3) were treated using the same cryosurgical techniques. At an average follow-up of 66 months, there were no deaths, and seventeen of the patients were disease free. Five patients had recurrent tumor at rebiopsy which was controlled with curettage and cryosurgery or *en bloc* resection. Complications of cryosurgery noted were: four fractures at the operative site, three cases of joint stiffness, one delayed wound healing, and four temporary nerve palsies, which became "minimal to completely absent with time".

The role of cryosurgery in the treatment of giant cell tumors is discussed in three of Marcove's papers (45,48,49). 52 patients were treated by direct application of liquid nitrogen. 26 patients had previous operative treatments and nine of these had had multiple procedures. The results were divided into the first 25 cases and the second 27 because of improvements in technique over time. The first group had nine recurrences at rebiopsy, and an average follow-up time of 66 months. The second group had three recurrences in the 25 who underwent rebiopsy, none of which were in the original area treated. The average time of follow-up was 22 months. All patients were free of disease at the time of report. Four patients had nerve palsies, three of which resolved completely. The fourth had a sciatic nerve palsy which had not yet resolved after a few months follow-up.

Marcove has demonstrated considerable success with these cryosurgical techniques. The results in 128 patients are described. The postoperative complications reported include skin necrosis (10 cases), wound infection (10), postoperative fractures (24), and temporary nerve palsies (12). Eleven of these twelve nerve palsies resolved over the period of follow-up reported. He describes the deficits as becoming "minimal to completely absent with time". The twelth was a sciatic nerve palsy which had not yet resolved after "a few months" of follow-up. His results indicate that in the human, functional

nerve regeneration following freeze injury can be accurate and complete.

Terao et al. (72) reported in 1983 the use of a spray of supercooled nitrogen gas at -196° C to freeze the posterior nasal branches in the pterygopalatine foramen in 102 cases of vasomotor rhinitis. All patients had failed to respond to antihistamines. At follow-up six months or more postoperatively, 77 of the 102 patients had a good to excellent response. Of the 25 failures of treatment, twelve were due to recurrence of symptoms after several months. This is presumably due to nerve regeneration. The authors also used the experimental procedure in dogs and found destruction of the epineurium, slight injury to the perineurium, and a varying extent of damage to the nerve fibers. The authors conclude that the cryosurgical technique developed provides adequate relief of vasomotor rhinitis. Longer follow-up would be useful to determine if more patients would have recurrence of symptoms due to nerve regeneration.

ii. Animal Models for Cryosurgery

Animal models have been used in an effort to evaluate the effect of varying the freezing temperature, number of freeze-thaw cycles, and cryoprobe surface area, as well as the results of freezing a tumor which has involved a peripheral nerve.

Neel *et al.* (56) reported on the freezing of tumors with a cryoprobe utilizing the mouse model. Three tumor-host systems were used and were introduced to the flank of the animal. The tumor was then frozen with a cryosurgical probe and the animals were observed for eight weeks. Increasing the number of freeze-thaw cycles and decreasing the probe temperature were found to increase tumor control. It was necessary to freeze the tissues to temperatures below -60^{o} C to acheive effective tumor control. The best results were obtained using multiple freeze-thaw cycles with a probe temperature of -180^{o} C and a larger probe tip to promote rapid heat transfer. The cure rate under these conditions was 83 percent and was 100 percent for two of the three tumor-host systems. They conclude that cryosurgery can effect good tumor control in the mouse model.

The results of Neel's research provide support for the use of cryosurgery for cancer, but the tumor was not implanted near any important structures. To further investigate the use of cryosurgery when tumors have involved peripheral nerve, Breidenbach *et al.* (11) used rats and implanted the Walker 256 tumor on the facial nerve. The tumor was frozen repeatedly with a cryoprobe 48 hours after implantation. Of the ten rats treated in this manner, five remained without tumor. These rats underwent repeat freezing of the tumor at four day intervals until no active tumor was evident. Five freezings or less were

necessary to control the tumor in these rats. All rats whose nerve was frozen had return of function within 21 days as demonstrated by return of whisker motion and loss of nose deviation to the unoperated side. They conclude that cryosurgery is effective for destruction of the tumor and preservation of the nerve.

B. Cryoanalgesia

Given the ability of freezing to produce a second degree nerve injury (70) which results in minimal inflammatory reaction and scarring (12,74), and a consistent return of normal function (5,12,71), cryogenic injury to peripheral nerve offers an interesting solution to the problem of providing prolonged anesthesia.

In 1974, Nelson et al. (57) reported the use of freezing of the intercostal nerves at the time of thoracotomy to reduce pain level in 38 patients. Five intercostal nerves were isolated from the adjacent vessels one to two cm lateral to the transverse process and visibly frozen with a cryosurgery probe (probe temperature is not given). The total dose of Demerol (meperidine) given over the first five postoperative days was compared to 38 control patients who had not received nerve freezing. The narcotic use was found to be significantly lower in the experimental group than in controls. Pain not masked by

the procedure included the drainage tube sites and shoulder pain referred from the pleura. The authors found a band of anesthesia about four inches wide in the area of the incision in which sensation began to return at six to eight months and is nearly normal at twelve months. No neuritis or neuromas were found in up to 24 months of follow-up. They conclude that intercostal nerve freezing provides a more effective alternative to the use of local intercostal block.

Lloyd, Barnard, and Glynn (33) described the freezing of peripheral nerves in 64 patients with intractible pain. Two freeze-thaw cycles to -60° C and both open and closed application of the cryoprobe was employed. The median duration of pain relief was eleven days, with a range of up to 224 days. Twelve patients received no relief. Occasional relief of pain was found even after the return of motor and sensory function. The authors proposed the term cryoanalgesia for the blocking of peripheral nerves by the application of a cryoprobe. This group of authors later reported on the use of cryosurgery in the management of intractable facial pain in 21 patients (3). In the eleven patients treated with cryosurgery alone, median duration of pain relief was 116 days and of sensory loss was 49 days. In 1980, they reported on 29 patients treated with cryoanalgesia at thoracotomy (22). The same freezing technique (to -60° C for two cycles of one minute using a cryoprobe) was employed. One intercostal nerve above and

below the incision was frozen at the time of surgery. They were found to require less narcotics ($p < 0.005$) than a matched group of 29 controls. 26 of the 29 patients did report pain away from the thoracotomy site. From these three studies, they conclude that nerve freezing is an effective and simple technique for providing long term relief for chronic pain and postoperative pain.

Katz et al. (27) also used cryoanalgesia for post-thoracotomy pain, confirming the results of the above study and also performing pulmonary function tests. Five to six intercostal nerves about the incision were frozen for two cycles. The fifteen patients treated had lower subjective pain scores and lower narcotic requirements. There was, however, no difference in mean forced vital capacity. They report a duration of pain relief of two to three weeks and full return of sensation by post-op day 30. This return of function is much more rapid than that reported by Nelson (57), who did not see near normal sensation until twelve months postoperatively. Katz does not mention the location of the freeze injury and it may have been much closer to the incision than the very proximal freeze employed by Nelson.

Maiwand and Makey (39) reported in 1981 on a project similar to the previous studies, in which 100 patients received cryogenic intercostal nerve block. Their results are also similar to previous work. 79 were reported to be pain free, twelve had some discomfort, and only nine had

severe pain requiring narcotics. Normal sensation in this series returned by three to six months.

Evans, Lloyd, and Green (19) explored the use of cryoanalgesia in the rat sciatic nerve model. Temperatures from 0 to -60° C and up to three freeze-thaw cycles were used. They found that freezing to temperatures of -20° C and below resulted in a consistent, prolonged sensory loss, lasting 38 days on average. They used a qualitative evaluation of the animals use of the limb. Return of motor function to the thigh muscles was apparent at a mean of 24.5 days and motor function to the distal musculature was complete at 38 days (mean). This is consistant with a rate of axonal growth of 1-1.5 mm per day. The authors found no effect of decreasing the freeze temperature to below -20° C or from increasing the number of freeze-thaw cycles. There was minimal fibrosis and scarring at the site of the injury. The authors conclude that nerve freezing offers an effective method to provide prolonged but reversible nerve block.

To summarize, freezing peripheral nerve in humans for the purpose of prolonged analgesia results in a consistent, long term deficit which resolves completely over a period of weeks to months (57,3,22,27,33,39). The animal study by Evans (19) points out the difference between a nerve freeze to temperatures of -20° C or below, which results in a prolonged loss of function, and the short term neuropraxia found after briefly cooling the nerve to temperatures of -

10° C or above, which can resolve over a period of minutes to hours.

III. Research on the Effect of Freezing on Peripheral Nerve Tissue

A. Mechanism of Cryogenic Injury

Hippocrates was the first to note the analgesic effect of extreme cold on peripheral nerve (25). Many investigators have reported on this matter since that time. In 1916-1919, Trendelenberg (74) noted the speed and quality of nerve fiber regeneration in man and animals following freeze injury. He advocated nerve freezing as the most gentle method of interupting nerve function and was impressed with the absence of neuroma formation and the excellence of regeneration. Bielschowsky and Valentin (6) continued these experiments and further defined the histopathology of these lesions at various times after freezing. After freezing the dog sciatic nerve for five minutes, they found all the fibers degenerated and regeneration was complete by 91 days. They felt circulatory stasis could be the mechanism of injury but could not obtain critical evidence on this point.

In 1945, Denny-Brown *et al.* (16) published a classic study detailing both the histological and functional changes following cooling and freezing the sciatic nerve in

cats. Cooling was accomplished using a metal jacket placed around the nerve and cooled with a circulating salt solution. The actual nerve temperature attained was measured with a thermocouple. Cooling the nerve to 8-11.6° C resulted in little to no clinical damage. Cooling to 5-6° C led to a long term loss of motor and sensory function with destruction of the large, myelinated fibers. They found that motor function was more sensitive to cold induced injury than sensory function, and that pain function was the most resistant. When the nerve was "frozen solid" (indicating a temperature of at least -4° C) with a CO_2 spray for over 45 seconds, they again observed a complete loss of motor and sensory function. Examination by light microscopy at 2-3 days revealed an intense inflammatory reaction with loss of Schwann cells and proliferation of fibroblasts. The axons were coiled, fragmented, and swollen. At 10-14 days, there was degeneration of myelinated nerve fibers and a regeneration of small diameter nerve fibers accompanied by proliferating Schwann cells and phagocytic histiocytes. At three months, regeneration was complete, showing excessive cellularity and the presence of Schwann cells. The axons and myelin sheaths were thinner than normal. To summarize, they found myelin and axon cylinders to be selectively damaged by cold, with large, myelinated fibers being more susceptible to injury than small, unmyelinated fibers. This is consistant with their observation that motor function is

more sensitive to cold injury than sensory or pain function. Severe freezing led to pan-necrosis of the entire nerve bundle.

Whittaker (76) confirmed these results in an experiment where he evaluated the histological changes caused by freezing the ventral epithelium of the tongue of hamsters. Wallerian degeneration in nerves was found to be accompanied by sensory loss. Sensation returned within 7 to 14 days. Recovery is more rapid than the more proximal lesion described by Denny-Brown (16) because the injury is close to the end organ and the distance over which the nerve must regenerate is much less.

It can be seen that nerve freezing leads to a long term loss of nerve function, which returns after nerve regeneration. The response of the nerve to cooling to temperatures above freezing was investigated by Basbaum (4). Compound action potentials were recorded before, during, and after cooling the cat sciatic nerve to 5^{o} C for two hours. After twelve minutes of cooling, a total nerve block was obtained. When the nerve was rewarmed, function was restored. In spite of this, the large axons were observed by light and electron microscopy to undergo Wallerian degeneration and segmental demyelination. The authors did not comment on function of the nerve over the course of this experiment, but one can assume that a prolonged nerve palsy ensued.

To investigate the changes in intraneural pressure and permeability following cryogenic lesions to peripheral nerve, Myers *et al.* (55) recorded intraneural fluid pressure and examined histological morphology in rat sciatic nerves frozen to -60° C. Maximum values of 23 cm H_2O (normal is 2 cm H_2O) were recorded at 90 minutes and a second peak occurred several days later. Perfusion studies with horseradish peroxidase demonstrated much extravasation within the nerve. The authors conclude that the first peak in pressure was due to vascular damage, and the second to the process of Wallerian degeneration.

A gradation in the response of peripheral nerve tissue to cooling can be seen. At exposure to temperatures above roughly 8 to 10° C, the nerve undergoes a temporary palsy which resolves over a period of minutes after rewarming. The nerve does not undergo lasting changes and Wallerian degeneration does not occur. Exposure to temperatures as high as 5° C or below has been shown to cause Wallerian degeneration and a loss of function for a period of weeks (16). Function is restored only after nerve regeneration has occurred. The degree of nerve injury increases as the temperature decreases, especially near the freezing point. For example, Lenz (31) found freezing to -25° C to be more damaging than freezing to -15° C. Nerve freezing leads to a consistent loss of nerve function followed by Wallerian degeneration of the nerve fibers (4,16,40,76). Several probable mechanisms of injury have been proposed:

dehydration and toxic concentration of electrolytes by removal of water from solution, intracellular ice formation, rupture of cell membranes, thermal shock, vasogenic edema, and denaturation of protein molecules within the cell membrane (4,21,32).

B. Regeneration Following Cryogenic Injury

Several experimenters have further explored the area of nerve freezing using histological examination. Mira (53,54) froze the rat sciatic nerve to -180° C and examined the regeneration in the nerve to the medial head of the gastrocnemious muscle. Regrowth began at 12 to 15 days and by one month the nerve contained a number of axons equal to or greater than the contralateral control nerve. At two years postoperatively, the nerve still contained 21 to 31 percent more axons than the contralateral nerve. Repeated freezings separated by three week intervals served to increase the number of axons present but also to decrease their size. A maximum occurred with three freezings, resulting in a 120 percent increase in the number of axons one month after the last freeze.

Nerves were also examined at various time intervals after three freezings three weeks apart (53). From the first to the third months, the number of nerve fibers decreased from 120 to 90 percent more than control. After that time, no further reduction in number was seen, but

there was a gradual increase in size and a gradual return to the bimodal distribution of size. At eighteen months, there was a lower percentage of large fibers and the average size of fibers had only reached 70 percent of control. Mira concludes that repeated freezing leads to a large and lasting increase in the number of myelinated fibers accompanied by a decrease in their size. The importance of the continuity of the basal lamina connective tissue sheaths in the regeneration process is also stressed.

Sjoberg *et al.*(68), using the rat sciatic nerve model, compared freezing injury to crushing in terms of growth rate, evaluated by a pinch test of the nerve and by immunocytochemical staining of neurofilaments. They found a growth rate of 3.3 mm/day for crush vs. a rate of 1.9 after freezing a 15 mm nerve segment. This was increased to 3.2 mm/day by decreasing the length frozen from 15 mm to 12 mm. Using tritiated thymidine incorporation, they found Schwann cell migration occuring from both proximal and distal ends of the injury, independent of axonal regeneration. They concluded that the living Schwann cells and/or other cells distal to the injury increase the rate of regeneration.

Other authors have used electrophysiological studies to examine the results of nerve freezing. Basbaum (4) investigated the response of the cat sciatic nerve to injury by cooling. Compound action potentials were

recorded before, during, and after cooling the nerve to 5° C for two hours. After twelve minutes of cooling, a total nerve block was obtained. When the nerve was rewarmed, function was restored. A return of 41 to 75 percent of the amplitude of the action potential and 85 to 100 percent of the conduction velocity was obtained after 30 minutes at 25° C. In spite of this, the large axons were observed by light and electron microscopy to undergo Wallerian degeneration and segmental demyelination. It can be assumed that nerve conduction was interrupted, but the author does not comment on sensory and motor function over time. Survival of small myelinated and unmyelinated fibers were evident. It was hypothesized that a small surface to volume ratio makes the large axons more susceptible to injury by cold, and that the mechanism of injury involves both vasogenic edema and enzymatic activation of the Schwann cell.

Makitie and Teravainen (40) recorded evoked spinal cord dorsum potentials after both cooling and freezing the sural nerve in rabbits. Although recording was performed in the central nervous system, their results are relevant to peripheral nerve function. Cooling to 12 to 2°C led to a maximal loss of about 40 percent of the evoked potential following distal stimulation. Cooling to 2° C to -2° C gave about a 70 to 80 percent loss. These changes normalized over the next 20 to 60 days. Freezing the nerve to -45° C led to the loss of response to distal stimuli.

There was a rapid return of response over day 50 to 100 and a slower return over day 150 to 450 to values over two times the original evoked potentials. The authors do not make a clear distinction between temporary neuropraxia and a prolonged loss of nerve function. The results, given in terms of evoked potentials, are not shown to be related to the ultimate functional outcome. This would have made their results much more meaningful.

Several experiments involve functional testing after nerve freezing. Carter *et al.* (12) froze the combined median-ulnar nerve in rats with a -100^{o} C probe. Return of muscular function began on the fourteenth day and was complete by day 25. Nerve conduction returned by day fourteen as confirmed by electromyography.

Functional recovery in the monkey and dog was examined by Beazley *et al.* (5), who froze the facial nerve in four rhesus monkeys (to -40^{o} C for 2 min x 2 with a cryoprobe) and followed regeneration with cinephotography. Evidence of regeneration began at the fifth week and was complete at the ninth week. They also froze the recurrent laryngeal nerve in dogs and monitored regeneration by observation of the position and motion of the vocal cord under direct laryngoscopy. Nine of the ten dogs had return of function in 36 to 56 days. Their conclusions are as follows. The rate of regeneration is 1.5 mm per day in both species, which is similar to the rate seen after crush injury and only slightly more rapid than following nerve repair. The

duration of palsy is directly related to the distance between the injury and the end organ, and that the duration of palsy is directly related to the time period that the nerve is below 0° C, to a maximum duration of 56 days (average was 47) for 4.5 minutes at -65° C.

To examine the possible absence of gamma innervation following nerve regeneration, Takano (71) used a ten minute application of dry ice to the sciatic nerve of the cat. The first functional signs of reinnervation were observed at three to four weeks. Functional recovery was followed by an informal gait analysis. At seven to eight weeks, the cats walked with a slight limp, and at nine weeks the gait was difficult to distinguish from normal. Conduction velocities were also tested and were found to be lower than the contralateral side, but they retained the normal bimodal distribution. None of the preparations tested showed any evidence of gamma innervation, as tested by three independent gamma-activating or gamma-blocking procedures (1. pinna stimulation, 2. selective blocking of small fibers by procaine, 3. intravenous application of diazepam [1 mg/kg]).

This is in contradiction to the results obtained by Scott (66), who tested conduction velocity in the common peroneal nerve in cats 140 days after freezing with dry ice. He also found a normal bimodal distribution but an overall decreased conduction velocity. By examining the response of muscle spindle primary endings to stretch, it

was determined that both efferent and afferent axons regenerate and have normal function. No evidence for selective loss of gamma axons was found, and he concludes that freezing with dry ice leads to degeneration of axons and myelin sheaths but leaves endoneurial tubes and connective structures intact. It is important to note, however, that only three cats were used in this experiment.

Bondoux-Jahan and Sebille (7,8) examined the effect of a prior conditioning lesion on rat sciatic nerve regeneration after nerve freezing. The conditioning lesion consisted of both section and freezing of the tibial nerve at the level of the ankle two to fourteen days prior to freezing of the sciatic nerve. Electrophysiological measurement of nerve regeneration was employed. Section of the tibial nerve seven days before freezing the sciatic nerve was found to be most effective, leading to a rate of regeneration of 5.6 mm per day vs. 4.5 mm per day if no conditioning lesion was administered (statistically significant, $p<.001$). Prior freeze of the tibial nerve was only helpful if performed 14 days ahead, and gave a rate of regeneration of 5.0 mm per day ($p<.05$ vs. controls). These rates are much higher than those reported by Sjoberg (68), who found rates of 1.9 to 3.2 mm/day, and Evans (19), who found a rate of 1 to 1.5 mm/day. Freezing was, however, more effective at reducing the delay before the start of regeneration (as determined by linear regression). With no conditioning lesion, the delay was 2.59 days. The minimum

delay with freezing was 1.45 days (conditioning lesion 7 days pre-op) vs. 2.01 days for prior section (conditioning lesion 4 days pre-op).

The authors examined the influence of the muscle cells on conditioning lesion effects in a second paper (8), in which they performed a prior tenotomy to a group of muscles supplied by the tibial nerve in an attempt to alter the status of those muscles. All the tendons of the foot sole muscles were cut at their insertion into the calcaneus 14 days before the conditioning lesion was performed. It was shown that prior tenotomy blocks the effect of a conditioning lesion. The authors conclude that this is a result of decrease in the ability of the muscular cell to perform protein synthesis, thereby decreasing synthesis of nerve trophic factors.

The effect of a conditioning lesion was also investigated by Hunt *et al.* (24), who looked at the results of transection and repair of the rabbit sciatic nerve with and without the administration of a proximal nerve freeze at the same time. They found no significant differences between the two groups in either axonal counts, muscle strengths, or muscle weights, and therefore no evidence to support the use of a conditioning lesion. The administration of the conditioning lesion at the time of nerve repair is not likely to improve the outcome, as there is no time to allow for the cell body to increase protein synthesis, the probable mechanism of increased axonal

growth rates (7). The optimal delay between performing the conditioning lesion and the test lesion was found by Bondoux-Jahan (7) to be seven to twenty-one days.

After cryogenic injury, the nerve regenerates over a period of time, typically to a degree consistent with complete functional recovery (5,12,71,76). The rate of regeneration in the rat sciatic nerve was found by Evans (19) to be 1 to 1.5 mm/day and by Sjoberg (68) to be 1.9 mm/day or 3.2 mm/day if a larger distal segment was left unfrozen and containing viable Schwann cells. Bondoux-Jahan (7) noted a rate of 4.5 mm/day which could be increased to 5.0 mm/day or more with the administration of a prior conditioning lesion. After regeneration, the number of axons is greater than normal. Mira (53,54) found a 21 to 31 percent increase in the axonal count one month after freezing and a 120 percent increase after three separate freezings.

C. Role of the Schwann Cell

The Schwann cell plays a crucial role in the ability of myelinated nerve fibers to rapidly conduct the action potential over long distances. The myelin sheath provides the axon with the characteristics of low capacitance and high resistance to current leakage between the nodes of Ranvier. This permits the passive spread of the action potential between nodes and allows saltatory conduction, the rapid leap of the signal from node to node. The

Schwann cell thus provides increased conduction velocities with decreased energy use.

Guttman and Sanders (23) reported the results of autografts and zenografts (dog and rat) in the peroneal nerve of the rabbit. After 15 to 25 days or more, the histological changes and the distance of growth of the host axons was examined. They concluded that the cells of the graft were destroyed as a result of the host immune reaction. They also demonstrated the survival of Schwann cells in autografts, which are seen to multiply and set up a channel for sprouting axons. This provides a good basis for recovery.

Verhoog and van Bekkum (75) implanted peripheral nerve segments under the kidney capsule in rats. The results were examined in terms of the number of round cells infiltrating the grafts. High dose (150,000 rads) eliminated this reaction in the grafts. They also froze grafts before implantation and noted inhibition of the immune response. Cryoprotection of the graft was shown to eliminate this effect. They concluded that the transplantation antigens responsible for the immune response are expressed only by living cells. Therefore, cell death was responsible for the inhibition of the immune response after high dose radiation or freezing without a cryopreservation agent.

Pollard and McLeod (61) detailed the fate of donor Schwann cells by implanting nerve grafts from normal mice

into trembler mice. These mice have a hereditary demyelinating neuropathy which allows distinction between the host and donor Schwann cells. By the use of predegenerated grafts free of myelin and normal myelinated grafts, it is show that the grafts are rejected in both the presence and absence of myelin. Normally myelinated axons were seen within the grafted segment. The authors conclude that Schwann cells are the chief antigen responsible for rejection. However, this conclusion is not warranted. One cannot implicate the schwann cell solely on the basis of rejection of both myelinated and nonmyelinated grafts.

Zalewski and Gulati (77) looked at the use of frozen allografts in the rat. Histological examination showed failure of both frozen isografts and fresh allografts at two and nine months, and at two weeks it was seen that freezing had killed most Schwann cells. They conclude that axonal regeneration through a long frozen nerve graft fails because of loss of the Schwann cells. In a later study, Zalewski *et al.* (78) explored the extent of new ingrowth into a rejected nerve allograft. Host axons were seen to migrate only into the proximal part of the allograft. They conclude that viable allogenic cells including fibroblasts and Schwann cells along with the connective tissue matrix provide the best framework to support axonal regeneration.

The Schwann cell can be seen to play an important role in nerve regeneration in grafts. Both Gutman and Sanders (23) and Zalewski et al. (77,78) concluded that the

presence of Schwann cells is important for axonal ingrowth. In the area of nerve freezing, Sjoberg (68) found that the rate of axonal ingrowth increased from 1.9 to 3.2 mm/day by leaving a larger unfrozen nerve segment distal to the freeze injury. They conclude that the living Schwann cells and/or other cells distal to the injury increase the rate of regeneration.

E. Cryopreservation

i. Effects of Cryopreservatives on Survival following Freezing

a. Historical Review

For centuries, studies and observation of biological systems at low temperatures have been performed. The recognition and use of chemical compounds which modify the freezing process, however, is a relatively modern occurance. In 1913, Keith (29) noted the improved survival of bacteria frozen in the presence of glucose, sucrose, milk, or glycerol. In 1938, Luyet (36) showed that exposure to sucrose improved the survival of frozen frog spermatozoa. Polge et al. (60) used twenty percent glycerol to successfully preserve motility of fowl spermatozoa after freezing. Pascoe (58) showed the protective effect of fifteen percent glycerol on the freezing of rat ganglia. Lovelock (34), in 1959, was the first to demonstrate the protective effect of dimethyl sulfoxide (DMSO) in the freezing of erythrocytes and spermatozoa. Ten to fifteen percent DMSO was found to afford nearly one hundred percent survival of erythrocytes. Glycerol was, however, more protective for bull semen.

b. Mechanism of Cryopreservation

Many researchers have used erythrocytes or blood components to investigate the freezing process and the use of cryoprotective compounds.

Doebbler and Rinfret (17) investigated the use of many cryoprotectants in the freezing (cooling rate was 4.6° C/min, rate of rewarming 2.6° C/min.) of erythrocytes. The most effective compounds tested were: disaccharides (which do not penetrate the cell membrane), providing 85 percent survival; polyvinylpyrrolidone, with 91 percent survival; and Dextran, with 96 percent survival. DMSO was not tested. All compounds were tested at concentrations of 0.3 M. They concluded that rapid freezing and rapid thawing led to better survival, and that the best cryoprotectants were those that form multiple hydrogen bonds.

Cavins et al. (13) froze human granulocytes in ten and fifteen percent DMSO (from 0 to -30° C at 1° C/min, -30 to -195° C at 19.5° C/min, rewarm rapidly in 37° C water bath). Viability was about five percent, which increased to 20.3-22.3 percent if the solution was diluted with autologous plasma to reduce the DMSO concentration. They concluded that there was little difference between ten and fifteen percent DMSO, and that dilution of the DMSO may decrease cellular damage.

Bouroncle (10) examined the freezing process in both normal and leukemic human white blood cells using DMSO. 12.5 percent DMSO was found to be the optimal concentration for cryopreservation, with normal lymphocytes frozen to -80° C having a viability of 93 to 96 percent.

Meryman *et al.* (52) explored the freezing of human blood using a variety of cryoprotective agents. They performed an elegant series of experiments designed to isolate the colligative effects of various cryoprotectants from other, non-colligative effects which have been attributed to them, such as stabilization of macromolecular structures by the strengthening of hydrophobic forces (51). Methanol, DMSO, and glycerol were found to act wholly in a colligative fashion, with hemolysis being solely a function of the salt concentration. They conclude that cryoprotectants function by two mechanisms: colligatively, by reducing the amount of ice formed, and kinetically, by increasing the viscosity of the water, and by decreasing the vapor pressure of adjacent ice, thereby increasing the time required for water to leave the cell.

Many other experimental models have been used to explore the effects of cryopreservation agents on the freezing of various tissues.

In 1964, Farrant (20) investigated the toxicity and cryoprotective effects of DMSO and other compounds on guinea-pig uteri smooth muscle. Freezing to -30 and -79° C in the presence of ten percent DMSO was partially

protective, with a resumption of spontaneous contractions but a decreased response to histamine. Exposure to ten percent DMSO alone induced a loss of response to histamines. Farrant concludes that, while providing partial protection to smooth muscle, DMSO caused non-specific osmotic damage.

Smith (69) looked at the survival of isolated mammalian chondrocytes frozen to -79° C in the presence of ten percent DMSO. Slow cooling and rapid rewarming conditions were employed. Microscopic evaluation showed activity for four to six hours after freezing for the rabbit and eight hours or more for the dog, monkey, and human preparations. Viability was tested in the rabbit by homografting to prepared defects in bone. At six weeks, normal appearing chondrocytes were found surrounded by well stained matrix. Smith concludes that isolated chondrocytes can remain viable after freezing and thawing in the presence of ten percent DMSO.

Karow, Carrier, and Holland (26) compared the survival of rat hearts cooled to -20° C after pretreatment with both fifteen and thirty percent DMSO. After a rinse in Ringer's solution, eight of the ten hearts frozen in fifteen percent DMSO resumed contractions. The hearts in thirty percent DMSO did not appear to freeze at -20° C, but only one of sixteen cooled in 30 percent DMSO resumed contractions. They conclude that fifteen percent DMSO is effective in maintaining viability of rat hearts cooled to -20° C.

Kawamoto and Barrett (28) developed methods of preservation of fetal rat central nervous tissue at low temperatures. Neuronal viability was maintained at 3-8° C for over a week and, with the use of five to ten percent DMSO, for several months at -70 to -90° C. Five percent DMSO was found to be adequate for good survival rates. Fifteen to twenty percent DMSO preserved neurons during freezing, but long exposure at temperatures above freezing led to vacuolation and poor neuronal growth. This emphasizes the importance of dilution of the cryoprotectant after freezing.

These experiments point out several steps to improve survival of cells or tissues following freezing. The concentration of DMSO used successfully ranged from five to thirty percent, but to avoid toxicity, a concentration of fifteen percent or below is more useful (26,28). Dilution of the DMSO following thawing improved survival (13). Mechanism of cryoprotection is probably due to both colligative and kinetic effects (52).

ii. Toxicity of Cryopreservatives

a. Effect of Cryopreservants on survival Above Freezing

As previously discussed, Bouroncle (10) examined the freezing process and DMSO toxicity in both normal and leukemic human white blood cells. The toxicity of DMSO

exposure without freezing was found to increase with increasing time or temperature. For leukemic cells, viability was found to be 70 percent after fifteen minutes exposure to 12.5 percent DMSO at 4° C and 45 percent at 20° C. Unfortunately, normal cells were not tested for survival after exposure to DMSO alone. The author concludes that to avoid problems with toxicity, the cryoprotectant must be added immediately prior to freezing and samples should be kept at 4° C.

b. Effect of Cryopreservants on Nerve Conduction

The action potential is a self-propagating depolarization which arises from a series of voltage dependent changes in the ionic permeability of the neuronal membrane. The voltage dependent behavior of the membrane is due to voltage gated sodium and potassium channels. The action potential is driven by the ionic gradients of sodium and potassium, maintained by the sodium-potassium pump. The value of the resting membrane potential is dependent upon the relative membrane permeabilities to sodium and potassium. The effects of DMSO on these processes is not entirely clear. In myocardium, DMSO has been shown to inhibit the sodium-potassium pump, stimulate adenyl cyclase, and to alter ion fluxes and mitochondrial respiration, as well as to inhibit cholinesterase activity (64,67). Several investigators have looked at the effects

of DMSO on both nerve conduction and electrical membrane properties alone and with freezing.

Pribor and Nara (62) measured the conduction velocity and action potential in frog sciatic nerves that were exposed to DMSO and glycerol or frozen with these agents. The effect of five percent DMSO alone was insignificant, while ten and fifteen percent solutions slightly decreased the action potential and conduction velocity. Glycerol was found to have more profound and less reversible effects on the nerve than DMSO. Freezing the desheathed nerve to -10° C with five percent DMSO was the least damaging, with retention of normal conduction velocity and 55 percent of the action potential. Freezing with DMSO concentrations of ten and fifteen percent was more toxic to the nerve.

They conclude that five percent DMSO provides the best protection for frog nerve freezing, but that this protection is not complete.

Sams (64) detailed the effects of DMSO alone on nerve conduction in the frog sciatic nerve. During exposure to six percent DMSO at 22° C, nerve conduction was slowed. This effect was totally reversible after at least two hours exposure. These results confirm the experience of Pribor and Nara (62), that low concentrations of DMSO are not toxic to frog peripheral nerve tissue.

In 1971, Menz (50) investigated the effect of freezing nerve, muscle, and leucocytes without a cryoprotectant, as well as the effect of DMSO alone. Freezing rat peripheral

nerve to -15° C or below led to a loss of excitability. The effect of DMSO on nerve tissue at 22°C after washing left one of four nerves with normal action potentials, two of four with relatively low values, and the fourth did not recover. At 2° C the two nerves tested both gave low values. Examination by electron microscopy showed shrinkage of axoplasm and Schwann cell cytoplasm. They feel that the mechanism of damage by DMSO may be related to an exosmosis of the axoplasm when the agent is added and removed rapidly. The study could have benefitted greatly from using a greater number of nerves, since the results of the DMSO toxicity experiments are not conclusive.

Asher (2) looked at the response of lobster ventral nerves to DMSO at room temperature and after freezing. DMSO alone decreased the conduction velocity and broadened the action potential. This effect was not completely reversible. No response was elicited from untreated nerves after freezing. Freezing to -20° C in the presence of DMSO resulted in a delayed compound action potential whose amplitude was about ten percent of the original signal. Freezing to -30° C further increased the stimulation threshold and the compound action potential became increasingly smooth, with the amplitude being slightly larger. To sum up, 2.5 percent DMSO was inadequate as a cryoprotectant, and no significant difference between five and ten percent solutions was noted at these temperatures.

Lenz (31) evaluated the use of DMSO and ethanol in the *in vivo* freezing of the rabbit sciatic nerve. In untreated nerves, some fibers retained the ability to generate an action potential after freezing to -15° C, while nerves pretreated with DMSO did so to -25° C. The pretreated group also had an earlier and more rapid return of the action potential. Typical degenerative histological changes were observed, with large, myelinated fibers suffering greater damage. They conclude that DMSO and ethanol were effective in increasing the threshold of freezing damage. Lowering the temperature, increasing the freezing time or increasing the number of freeze-thaw cycles led to quantitatively greater damage.

c. Effect of Cryoprotectants on Electrical Membrane Properties

Pasic and De Sa Faria (59) examined the response of the abdominal ganglia of the sea hare *Aplysia* to DMSO and glycerol alone and with freezing. The cryoprotectants alone led to a decrease in the amplitude and frequency of spontaneous spike activity. With both ten and twenty percent DMSO, these changes were irreversible after a rinse in sea water. The effects of glycerol were completely reversible. Using intracellular recording, both led to membrane depolarization, DMSO to a greater extent than glycerol. Freezing the ganglia to -20° C resulted in decreased action potential amplitude except in the case of

twenty percent glycerol. Freezing to -196° C without a cryoprotective agent resulted in no functional recovery. Most preparations frozen to this temperature with a cryoprotectant demonstrated spontaneous spike generation. The action potential frequency and amplitude was invariably depressed. They conclude that glycerol is the better cryoprotectant for *Aplysia*, but that the protection is not complete.

Scott (65) used mouse dorsal root ganglia cell cultures to investigate the effects of cryoprotectants and freezing on various electrical membrane properties. Freezing to -196° C (from 0 to -15° C at 1° C/min, then transferred to liquid nitrogen) led to an increase in membrane resistance and duration of action potential and a decrease in resting membrane potential. Exposure to DMSO alone led to changes in EMP which were different than those due to freezing, but included an increase in the duration of the action potential. They conclude that neurons can survive freezing and thawing and exhibit electrical excitability and the ability to generate action potentials. The use of DMSO and a cooling rate of 1° C/min is advocated.

d. Effect of Stepwise Cooling

In an effort to decrease the adverse effects of freezing, Farrant *et al.* (21) used a two step cooling

procedure with five percent DMSO in the preservation of hamster lung fibroblasts. They measured cell survival with a cell colony assay and also used freeze substition techniques to examine the degree of cellular shrinkage and amount of intracellular ice. They found that cell shrinkage induced by holding the cells at -25° C and -35° C for ten minutes before rapid cooling to -196° C increased cell survival from zero to 81 percent. Rapid thawing was noted to be more easily tolerated than slower rates. It was concluded that injury occurred during rewarming and was osmotic in nature.

Tomford *et al.* (73) investigated the effects of DMSO and glycerol on cartilage as well as the viability of the cell after freezing. They found no toxic effect of these agents if the concentration is below twelve percent and the exposure time is less than ninety minutes. The best freezing technique used was cooling the chondrocytes to -40° C for five minutes before rapid cooling to -80° C, which resulted in survival rates of 90-95 percent. Surviving cells were cultured and found to produce aggregated proteoglycans similar to those made by fresh chondrocytes. They conclude that chondrocytes can survive freezing and remain capable of functioning like fresh chondrocytes.

e. Summary of the Literature Involving Cryoprotectants

It can be seen that DMSO is an effective cryoprotectant. The concentration required for adequate protection varies from five to ten percent in various experimental models. Toxicity can occur at concentrations of about ten percent or above. In nerves, the changes which occur include a decrease in both conduction velocity and action potential amplitude (62,64). Scott (65) described various changes in electrical membrane properties on exposure of dorsal root ganglia cells to DMSO, including an increase in the duration of the action potential. The mechanism of cryoprotection by DMSO is probably a combination of kinetic effects, increasing the time required for water to leave the cell, and colligative effects, reducing the amount of ice formed (52).

IV. Animal Model

A. Extrapolation from the Animal Model to Humans

Kline *et al.* (30) looked at nerve severance in dogs, monkeys, baboons, and chimpanzees, and followed the results with electrophysiological studies, axonal counts, and histological studies. They concluded that differences exist between species in their response to nerve injury. Connective tissue proliferation was found to be greater in

phylogenetically higher animals. It is important to note, however, that only one or two animals of each species was used, and the results were not statistically significant. Mackinnon *et al.* (38) has questioned the use of the rat model for the study of peripheral nerve injury, repair, and regeneration because of these reported inter-species differences, and the reported extraordinary regenerative capacity of the rat. To investigate this, they resected the sciatic nerve in 30 Lewis rats. Five months later, they found an average ingrowth of 23.7 mm.

On the other hand, Lundberg *et al.* (35) found that after resection of a one cm. segment of the sciatic nerve in Sprague-Dawley rats, there was no evidence regeneration through the distal segment. Ruwe (63), and Easterling (18) also found no evidence of regeneration twelve weeks after resection of a two cm. segment of the sciatic nerve. In contrast to injuries which sever the nerve, nerve freezing leaves the nerve architecture intact, fascilitating a rapid and complete recovery. In the case of humans, it has been shown repeatedly that complete recovery can be expected after nerve freezing, such as administered for pain relief following thoracotomy (22,27,33,39,57). For this reason, it is reasonable to explore the area of nerve freezing utilizing the rat model.

B. Testing recovery following Nerve Freezing

i. Functional Testing

Recovery of function is the ultimate proof of recovery from nerve injuries. It has proven difficult to quantify functional recovery in animal models, so many experimentors have relied on histological examination and electrophysiological studies. These studies are valuable, but they do not provide information about the ultimate functional result of regeneration following nerve injury.

To address this deficiency, deMedinaceli, Freed, and Wyatt (15) developed a method for evaluating the sciatic nerve function in rats from measurements of the hind foot prints of walking rats. They used rats in which one sciatic nerve had been administered a crush injury, and compared measurements taken from the prints of the injured limb to those of the normal side. These were converted into percent deficits, and combined into the "sciatic functional index". Their method is as follows. A piece of unexposed x-ray film was placed on the floor of a confined walkway with a dark shelter at one end. The hind feet of the rat were dipped in developing fluid, and as the rat walked over the film, the developer caused permanant footprints to appear on the film. Four parameters were measured for both feet. Distance to opposite foot is the orthogonal projection of the distance from the tip of the print to the tip of the next contralateral toes. This

measures the ability of the limb to support the animal's weight. The print length measures the ability of the animal to walk in the normal fashion, with the foot plantar flexed. Total spread is the width of the print from the first to the fifth toes, and intermediary toes is the distance from the second to the fourth toes. These two variables assess the function of the peroneal branch of the sciatic nerve. The authors then made the assumption that these four variables are of equal importance and devised an emperic formula. The index was then applied to rats with the following procedures performed on the sciatic nerve: a sham operation, an injection of Ringer's solution, a crush injury, and transection with immediate repair. They found the measured rate of functional recovery to be consistent with other established methods (23), and concluded that the sciatic functional index provides a sensitive and reliable test for recovery in the rat sciatic nerve model. Easterling (18) notes the problems which arise when the SFI is applied to recovery following large nerve grafts, particularly the lack of reinnervation to the intrinsic muscles of the foot precluding the ability of the rat to spread its toes. In the case of nerve crushing or freezing, where the nerve architecture is intact, the SFI should work as predicted by deMedinaceli (15). The SFI will be used to evaluate the results of the present study.

ii. Axonal counts

Following destruction of the axon, regeneration begins with the outgrowth of many fine axonal sprouts. In the case of freeze injury where the basal lamina remain intact, all of these branches are restricted to the path of the original axon. During regeneration after nerve injury, the area previously occupied by a single large axon is replaced by a cluster of these small, regenerating axons. All of the branches do not survive and eventually one or more larger myelinated axons will remain. Since a necessary condition for reinnervation following nerve injury is the presence of myelinated axons in continuity from the proximal to distal nerve segments, many authors have used axonal counts as a method of assessment of regeneration following nerve injury. Axonal counts will be performed and their relation to the results of functional testing will be examined.

iii. Muscle Weights

Functionally significant reinnervation to muscle should reverse the process of atrophy and fibrosis associated with deinnervation. MacDonald *et al.* (37) have shown that increasing collagen content and loss of muscle fiber mass is indicative of worst functional recovery. Wet muscle weight will be measured at the time of sacrifice and compared to the results of direct functional testing.

iv. Electrophysiological Studies

Due to the problems inherent in functional testing in animals, many investigators have used electrophysiological studies to draw conclusions about functional recovery following nerve injury. Most researchers have tested nerve conduction velocities alone or in combination with compound nerve action potentials. It is important to note that a small number of intact nerve fibers can lead to a normal measured conduction velocity. The significance of these measurements in terms of functional recovery remains unclear. Almquist and Eeg-Ologsson (1) addressed this question in a 1970 study of 19 patients with nerve laceration and subsequent suture repair. They compared nerve recovery tested by two point discrimination with the results of measured conduction velocities. There was no correlation. Since no correlation between conduction velocity and functional recovery has been demonstrated, electrophysiological studies will not be used in the present study.

Methods and Materials

Animal Model

Adult male Sprague-Dawley rats weighing from 300 to 320 grams (CAMM, Wayne, NJ) served as the experimental animal. They were kept in plastic cages in the animal care facility (FMB 05) and were given rat chow and water ad libitum. The diurnal cycle was twelve hours of light and twelve hours of darkness. The animals were divided randomly into three groups. The first experimental group had a one centimeter segment of the nerve frozen. The second experimental group received a ten minute application of a ten percent solution of DMSO and then the nerve was frozen. The third group was a control and received a sham operation. Each group consisted of ten rats in the initial experiment. To examine axonal ingrowth over time, an additional nine rats were used in the two experimental groups in the serial histology.

Surgical Procedure

The left or right hind leg was chosen randomly, with the contralateral limb serving as an internal control. The anesthetic used was pentabarbitol sodium (Abbott

Laboratories, North Chicago,Illinois), 30 to 40 mg/kg, administered intraperitoneally. The sciatic nerve was exposed from the sciatic notch proximally to the distal muscular branches using a gluteal splitting incision. The nerve was dissected carefully from the surrounding fascia using an operating microscope (J.K. Hoppl Co., Model 029001) and microsurgical instruments. In the first experimental group, the nerve was elevated and inserted in a slot cut in the end of a one cm diameter plastic test tube located 1.5 cm from the sciatic notch. The slot was sealed with moist gelfoam (Upjohn Co., Kalamazoo, Michigan) and the test tube was elevated and insulated from the surrounding muscle bed with a layer of styrofoam. The tube was then filled with liquid nitrogen for a period of thirty seconds. The nerve was thawed by the application of normal saline after forty five seconds of freezing time.

In the second experimental group, the nerve was placed on a layer of styrofoam and covered with gelfoam soaked in a ten percent solution of DMSO. The gelfoam was changed after five minutes and removed after ten minutes. The nerve was then frozen as outlined above.

In the control group, the nerve was exposed and freed from the surrounding fascia as above. In all groups, the gluteal muscle was then approximated and the skin closed with 3-0 Dexon (Davis and Geck, Danbury, Connecticut). The operations were performed using aseptic technique in the Orthopedic Surgery Operating Laboratory.

Functional Testing

The animals were followed by gait testing and three qualitative functional tests pre- and post-operatively and weekly for ten weeks. The gait testing was conducted in a confined walkway measuring 12 cm wide by one meter long, with a peanut butter reward at one end. This is a modification of one described by deMedinaceli (15). The rats initially required one or two trials to become accustumed to the walkway, but soon became more cooperative and walked steadily to the end. A piece of unexposed x-ray film (Kodak X-omat TL Film) was placed on the floor of the walkway and the hind feet of the rat were dipped in undiluted developer (Dectol, Eastman Kodak, Rochester, N.Y.). The rat was allowed to walk over the film on the way to the reward. Three to five prints of each foot then appeared on the film. Four parameters were measured, all to the nearest mm, for both the left and right feet. Distance to opposite foot (TOF) is the orthogonal projection of the distance from the tip of the print to the tip of the next contralateral toes. This value was measured and an average of two to three values was obtained if the measurements were not uniform. Print length (PL) was also measured . Total spread (TS) is the width of the print from the first to the fifth toes. Intermediary toes (IT) is the distance from the second to the fourth toes.

The values were then labeled as being from the experimental (E, for example ETOF) or normal (N, for example NTOF) and entered into the following formula to arrive at a Sciatic Functional Index (69).

$$SFI = \frac{ETOF-NTOF}{NTOF} + \frac{NPL-EPL}{EPL} + \frac{ETS-NTS}{NTS} + \frac{EIT-NIT}{NIT} * \frac{220}{4}$$

Three qualitative tests were also utilized to evaluate the function of the nerve. The first two were administered while holding the rat in one hand. The sensory score was a pinch to the palmar aspect of the toes using an Addison forcep. No reaction ranked a zero, a mild reaction, i.e. withdrawl of the foot less than one cm. was a one, and a vigorous reaction, withdrawl greater than one cm. was a two. The second, a measure of tone, involved pressing a finger against the foot of the rat. On the control side, the rat would grasp the finger and provide resistance to pressure. This reaction on the experimental side ranked a two, while no reaction was a zero and an intermediate reaction was a one. The third was a ranking of the ability of the rat to ambulate, again from zero to two. No use of the limb was a zero, use of the limb with a limp scored a one, and normal use was a two.

Histological Studies

Ten weeks after the initial procedure, the animals were sacrificed by lethal pentabarbitol injection. In the serial histology, one animal from each experimental group was sacrificed at 36 hours postoperatively and weekly for six weeks. Two animals per group were used at the fourth and fifth weeks. The nerves were reexplored and a 2.5 cm segment was harvested using an operating microscope (JK Hoppl Co., model 029001) and microsurgical instruments. The segment was divided into five pieces to allow adequate penetration of the fixative. They were fixed in Karnovsky's fixative (pH 7.4) overnight, then washed with cacodilate buffer with dextrose and post-fixed with 1.5 percent osmium tetraoxide in cacodilate buffer, dehydrated in alcohol, and embedded in epon by Lufts formula number one. Sectioning was performed with an ultra-microtome (LKB Huxley, AB Bromma, Sweden) using a glass knife. Sections of 1 micron were prepared and examined by light microscopy.

Axonal Counts

Axonal counts were performed in Yale University's Peabody museum Morphometrics Laboratory using an Eyecom II (Spacial Data, Melbourne, Fla.) with software developed by Alan Pooley, PhD. for calculation of area and counting points. A section was chosen from a segment proximal to

the injury and from a segment near the center of the injury. Two to three representative areas were counted in each section, comprising approximately ten percent of the area. The total number of axons counted divided by the total area counted was multiplied by the total area of the section to arrive at the total number of axons per section. The results were compared as a percentage of the proximal section.

Tibialis Anterior Muscle Weight

At the time of sacrifice, following nerve harvest, the tibialis anterior muscle was removed bilaterally. The anterior compartment was entered through a midline incision from the superior pole of the patella to the first web space. The tibialis anterior muscle was removed from the origin to the level of the inferior extensor retinaculum. The muscle was weighed to the nearest 0.0001 gram (Sartorius Analytical Balance, Model 2603, West Germany). The results were reported as a percentage of the non-operated side.

Statistical Analysis

The results of gait testing and tibialis muscle weights were analysed using a one way analysis of variance (ANOVA). The results of the qualitative testing were

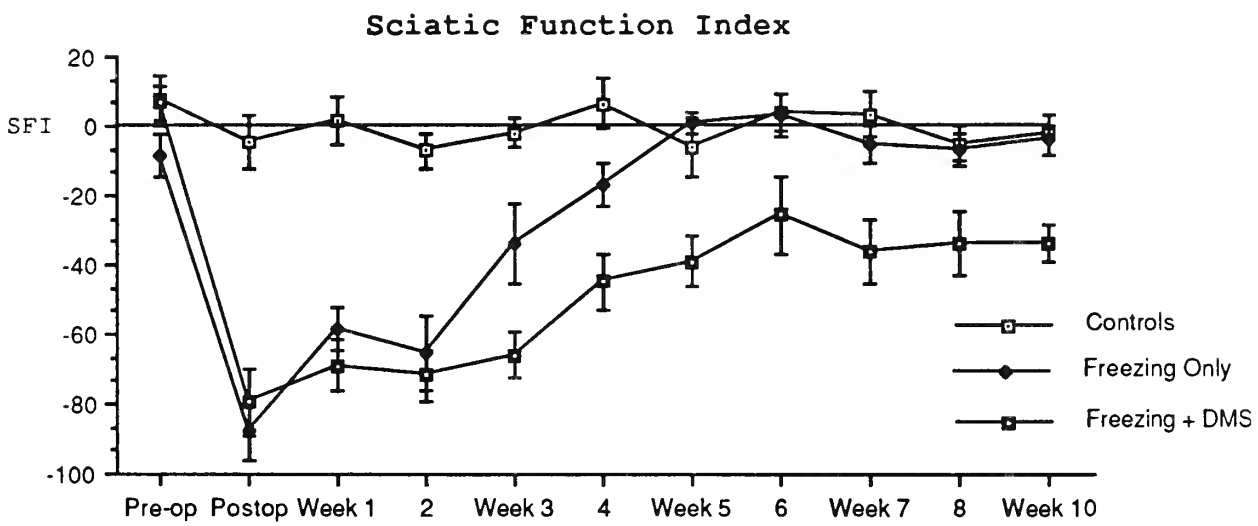
summed to a score of zero to six and were analysed using a one way Kruskal-Wallis test for significance (a non-parametric equivalent to analysis of variance). Weekly variations were analysed using the one way ANOVA. When applicable, results were reported as mean +/- standard error. The statistical analyses were done using the Statview 512+ package on a Macintosh SE.

Results

Functional Testing

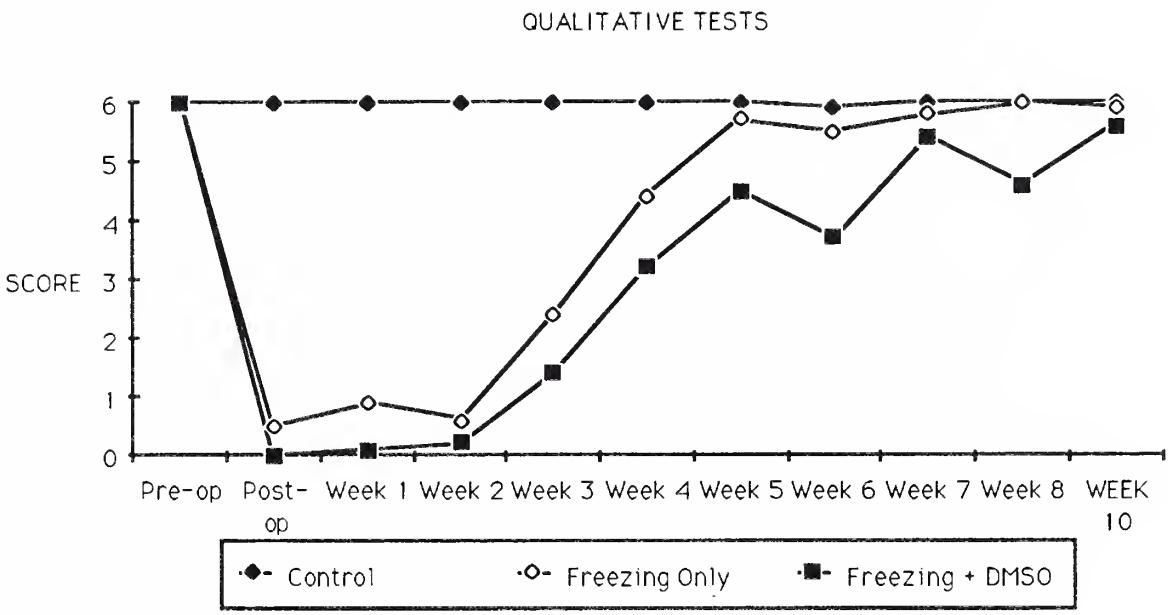
In contrast to animals suffering a severe nerve injury such as nerve transection, the method of gait testing developed by deMedinaceli (15) proved to be very suitable for the evaluation of nerve function after freeze damage to the nerve. The preoperative SFI value for all 30 rats was 1.86 +/- 3.81. At 24 hours postoperatively, the values for the freezing only and the freezing + DMSO groups were -87.81 +/- 8.56 and 79.37 +/- 9.71, respectively. The difference between these values is not statistically significant. The control group, which underwent a sham operation, remained essentially unchanged at -4.79 +/- 7.8 .(See graph) There is a small rebound at week 1, to -58.4 +/- 6.1 for the freezing only group, and -69.1 +/- 7.2 for the freezing + DMSO group. There is a rapid return of function beginning between week two and three in the freezing only group and between week three and four in the freezing + DMSO group. At five weeks and beyond, the freezing only group becomes statistically indistinguishable from the controls. The freezing + DMSO group does not do as well. At week 5 the SFI value is - 38.9 +/- 7.1 and it remains between -25.6 and -36.2 over the next 5 weeks,

finishing at - 34.1 +/- 5.5 at week 10. These values are statistically different from both the controls and the freezing only group to a confidence level of 99.5 percent at weeks 5,7 and 10, and at a 95 percent level from week three on.



The qualitative tests were performed at the same time as the gait analysis and, as previously described, the scores were zero for no function, two for normal function, and one for an intermediate level of function. For the

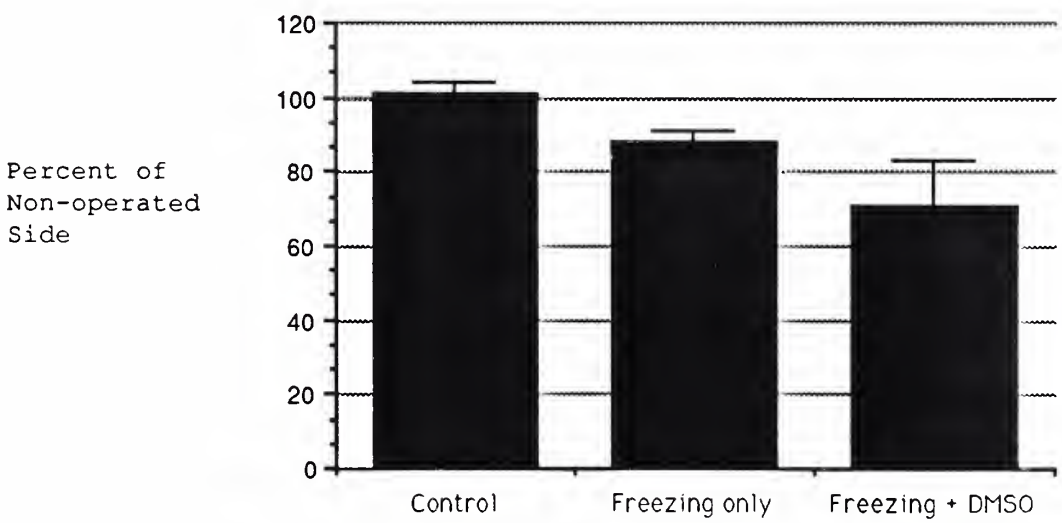
purpose of analysis, the sensory, motor, and tone scores were added for each individual rat to come out with a overall qualitative score from zero to six. The preoperative values for all rats were six. At 24 hours postoperatively, the freezing only and freezing + DMSO groups averaged 0.5 and 0.0 , respectively. There was no change in the control group. There is then a rapid return of function beginning between week two and three in both experimental groups. At week five and beyond, the freezing only group becomes statistically indistinguishable from the controls. At week seven and at week ten, all groups are near an average score of six and are statistically indistinguishable. The freezing + DMSO group's scores are somewhat worse than the freezing only group for the last three measurements. The freezing only group averaged 5.9 for the last three measurements versus 5.2 for the freezing + DMSO group. Overall, both experimental groups had very good functional recovery as tested by both quantitative tests and the sciatic function index.


QUALITATIVE TESTS
SCORE
6
5
4
3
2
1
0
Pre-op
Post-op
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Week 8
WEEK 10
Control
Freezing Only
Freezing + DMSO

Tibialis Anterior Muscle Weights

At the time of sacrifice, the tibialis anterior muscle was removed bilaterally, stripped of connective tissue, and weighed to the nearest .0001 gram. The results are reported as a percent of the non-operated side. The control group averaged 100.9% +/- 1.0 of the non-operated side. The freezing only group averaged 88.2% +/- 1.0 , while the freezing + DMSO group averaged 70.6% +/- 3.9 of the non-operated side. All of these groups proved to be statistically different at a 99.5 % confidence level by the ANOVA analysis. It can be seen that the freezing + DMSO group had significantly less reinnervation than the group which received freezing only.

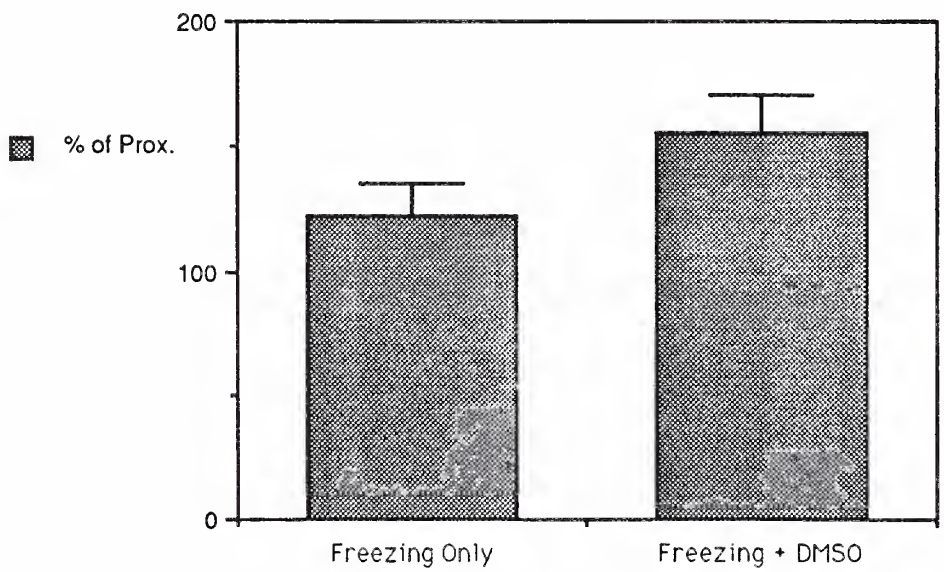
Tibialis Anterior Muscle Weight



Axonal Counts

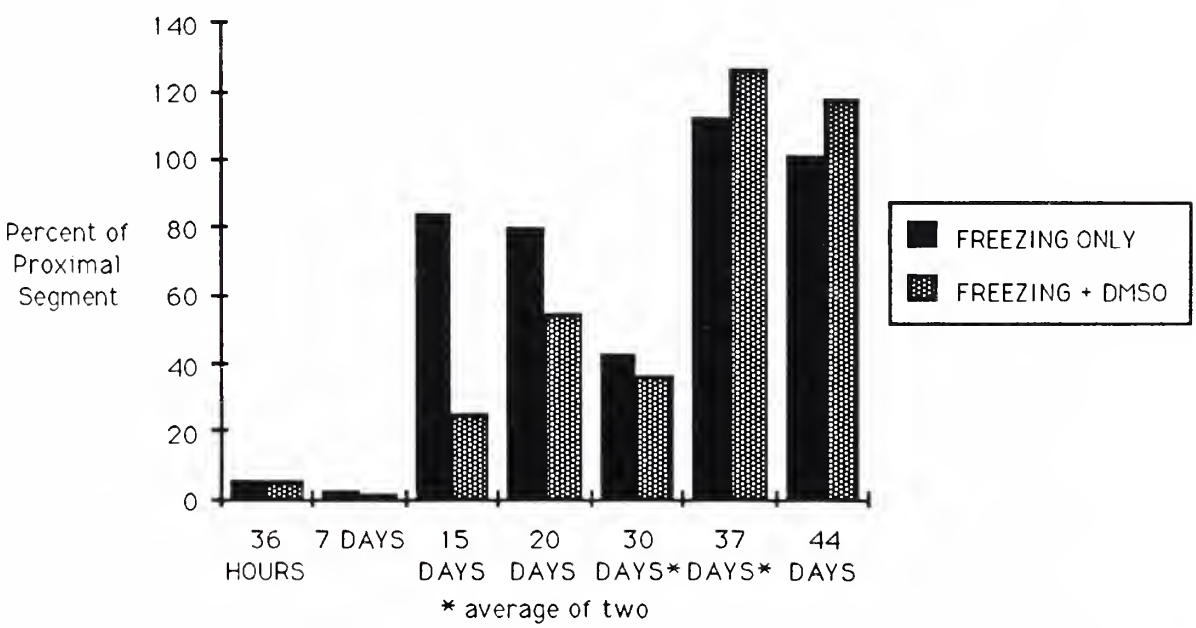
Ten weeks after the initial procedure, the nerves were reexplored and a 2.5 cm segment was harvested. Sections were prepared and examined by light microscopy. There were eight nerves examined in each group (See Fig. 3-5). The average axonal count in controls was 8298 +/- 485. The average in the freezing only group was 7048 +/- 513 in the proximal segment and 8273 +/- 587 in the frozen segment. The averages for the freezing + DMSO group were 7111 +/- 379 and 11051 +/- 1112. Thus the freezing only group had 22 percent more axons in the injured segment than proximally. The freezing + DMSO group had 56 percent more.

Axonal counts:
Injured segment as a percent of proximal



Serial Axonal Counts

The second step of the study was carried out to quantify the extent of axonal ingrowth into the injured segment of the nerve. The surgical procedure was the same as previously described for each of the experimental groups. One animal from each group was sacrificed at 36 hours, and weekly for six weeks. Two animals per group were used at the fourth and fifth weeks. By five weeks time, the injured section of the nerve contains a number of myelinated axons equal to or greater than the proximal, uninjured section (Fig. 1 and 2).



Discussion

The intention of this study was to utilize an experimental model of peripheral nerve freezing and the use of cryopreservative agents which is meaningful in terms of functional recovery. In animal studies, many previous authors have performed in vitro studies or have utilized histological examination, electrophysiological studies, alone or in combination with a single qualitative assessment of function to evaluate nerve damage or regeneration after freezing (2,4,7,8,31,40,50,53,54,62,64). These studies have contributed greatly to our understanding of nerve regeneration and the response to cryoprotective agents, but the theraputic significance of their results is often unclear. Almquist (1) has discussed the limited significance of histological and electrophysiological measurements when they are extrapolated to humans, where muscular function is the chief determinant of success.

Many human studies have been performed utilizing cryosurgery. In those involving nerve freezing for the relief of pain, return of function is typically assessed by return of sensation (27,39,51), and regeneration is reported to be complete over a period of less than one month to over twelve months. Marcove reported repeatedly on the reliable return of function in nerves frozen incidently during cryosurgery for

cancer(42,43,45,47,48,49), with at least 11 of 12 cases of nerve palsies resolving over time. However, he does not describe in detail this recovery or how it is assessed. No study in humans has attempted to quantify the return of motor function after cryogenic injury to peripheral nerve.

Research in animal models has provided much useful information about nerve freezing. If nerve temperature remains below approximately 5° C longer than momentarily, or goes below the freezing point, the nerve will undergo Wallerian degeneration and death of Schwann cells will occur (16,75,76). A long term deficit will ensue. Regeneration occurs over a period of weeks and is typically complete, leaving no functional deficit (5,12,19,71,76). Long term increases in axonal counts are seen (53,54). The Schwann cell is important for axonal ingrowth (23,68,77,78).

Cryopreservatives can increase survival in some isolated cell systems to nearly 100 percent (10,17). More limited success has been acheived with intact tissues (20,26). The primary effects of DMSO on peripheral nerve tissue above freezing are a consistent decrease in conduction velocity and action potentials (50,62,64). This effect is at least partially reversible (62,64). The concentration of DMSO required for cryoprotection ranges from about five to ten percent and toxicity occurs in most systems at a concentration of about ten percent or above. The mechanism of protection by DMSO appears to be an

combination of kinetic and colligative effects. However, no author has described functional, long term survival of the nerve after freezing either with or without cryopreservatives.

Animal models of peripheral nerve regeneration following cryogenic injury show very similar results to those in these human studies. Regeneration is typically complete, with no residual functional deficits. The rate of regeneration has been found to be 1.5 mm/day in both the dog and monkey (5), and in the rat, rates from 1-1.5 mm/day (19) to as high as 4.5 mm/day (7) have been reported. Excluding the most rapid rate, these rates are similar to those following nerve crushing and only slightly more rapid than after nerve repair. Because of these similarities between both the ultimate functional result of nerve regeneration and the rate of regeneration, it is reasonable use the rat model to explore the area of regeneration following cryogenic injury to peripheral nerve.

In this project, our purpose was to quantify the return of function after nerve freezing as accurately as possible, and to correlate these results with those of other commonly used assesssments of nerve regeneration. To do this, both the sciatic functional index and three qualitative indices of nerve function were employed. Tibialis anterior wet muscle weights were measured. Axonal counts were also used, but with the recognition of their limited significance. Electrophysiological studies

were omitted because they have not been shown to correlate with return of function (1).

Functional Studies

In contrast to the results seen by Ruwe (63) and Easterling (18), who found the sciatic functional index to be poorly suited to the assessment of recovery following nerve grafting of a large gap, this gait study was found to be quite appropriate for the assessment of recovery after nerve freezing. This injury is comparable to the crush injury that the sciatic functional index was originally shown to work well for (15). There is a clear correlation between the results obtained with the use of these gait studies and the three quantitative tests of sensory and motor function and limb tone. Both methods show a complete loss of function postoperatively and a rapid return of function beginning near the third week. By the fifth week, there is no statistical difference between the controls and the freezing only group. At ten weeks, the freezing + DMSO group still lags the controls and the freezing only group by about 30 SFI units. The sciatic functional index may be the more sensitive in that it shows freezing with ten percent DMSO to be a somewhat worse injury than freezing alone. This result is supported by the tibialis anterior muscle weights and the axonal counts. The results of functional testing have been emphasized over other methods of assessment of nerve regeneration because functional

return forms the ultimate proof of recovery from nerve injuries.

Muscle Weights

Decreased muscle fiber mass has been shown to correlate with a worse functional recovery (37). The wet muscle weights were lower in both the experimental groups than the controls. The freezing only group averaged nearly ninety percent of the unoperated side, while the freezing + DMSO group averaged only seventy percent of the contralateral side. Controls, as expected, averaged one hundred percent of the unoperated side. This supports the idea that the freezing only group had received earlier or more complete reinnervation than the group pretreated with DMSO.

Axonal Counts

Higher axonal counts of small axons have been shown to correlate with increasingly destructive injuries (53,54). The freezing only group averaged 22 percent more than the proximal segment, while the group pretreated with DMSO averaged 56 percent more than control. These results seem to correlate with the somewhat worse functional result obtained in the group pretreated with DMSO. This increase in numbers was accompanied by a decrease in axonal diameter and an increased space between the axons within the nerve.

Conclusions

The results of this study are consistent with the existing literature in that nerve freezing led to a

functional deficit which resolved completely over five weeks. Weight of muscle innervated by the nerve had reached 90 percent of the contralateral side by ten weeks. Axonal counts showed an equal or greater number of axons in the injured segment as compared to the proximal segment at five weeks, and a 22 percent increase at ten weeks. This agrees with Mira's results (54), where a 21-31 percent increase was seen two years postoperatively, and an equal or greater number at four weeks. Cryoprotection was employed in an attempt to prevent damage to Schwann cells and axons. The use of ten percent DMSO for the pretreatment of the nerve had a negative effect on recovery. Both functional tests showed less recovery than in the group treated with freezing only, the SFI to a greater extent than qualitative testing. Muscle weights were significantly lower than the freezing only group, and axonal counts were significantly increased. It appears that in the present study, pretreatment with ten percent DMSO did not preserve the Schwann cell under the conditions of rapid freezing to -196° C followed by rapid thawing. One improvement in technique would be to wash the DMSO from the nerve for a longer period of time. Dilution of DMSO has been shown to improve cell survival (13,28). The DMSO may have caused localized toxic effects. It is possible that DMSO in fact created a defect in myelin or in the basement membranes, leading to a worse long term prognosis for recovery.

To summarize, when the rat sciatic nerve was frozen to -196° C after local pretreatment with ten percent dimethyl sulfoxide, good reinnervation resulted, as measured by axonal counts, muscle weights, and functional testing. Rats in which the nerve was frozen only enjoyed better results. Excellent regeneration of the nerve was seen, as measured by these criteria. From a functional standpoint, the operated limb was indistinguishable from the contralateral side in this group five weeks after freezing.

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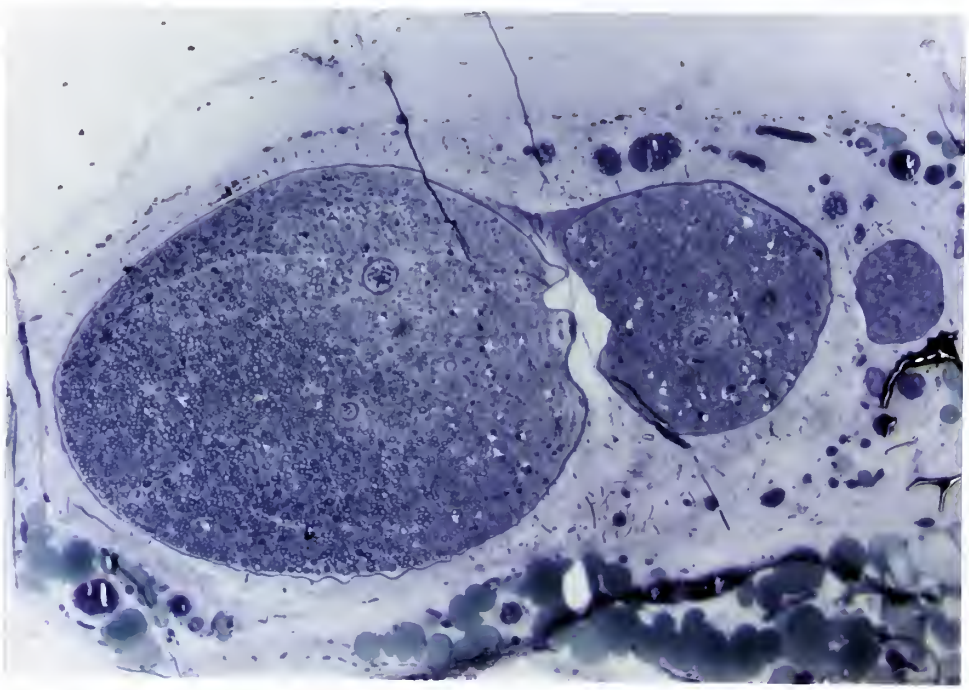
Figures

Figure 1: Nerve treated with freezing only, 5 weeks post-op.
Section of entire nerve (40x) and view of nerve (200x)



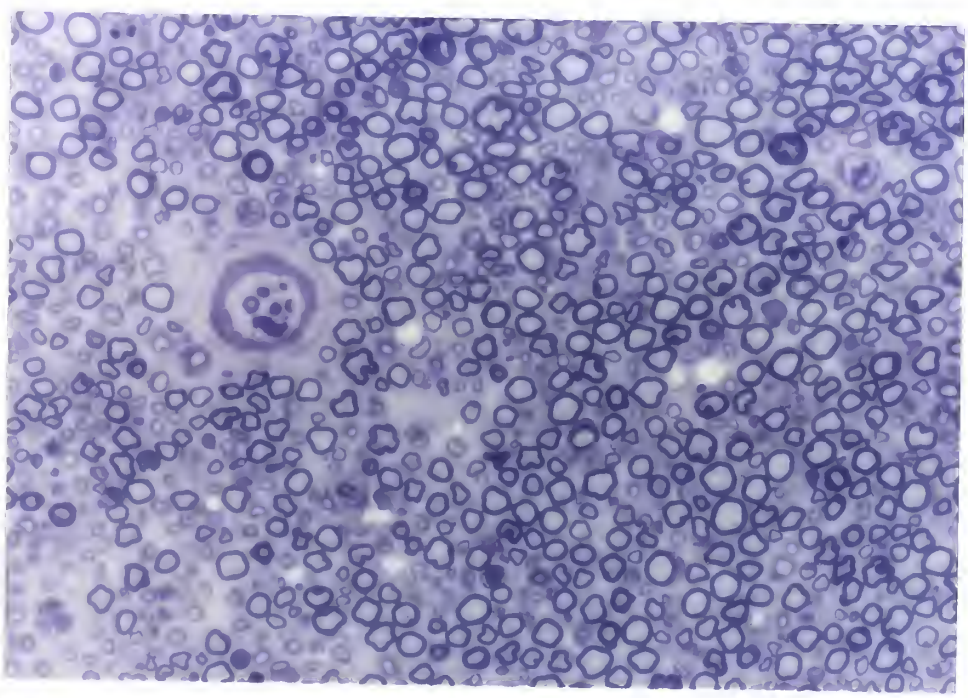


Figure 2: Nerve treated with freezing + DMSO, 5 weeks post-op. Section of entire nerve (40x) and view of nerve (200x)

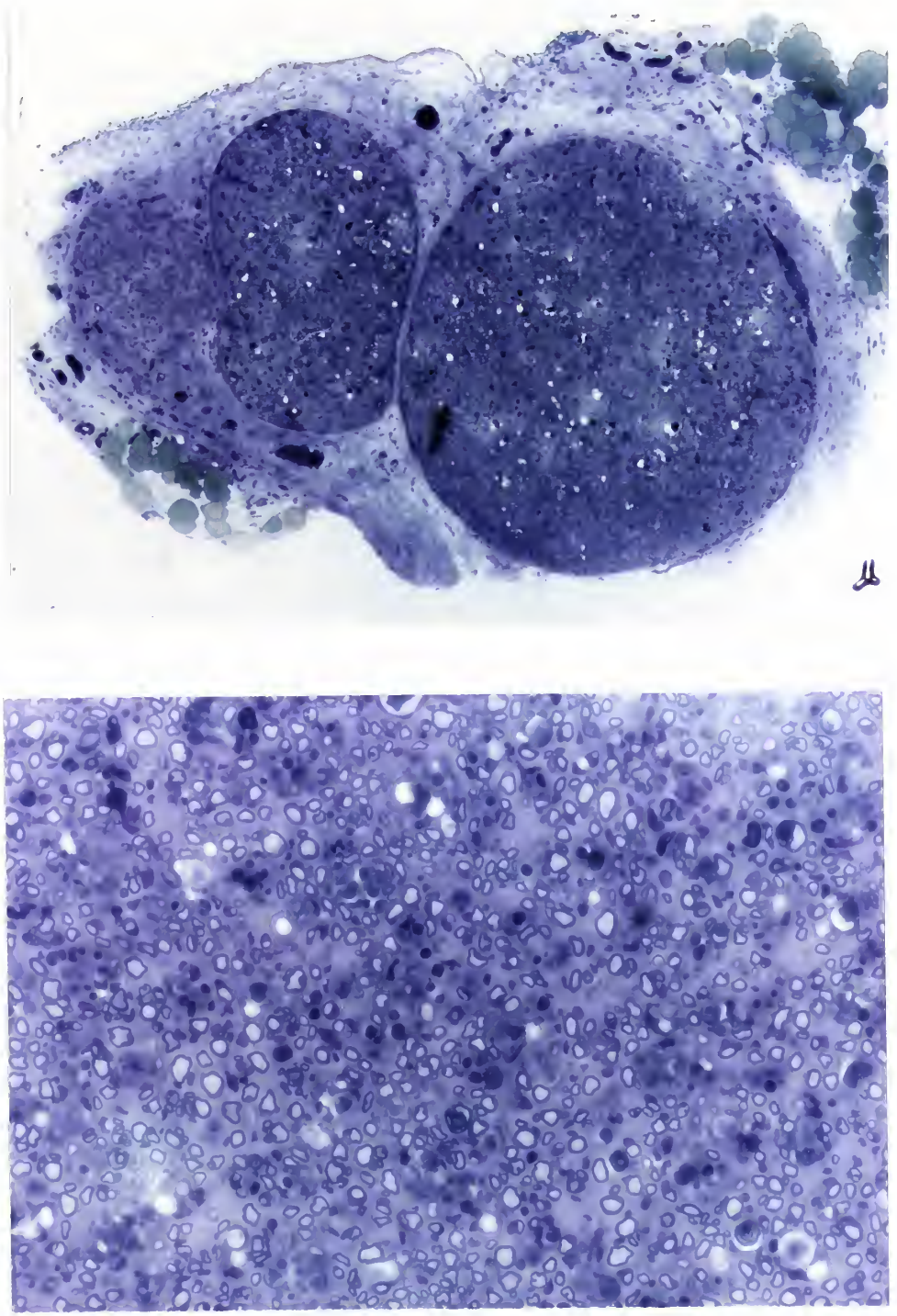
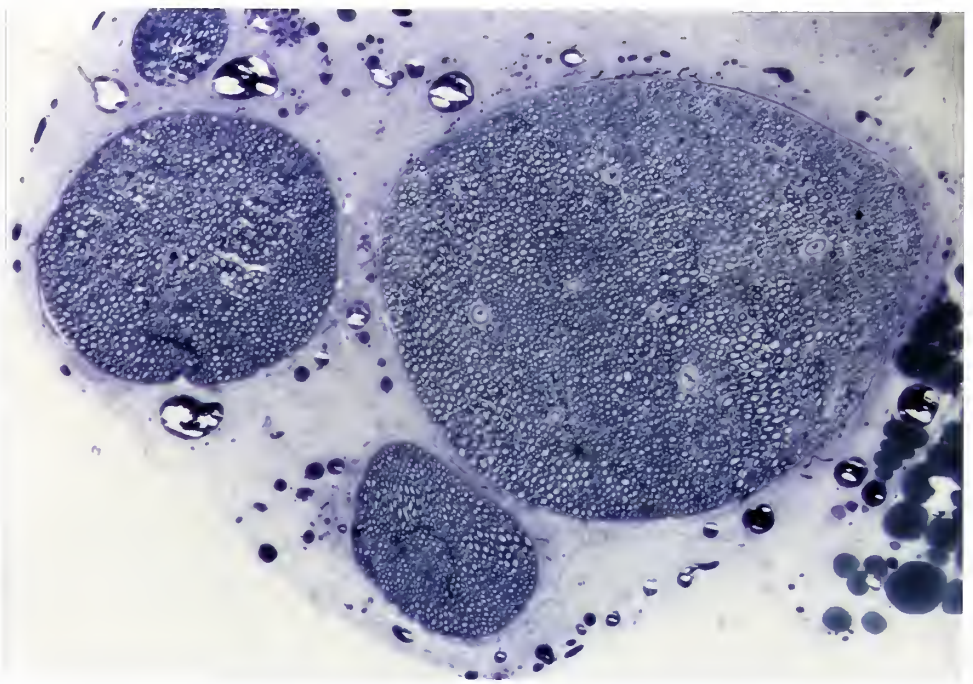


Figure 3: Nerve treated with a sham operation, 10 weeks post-op. Section of entire nerve (40x) and view of nerve (200x)



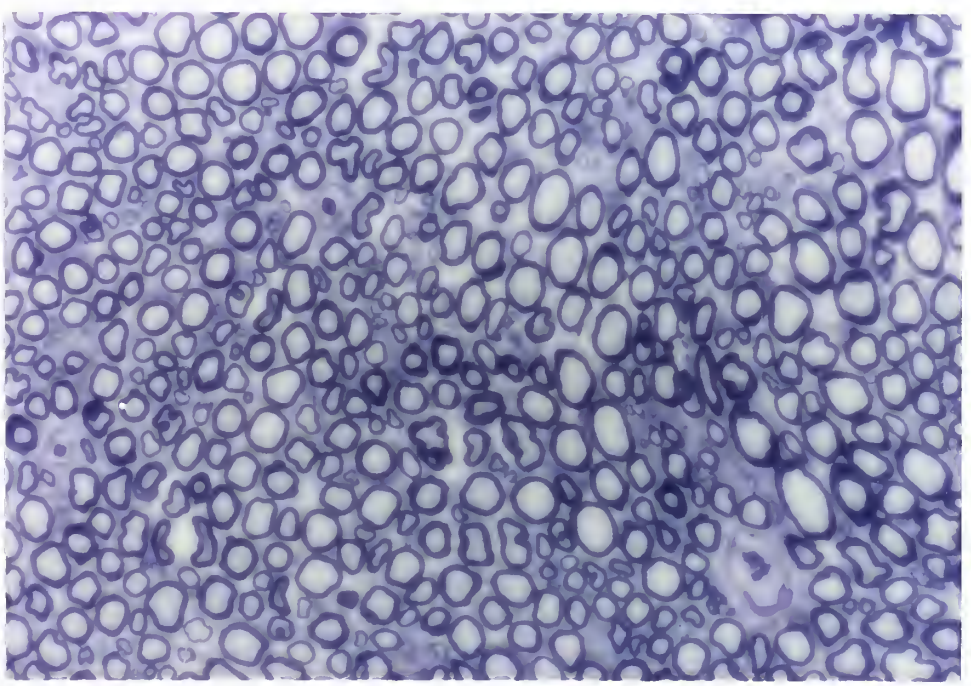


Figure 4: Nerve treated with freezing only, 10 weeks post-op.
Section of entire nerve (40x) and view of nerve (200x)

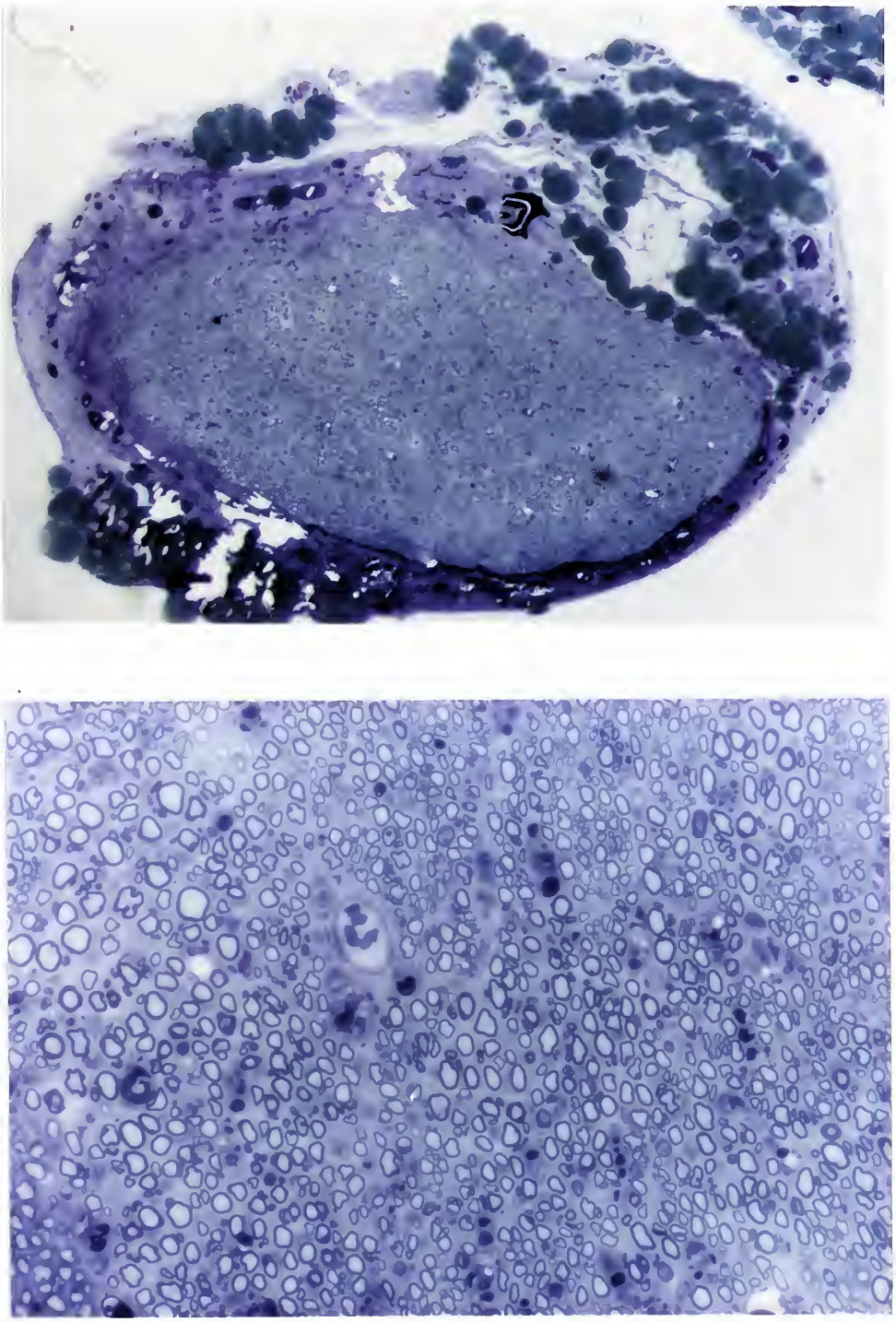
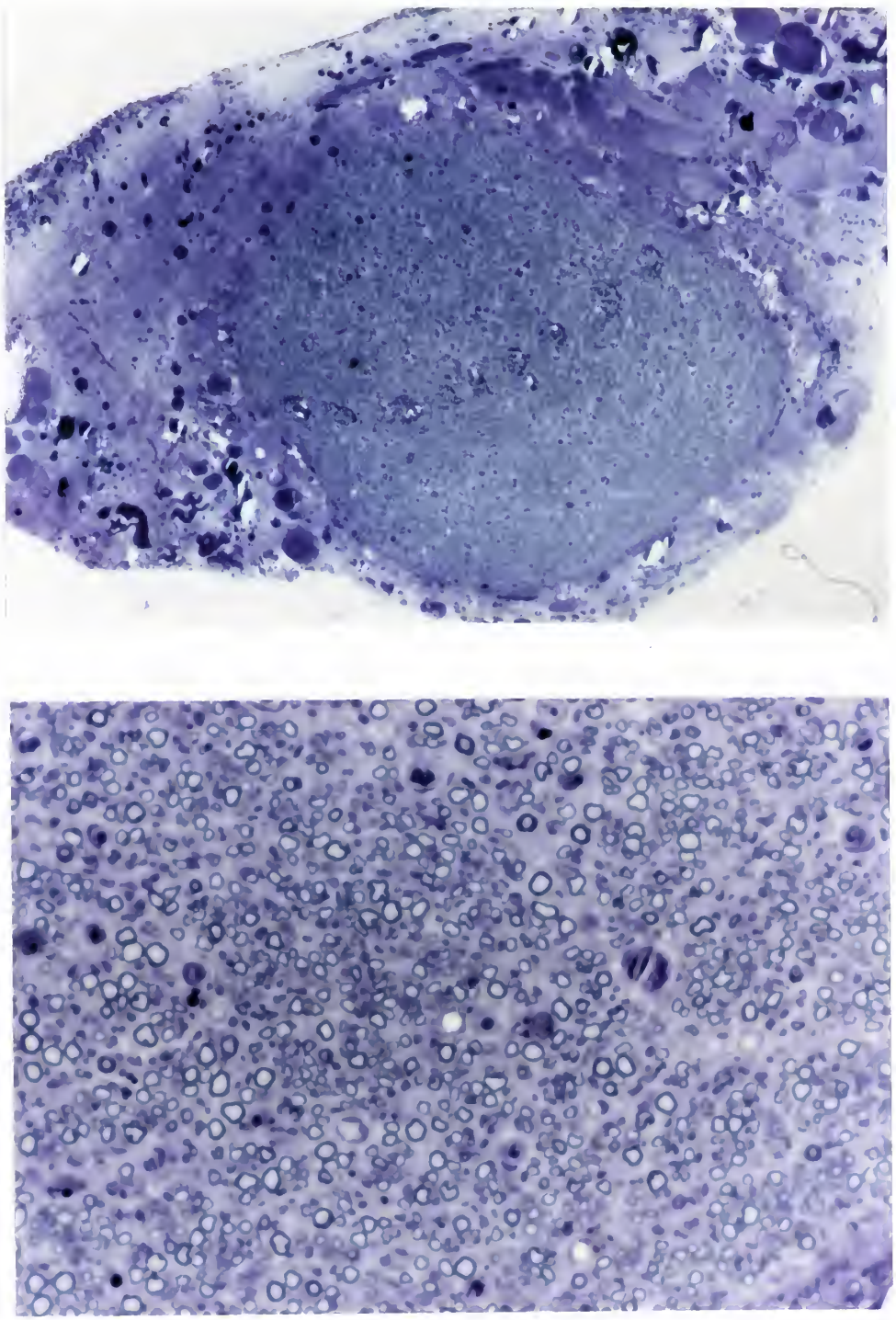


Figure 5: Nerve treated with freezing + DMSO, 10 weeks post-op. Section of entire nerve (40x) and view of nerve (200x)



Raw Data

Axonal Counts

ontrol	Rat Number			
	3	9868		
	9	7365		
	15	8514		
	18	9846		
	21	9672		
	24	7095		
	27	6403		
	30	7620		
	Average	8298		
	Std. Dev.	1371		
	Std. Error	485		
reezing Only	Rat Number	Proximal	Injury	Percent of Proximal
	1	5059	9362	185
	4	7005	8146	116
	7	5620	5497	98
	10	8993	6797	76
	16	6688	11042	165
	19	7585	8833	116
	22	6371	8575	135
	25	9062	7931	88
	Average	7048	8273	122
	Std. Dev.	1450	1659	38
	Std. Error	513	587	13
reezing + DMSO	Rat Number			
	2	8679	10757	124
	5	5646	9390	166
	11	7378	10498	142
	14	7636	12335	162
	20	5667	8104	143
	23	6700	8808	131
	26	8037	10373	129
	29	7146	18145	254
	Average	7111	11051	156
	Std. Dev.	1073	3146	42
	Std. Error	379	1112	15
			t=	1.698

Axonal Counts

Control	Rat Number			
	3	9868		
	9	7365		
	15	8514		
	18	9846		
	21	9672		
	24	7095		
	27	6403		
	30	7620		
	Average	8298		
	Std. Dev.	1371		
	Std. Error	485		
Freezing Only	Rat Number	Proximal	Injury	Percent of Proximal
	1	5059	9362	185
	4	7005	8146	116
	7	5620	5497	98
	10	8993	6797	76
	16	6688	11042	165
	19	7585	8833	116
	22	6371	8575	135
	25	9062	7931	88
	Average	7048	8273	122
	Std. Dev.	1450	1659	38
	Std. Error	513	587	13
Freezing + DMSO	Rat Number			
	2	8679	10757	124
	5	5646	9390	166
	11	7378	10498	142
	14	7636	12335	162
	20	5667	8104	143
	23	6700	8808	131
	26	8037	10373	129
	29	7146	18145	254
	Average	7111	11051	156
	Std. Dev.	1073	3146	42
	Std. Error	379	1112	15
			t=	1.698

Pre-op

	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
Control	3	12.4	2	2	2	6
	6	-9.1	2	2	2	6
	9	9.4	2	2	2	6
	12	38.4	2	2	2	6
	15	40.7	2	2	2	6
	18	-25.5	2	2	2	6
	21	-18.3	2	2	2	6
	24	5.2	2	2	2	6
	27	-10.9	2	2	2	6
	30	31.3	2	2	2	6
	Average =	7.4	2	2	2	6
	Std. Dev. =	23.6	0	0	0	0
	Std. Error=	7.5	0	0	0	0
Freezing Only	1	0.6	2	2	2	6
	4	0.9	2	2	2	6
	7	-17.1	2	2	2	6
	10	5.9	2	2	2	6
	13	2.6	2	2	2	6
	16	-33.9	2	2	2	6
	19	0.6	2	2	2	6
	22	-37.7	2	2	2	6
	25	-30.9	2	2	2	6
	28	23.4	2	2	2	6
	Average =	-8.6	2	2	2	6
	Std. Dev. =	20.2	0	0	0	0
	Std. Error=	6.4	0	0	0	0
Freezing + DMSO	2	29.8	2	2	2	6
	5	-4.8	2	2	2	6
	8	15.2	2	2	2	6
	11	20.4	2	2	2	6
	14	-14.6	2	2	2	6
	17	-14.1	2	2	2	6
	20	-2	2	2	2	6
	23	25.7	2	2	2	6
	26	12.3	2	2	2	6
	29	0.1	2	2	2	6
	Average =	6.8	2	2	2	6
	Std. Dev. =	16.1	0	0	0	0
	Std. Error=	5.1	0	0	0	0

Post-op

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	11.5	2	2	2	6
	6	-40.6	2	2	2	6
	9	27.6	2	2	2	6
	12	6.7	2	2	2	6
	15	2	2	2	2	6
	18	6.1	2	2	2	6
	21	-10.8	2	2	2	6
	24	-34.3	2	2	2	6
	27	20.8	2	2	2	6
	30	-36.9	2	2	2	6
	Average =	-4.8	2	2	2	6
	Std. Dev. =	24.7	0	0	0	0
	Std. Error=	7.8	0	0	0	0
reezing Only	1	-99.2	0	0	0	0
	4	-75.6	0	0	0	0
	7	-105.5	0	0	0	0
	10	-35.4	2	1	1	4
	13	-89.5	0	0	0	0
	16	-76.8	0	0	0	0
	19	-71.4	0	0	0	0
	22	-88.9	1	0	0	1
	25	-94.9	0	0	0	0
	28	-140.9	0	0	0	0
	Average =	-87.8	0.3	0.1	0.1	0.5
	Std. Dev. =	27.1	0.7	0.3	0.3	1.3
	Std. Error=	8.6	0.2	0.1	0.1	0.4
reezing + DMSO	2	-24.3	0	0	0	0
	5	-71.1	0	0	0	0
	8	-56.7	0	0	0	0
	11	-92.2	0	0	0	0
	14	-83.5	0	0	0	0
	17	-100	0	0	0	0
	20	-123.7	0	0	0	0
	23	-73.9	0	0	0	0
	26	-50.8	0	0	0	0
	29	-117.5	0	0	0	0
	Average =	-79.4	0	0	0	0
	Std. Dev. =	30.7	0	0	0	0
	Std. Error=	9.7	0	0	0	0

Week 1

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-17.6	2	2	2	6
	6	15.6	2	2	2	6
	9	15.4	2	2	2	6
	12	3.7	2	2	2	6
	15	-19.2	2	2	2	6
	18	-25.2	2	2	2	6
	21	9.6	2	2	2	6
	24	-19.2	2	2	2	6
	27	4	2	2	2	6
	30	48.3	2	2	2	6
	Average =	1.5	2	2	2	6
	Std. Dev. =	22.6	0	0	0	0
	Std. Error=	7.1	0	0	0	0
Freezing Only	1	-47.8	0	1	1	2
	4	-74.8	0	0	0	0
	7	-74.6	0	0	0	0
	10	-22	2	2	2	6
	13	-87.7	0	1	0	1
	16	-61.7	0	0	0	0
	19	-66.7	0	0	0	0
	22	-47.3	0	0	0	0
	25	-39.7	0	0	0	0
	28	-62	0	0	0	0
	Average =	-58.4	0.2	0.4	0.3	0.9
	Std. Dev. =	19.4	0.6	0.7	0.7	1.9
	Std. Error=	6.1	0.2	0.2	0.2	0.6
Freezing + DMSO	2	-20.5	1	0	0	1
	5	-92.7	0	0	0	0
	8	-68.8	0	0	0	0
	11	-82.6	0	0	0	0
	14	-86.6	0	0	0	0
	17	-84.7	0	0	0	0
	20	-78.8	0	0	0	0
	23	-42.9	0	0	0	0
	26	-55.8	0	0	0	0
	29	-77.8	0	0	0	0
	Average =	-69.1	0.1	0	0	0.1
	Std. Dev. =	22.8	0.3	0	0	0.3
	Std. Error=	7.2	0.1	0	0	0.1

Week 2

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	5.1	2	2	2	6
	6	-11.4	2	2	2	6
	9	-32.3	2	2	2	6
	12	-6.7	2	2	2	6
	15	1.8	2	2	2	6
	18	-13.5	2	2	2	6
	21	-22.8	2	2	2	6
	24	-18.9	2	2	2	6
	27	18.1	2	2	2	6
	30	10.3	2	2	2	6
	Average =	-7	2	2	2	6
	Std. Dev. =	15.8	0	0	0	0
	Std. Error=	5	0	0	0	0
Freezing Only	1	-84.5	0	0	0	0
	4	-59.3	0	0	0	0
	7	-65.6	0	0	0	0
	10	20.5	2	2	2	6
	13	-79.3	0	0	0	0
	16	-64.3	0	0	0	0
	19	-93	0	0	0	0
	22	-73.7	0	0	0	0
	25	-56.2	0	0	0	0
	28	-98.3	0	0	0	0
	Average =	-65.4	0.2	0.2	0.2	0.6
	Std. Dev. =	33.3	0.6	0.6	0.6	1.9
	Std. Error=	10.5	0.2	0.2	0.2	0.6
Freezing + DMSO	2	-40.5	1	0	0	1
	5	-73.1	0	0	0	0
	8	-79.5	0	0	0	0
	11	-65.1	0	0	0	0
	14	-104.3	0	0	0	0
	17	-98	1	0	0	1
	20	-52.5	0	0	0	0
	23	-88.5	0	0	0	0
	26	-39.4	0	0	0	0
	29	-77.6	0	0	0	0
	Average =	-71.9	0.2	0	0	0.2
	Std. Dev. =	22.5	0.4	0	0	0.4
	Std. Error=	7.1	0.1	0	0	0.1

Week 3

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-1.6	2	2	2	6
	6	8.4	2	2	2	6
	9	12.1	2	2	2	6
	12	-1.4	2	2	2	6
	15	20.2	2	2	2	6
	18	-19.2	2	2	2	6
	21	-3.7	2	2	2	6
	24	-22.4	2	2	2	6
	27	-3.2	2	2	2	6
	30	-11.7	2	2	2	6
	Average =	-2.3	2	2	2	6
	Std. Dev. =	13.4	0	0	0	0
	Std. Error=	4.2	0	0	0	0
Freezing Only	1	-39.9	0	0	0	0
	4	1.2	0	1	1	2
	7	-8.6	1	1	1	3
	10	43.3	2	2	2	6
	13	-63.3	0	0	1	1
	16	-68.2	0	0	1	1
	19	-45.6	1	2	1	4
	22	-63.2	1	1	1	3
	25	-26.1	1	1	2	4
	28	-65.5	0	0	0	0
	Average =	-33.6	0.6	0.8	1	2.4
	Std. Dev. =	36.5	0.7	0.8	0.7	2
	Std. Error=	11.5	0.2	0.3	0.2	0.6
Freezing + DMSO	2	-64	1	0	0	1
	5	-89.9	1	1	0	2
	8	-51	0	1	1	2
	11	-64.9	1	0	0	1
	14	-70.1	0	0	0	0
	17	-35.5	0	1	1	2
	20	-64.6	1	1	0	2
	23	-89.5	1	0	0	1
	26	-36.1	1	1	1	3
	29	-93.3	0	0	0	0
	Average =	-65.9	0.6	0.5	0.3	1.4
	Std. Dev. =	20.9	0.5	0.5	0.5	1
	Std. Error=	6.6	0.2	0.2	0.2	0.3

Week 4

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-20.1	2	2	2	6
	6	-0.8	2	2	2	6
	9	21.2	2	2	2	6
	12	12.3	2	2	2	6
	15	-5	2	2	2	6
	18	-21.6	2	2	2	6
	21	46.8	2	2	2	6
	24	39.8	2	2	2	6
	27	-9.8	2	2	2	6
	30	0.4	2	2	2	6
	Average =	6.3	2	2	2	6
	Std. Dev. =	23.5	0	0	0	0
	Std. Error=	7.4	0	0	0	0
Freezing Only	1	-20.1	0	1	1	2
	4	9.6	1	1	2	4
	7	-25.1	2	1	1	4
	10	-24.8	2	2	2	6
	13	-22.8	1	2	2	5
	16	-25.9	2	1	1	4
	19	-12.1	1	2	2	5
	22	12.3	1	2	1	4
	25	-5.3	2	2	1	5
	28	-55.9	1	2	2	5
	Average =	-17	1.3	1.6	1.5	4.4
	Std. Dev. =	19.7	0.7	0.5	0.5	1.1
	Std. Error=	6.2	0.2	0.2	0.2	0.3
Freezing + DMSO	2	-53.3	2	1	0	3
	5	-83	2	1	1	4
	8	-22.3	1	1	2	4
	11	-40.4	1	1	1	3
	14	-73.1	1	1	0	2
	17	2.8	2	2	1	5
	20	-46	1	2	2	5
	23	-41.6	0	0	0	0
	26	-29.3	1	2	2	5
	29	-62.6	1	0	0	1
	Average =	-44.9	1.2	1.1	0.9	3.2
	Std. Dev. =	25.1	0.6	0.7	0.9	1.8
	Std. Error=	7.9	0.2	0.2	0.3	0.6

Week 5

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-1.8	2	2	2	6
	6	-6.9	2	2	2	6
	9	23.5	2	2	2	6
	12	-13.9	2	2	2	6
	15	29	2	2	2	6
	18	-48	2	2	2	6
	21	-0.5	2	2	2	6
	24	-48.3	2	2	2	6
	27	16.1	2	2	2	6
	30	-8.9	2	2	2	6
	Average =	-6	2	2	2	6
	Std. Dev. =	26.4	0	0	0	0
	Std. Error=	8.3	0	0	0	0
Freezing Only	1	13.6	1	1	1	3
	4	-1	2	2	2	6
	7	0.6	2	2	2	6
	10	-7.5	2	2	2	6
	13	8.1	2	2	2	6
	16	11	2	2	2	6
	19	-2.5	2	2	2	6
	22	11	2	2	2	6
	25	-10.9	2	2	2	6
	28	-12.2	2	2	2	6
	Average =	1	1.9	1.9	1.9	5.7
	Std. Dev. =	9.5	0.3	0.3	0.3	0.9
	Std. Error=	3	0.1	0.1	0.1	0.3
Freezing + DMSO	2	-70.8	2	1	0	3
	5	-30.8	2	2	1	5
	8	-22.1	1	1	1	3
	11	-38	1	1	1	3
	14	-53.7	2	2	1	5
	17	-36.6	2	2	1	5
	20	-55.2	2	2	2	6
	23	-10.8	2	2	1	5
	26	-4.5	2	2	2	6
	29	-66.9	1	2	1	4
	Average =	-38.9	1.7	1.7	1.1	4.5
	Std. Dev. =	22.6	0.5	0.5	0.6	1.2
	Std. Error=	7.1	0.2	0.2	0.2	0.4

Week 6

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-11.4	2	2	2	6
	6	-18.9	1	2	2	5
	9	11.1	2	2	2	6
	12	-2.4	2	2	2	6
	15	2.5	2	2	2	6
	18	-8.1	2	2	2	6
	21	33	2	2	2	6
	24	27.9	2	2	2	6
	27	11.4	2	2	2	6
	30	-7.4	2	2	2	6
	Average =	3.8	1.9	2	2	5.9
	Std. Dev. =	17	0.3	0	0	0.3
	Std. Error=	5.4	0.1	0	0	0.1
Freezing Only	1	37	2	1	1	4
	4	1.6	2	2	2	6
	7	-4.6	2	2	2	6
	10	3.8	2	2	2	6
	13	-17.7	2	2	2	6
	16	-17.1	2	2	2	6
	19	-4.5	2	2	2	6
	22	1.9	2	2	2	6
	25	-9.4	0	2	1	3
	28	38.8	2	2	2	6
	Average =	3	1.8	1.9	1.8	5.5
	Std. Dev. =	19.8	0.6	0.3	0.4	1.1
	Std. Error=	6.3	0.2	0.1	0.1	0.3
Freezing + DMSO	2	-62.9	1	1	0	2
	5	-56.6	0	0	0	0
	8	5.4	1	2	1	4
	11	-21.3	2	1	2	5
	14	-20	2	1	2	5
	17	-61.2	0	1	1	2
	20	-33.4	2	2	2	6
	23	15.8	2	1	1	4
	26	37	2	2	2	6
	29	-58.7	1	1	1	3
	Average =	-25.6	1.3	1.2	1.2	3.7
	Std. Dev. =	35.6	0.8	0.6	0.8	1.9
	Std. Error=	11.3	0.3	0.2	0.3	0.6

Week 7

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	13.8	2	2	2	6
	6	2.8	2	2	2	6
	9	26.8	2	2	2	6
	12	-13.6	2	2	2	6
	15	-11.1	2	2	2	6
	18	-24.2	2	2	2	6
	21	17.3	2	2	2	6
	24	-24.9	2	2	2	6
	27	18	2	2	2	6
	30	29.6	2	2	2	6
	Average =	3.5	2	2	2	6
	Std. Dev. =	20.6	0	0	0	0
	Std. Error=	6.5	0	0	0	0
Freezing Only	1	-17.6	2	2	1	5
	4	17.2	2	2	2	6
	7	17.1	2	2	1	5
	10	-38.4	2	2	2	6
	13	-4	2	2	2	6
	16	9.4	2	2	2	6
	19	1.1	2	2	2	6
	22	-13.1	2	2	2	6
	25	-13.2	2	2	2	6
	28	-11.4	2	2	2	6
	Average =	-5.3	2	2	1.8	5.8
	Std. Dev. =	17.2	0	0	0.4	0.4
	Std. Error=	5.4	0	0	0.1	0.1
Freezing + DMSO	2	-53.1	2	1	0	3
	5	-82	2	2	0	4
	8	-30.2	2	2	2	6
	11	-20.3	2	2	2	6
	14	-32.4	2	2	2	6
	17	-57.1	2	2	2	6
	20	-42.4	2	2	2	6
	23	-2.3	2	1	2	5
	26	19.4	2	2	2	6
	29	-61.2	2	2	2	6
	Average =	-36.2	2	1.8	1.6	5.4
	Std. Dev. =	29.9	0	0.4	0.8	1.1
	Std. Error=	9.5	0	0.1	0.3	0.3

Week 8

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-1.6	2	2	2	6
	6	-13.9	2	2	2	6
	9	-13.9	2	2	2	6
	12	12.9	2	2	2	6
	15	-12	2	2	2	6
	18	-17.2	2	2	2	6
	21	2.1	2	2	2	6
	24	-32.3	2	2	2	6
	27	0.1	2	2	2	6
	30	25.3	2	2	2	6
	Average =	-5.1	2	2	2	6
	Std. Dev. =	16.4	0	0	0	0
	Std. Error=	5.2	0	0	0	0
Freezing Only	1	-8.2	2	2	2	6
	4	10.1	2	2	2	6
	7	-16.2	2	2	2	6
	10	17.4	2	2	2	6
	13	-15.5	2	2	2	6
	16	-29.6	2	2	2	6
	19	6.6	2	2	2	6
	22	-14.5	2	2	2	6
	25	-5.8	2	2	2	6
	28	-16.9	2	2	2	6
	Average =	-7.3	2	2	2	6
	Std. Dev. =	14.5	0	0	0	0
	Std. Error=	4.6	0	0	0	0
Freezing + DMSO	2	-69.2	2	1	1	4
	5	-40.1	2	2	0	4
	8	-8.6	1	2	2	5
	11	-18.9				0
	14	-28.9	2	2	2	6
	17	-46.5	2	2	2	6
	20	-36.3	2	2	2	6
	23	-10.8	2	0	2	4
	26	9	2	2	2	6
	29	-86	2	1	2	5
	Average =	-33.6	1.9	1.6	1.7	4.6
	Std. Dev. =	28.7	0.3	0.7	0.7	1.8
	Std. Error=	9.1	0.1	0.2	0.2	0.6

Week 10

Controls	Rat Number	SFI	Sensory	Motor	Tone	Sum of Qual.
	3	-20.1	2	2	2	6
	6	0.7	2	2	2	6
	9	8.9	2	2	2	6
	12	-13.8	2	2	2	6
	15	-10.2	2	2	2	6
	18	-15.9	2	2	2	6
	21	-9.8	2	2	2	6
	24	-2.7	2	2	2	6
	27	-4.2	2	2	2	6
	30	42.8	2	2	2	6
	Average =	-2.4	2	2	2	6
	Std. Dev. =	18	0	0	0	0
	Std. Error=	5.7	0	0	0	0
Freezing Only	1	14.6	2	2	2	6
	4	10	2	2	2	6
	7	-21	2	2	1	5
	10	8.4	2	2	2	6
	13	0.8	2	2	2	6
	16	-3.6	2	2	2	6
	19	-2.4	2	2	2	6
	22	-14.6	2	2	2	6
	25	-17	2	2	2	6
	28	-17.2	2	2	2	6
	Average =	-4.2	2	2	1.9	5.9
	Std. Dev. =	12.8	0	0	0.3	0.3
	Std. Error=	4	0	0	0.1	0.1
Freezing + DMSO	2	-55	2	1	1	4
	5	-56.5	2	2	1	5
	8	-24.3	2	2	2	6
	11	-36.7	2	2	2	6
	14	-35.8	2	2	2	6
	17	-18.5	2	2	2	6
	20	-35.1	2	2	2	6
	23	-26.6	2	2	1	5
	26	-1.7	2	2	2	6
	29	-51.2	2	2	2	6
	Average =	-34.1	2	1.9	1.7	5.6
	Std. Dev. =	17.3	0	0.3	0.5	0.7
	Std. Error=	5.5	0	0.1	0.2	0.2

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