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Regenerative capabilities of the rat seventh nerve using suture and collagen splint techniques: a comparative study

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REGENERATIVE CAPABILITIES OF THE RAT SEVENTH NERVE USING SUTURE AND COLLAGEN SPLINT TECHNIQUES A COMPARATIVE STUDY

INDEPENDENT STUDY PROJECT

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INTRODUCTION

Nerve regeneration following injury and subsequent repair has been studied over the years in an attempt to find the procedure which best facilitates new growth and restores function. Laboratory animals, such as the rat, have been used as models for procedures to be used on humans. Such experimentation is not possible on a large scale in human subjects for ethical reasons. Therefore, studies on animal models provide the basis for application to human clinical practice. Of overlooked importance, however, are the similarities those model systems possess to humans. Ideally then, the nerves studied should resemble as closely as possible the human counterpart to which they are being compared. This will help to assure success when transfering the procedure to humans.

Many recent studies have looked at peripheral nerve regeneration using the rat sciatic nerve, a spinal motor nerve. The applicability of these procedures to human cranial nerves is questionable. Studies on sciatic nerves in rodents may not be as meaningful because the sciatic nerve and cranial nerves differ in many respects. The long segment of the rat sciatic nerve is easy to expose and is already separated into distinct abductor and adductor fascicles when it emerges from beneath the sacrum. The main trunk of the facial nerve is only 6 mm long and is amenable to manipulation. But this is complicated by the fact that the posterior branch of the external jugular vein obscures the more proximal portion, leaving no more than one to two mm of the main trunk visible before the nerve

divides into its main branches. It is often these branches which are to be manipulated. The buccal branch, for instance, follows the groove between the masseter and temporalis muscles, passes beneath the eye toward the nose, sending branches to the upper and lower lips (see Figure 1). Its function is very specific, and its path very complex. The sciatic nerve's function is less specific, and its path direct. It would seem then that the rat facial nerve provides a more suitable model for the human facial nerve.

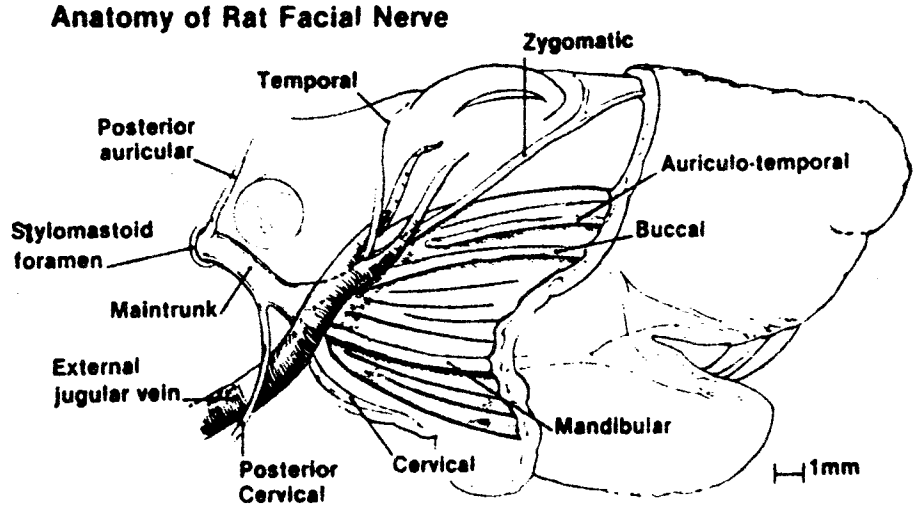


Figure 1. Schematic representation of the rat facial nerve. The main trunk exits from the mastoid foramen. Rather than forming distinct trunks, the branching of the main trunk occurs as one main furcation. The auriculotemporal nerve is seen coming from behind the masseter muscle and travels with the buccal branch of the facial nerve.

Unfortunately, there appears to be no ideal animal model for studies of facial nerve regeneration. The cat [1,2], rabbit [3,5], guinea pig [4], and rat [6] sciatic and facial nerves have been used in studies involving nerve suture, grafting. Mattox and Felix [7] in 1987 looked at similarities and differences between the human and rat facial nerves. Branches of the rat facial nerve amenable to experimental manipulation, as well as a detailed peripheral branching pattern, were described, and actual axonal counts were performed. The rat facial nerve was found to have many similarities with that of other non-primate mammals. Equally encouraging is the fact that the tissues of the rat are easily dissected and the main trunk and peripheral branches of the facial nerve are readily identifiable.

While division of the rat facial nerve into fascicles occurs at the region of identifiable branching, the main trunk is histologically monofascicular. This contrasts with the human, where the perineurium divides the facial nerve into fascicles extemporally. Therefore, regeneration studies with rat facial nerve may not exactly replicate the human clinical situation, because the regenerating nerves' growth is more directed once it has entered a fascicle. It also was found that within the first 6 to 8 mm of the buccal branch the exact site of sampling is not critical. Another advantage to using the rat includes its ability to tolerate facial paralysis produced by facial nerve transection without feeding problems [7].

Disadvantages include the relatively short segment of main trunk available to use, as well as a rich arterial and venous network around

the furcation. Even considering these disadvantages, Mattox and Felix [7] concluded that the main trunk was easily amenable to and appropriate for experimental surgical manipulation.

In their axon counts, Mattox and Felix [7] found that there is an average of 4650 axons in the main trunk of the rat facial nerve, although Martin [27] has reported 5353 fibers. The number of axons in the mandibular and buccal branches was more variable (buccal averaged 1955 with a standard deviation of 279); however, the authors felt that the standard deviations were consistent enough to allow comparisons without excessive numbers of animals (n=6).

Current Clinical Applications

The main focus of any nerve repair procedure is to maximize the number of fibers that regenerate past the suture line. Current standard clinical practice for the repair of human peripheral nerve injuries favors, if at all possible, the direct anastomosis of proximal and distal nerve stumps [8,9,10]. In addition, removing the epineurium, limiting the number of sutures, increasing anastomosis surface area by making an oblique cut, and minimizing tension on distal and proximal nerve ends also aids in maximizing regeneration. Although these procedures are of benefit, they are not without shortcomings. Oftentimes in nerve injury, a gap between the distal and proximal nerve stumps is too great for the ends to be joined. In such cases, grafting has been attempted. However, it is felt that

microsurgery of this nature has been improved to its fullest capabilities without providing satisfactory recuperation [11-15].

Alternatives to this technique have likewise been studied. Entubulation as an aid to regeneration has been studied for a number of years [14,16,22-24]. However, this procedure has not been perfected. Gap length [13,17], spatial and temporal growth of nerve fibers within a chamber [18-20], and accumulation of trophic factors within the chamber [21] have been described.

The "bottom line" however, to any technique, is the degree of function restored. To look at this aspect quantitatively, researchers have used fiber counts that are taken distal to the suture line. In addition, researchers have relied on electrophysiologic methods to provide further information concerning function in conjunction with fiber counts.

In humans, the amount and degree of restored function is judged behaviorally. The physician rates the recovery of certain facial areas by assigning a number from one to six which indicates the degree of function. In the rat model, useful behavioral measures do not appear to be feasible. Therefore, nerve fiber counts and electrophysiological measures are made to assess function. Although electrophysiological measures have been reported as being unreliable because of the variability in technical factors associated with the recording configuration [26] (i.e., the position and type of recording electrodes, interelectrode distance, the density of connective tissue, current

shunts, etc.), this type of recording is commonly used and presented in the literature. It was felt that its inclusion in this study would provide information to allow for comparisons across other studies. In addition, electromyographic (EMG) recordings also are performed on humans as a functional measurement.

The purpose of this study is to compare the effectiveness of repair procedures on the rat facial nerve using the current clinical surgical procedure of suturing nerve ends and a proposed technique utilizing splints made of collagen.

METHODS AND MATERIALS

This experiment was carried out on twelve adult female rats of the Sprague-Dawley strain. The rats were equally divided into two groups. Each rat underwent an initial operation where it was pre-anesthetized using 0.1 ml atropine and 20 mg/kg ketamine (i.m.) followed by deep anesthetization using 30 mg/kg sodium pentobarbital (i.p.). The right side of the face was incised in a dorsal to ventral manner, posterior to the mandible line. The skin was reflected inferiorly and the muscle posteriorly moved, exposing the VIIth nerve (see Figure 1). The buccal branch of the right VIIth nerve was transected unilaterally approximately 3 mm superiorly from the point of bifurcation in the neck region and the epineurium was pulled back. In two of the six "suture only" rats, the temporal mandibular branch was transected instead of the buccal branch. The two nerve stumps were then anastomosed using one of two surgical techniques: suture only (controls), or collagen splint. In the suture only technique, the cut nerve ends were carefully reapproximated and sutured using a 10/0 nylon suture. An attempt was made to minimize trauma by making only two passes through each nerve. For the collagen splint procedure, the cut nerve ends were carefully reapproximated and wrapped lengthwise with a cut collagen cuff which had been rinsed in Ringer's solution. The cuff covered 3 mm of each nerve end. No gap was left between the nerve ends in five of the six rats under this procedure. An unavoidable gap of less than one mm was left in one animal. After surgery, the wound was

closed, a topical antibiotic ointment (Panalog) was applied, and 30,000 units of penicillin was administered.

Three months were allowed for recovery, which provided ample time for healing and neuroma development. Functional results of the anastomosis were measured electrophysiologically and recorded on an IBM AT computer using a MI2 A/D converter/control unit, a Grass P-15 pre-amplifier and a Tektronix oscilloscope. The nerve was lifted off the muscle by two electrodes. A Grass S-1 Stimulator set at 150 mV (2/sec) was used to stimulate the nerve proximal to the anastomosis with the recording electrode placed 15 mm distal to this point for nerve potential recording. For muscle potential, a recording electrode was placed in the snout. The function of the nerve was then analyzed based on latency and amplitude measures and compared to the normal contralateral side which was handled in an identical manner. All measured responses represented the average of 100 stimulus presentations.

The rats were then sacrificed using an overdose of pentobarbital. Portions of the experimental and normal nerves were removed by placing sutures proximal and distal from the point of repair, and then cutting the nerve at this point. The nerve was then fixed overnight in a buffered 2% glutaraldehyde/1% paraformaldehyde solution at $4^{o}C$ and rinsed in buffer. The nerve was post-fixed in a 2% solution of osmium tetroxide for two hours at $23^{o}C$, dehydrated, and embedded in Historesin (LKB) plastic. A cross-sectional cut was taken using a rotary microtome and the section mounted and stained

using a 1:4 Paragon/70% ethanol solution [25]. The number of nerve fibers were counted using a Nikon Optiphot microscope, AT-clone computer, and Image Measure (Microscience) software. Statistical analyses were performed using StatView 512+ (Brainpower).

RESULTS

Visual Observations

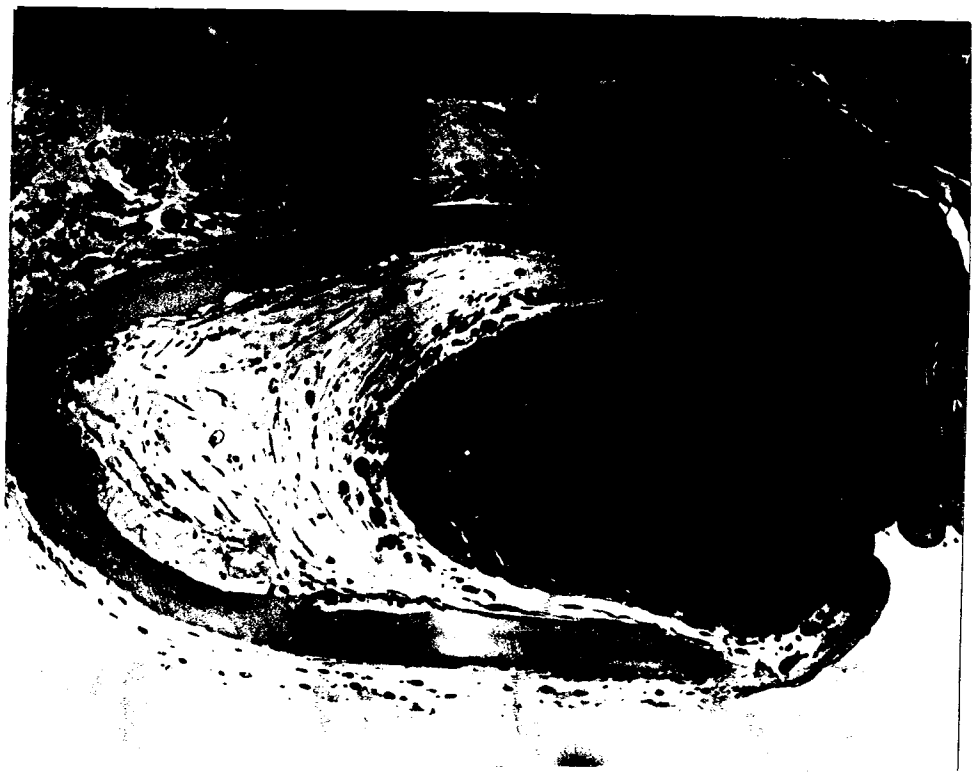
Upon reopening the face, the nerve was examined visually before electrophysiological measures were taken and the nerve removed.

Collagen Splint Group - (CS). In all six rats, the splint was visible. However, in two the splint was no longer completely around the nerve. This may be attributable to increased nerve growth, as it appeared that nerve was now too large to be encompassed by the cuff (see Figure 2).

The anastomosis site appeared well healed in five of the six rats. On one rat (CS-4), the splint appeared almost empty, even though the nerve was evident both proximal and distal to the site. It was felt that this might indicate a decreased degree of regeneration.

Suture Only Group - (SO). The sutures were evident in all six rats. Two of the six displayed right nerves which were abnormal in appearance; mottled or granular looking near the point of anastomosis. The anastomosis sites appeared healed in all animals.

Figure 2. Right proximal nerve section of rat #5 in the collagen splint group. The collagen splint is clearly visible. Note the outgrowth of nerve and oblique growing fibers.



Nerve Fiber Counts

Examination of the nerve sections under the microscope revealed various degrees of robustness of the axons. Only the objects which were identifiable as axons were counted; degenerating profiles were not counted.

Collagen Splint Group - (CS). In this group, one of the six rats displayed fibers which were growing obliquely on both the right and left sides. Right proximal fiber counts averaged 1532 fibers with a standard deviation of 481 (see Figure 3). Distally, 2381 $\pm$ 1779 fibers were counted (see Figure 4) and the number of left nerve fibers averaged 1385 $\pm$ 336 (see Figure 5). For one animal in particular (CS-5), the left nerve fiber count was unusually low at 955. For the CS-4 rat which appeared to have an empty collagen cuff, the left and right proximal axon counts were similar to other rats. However, the right distal axon count was 929, the lowest of all six rats.

Suture Only Group - (SO). The average number of fibers counted proximally was 1822 with a standard deviation of 335 (see Figure 6). Fiber counts taken distally averaged 1948 $\pm$ 858 (see Figure 7). Left nerve fibers averaged 1966 $\pm$ 581.

Comparison of CS and SO Groups. There was no significant difference between the means for the right proximal nerves of either CS or SO groups ($p=.45$). Table 1 and 2 display the raw data for both groups. For the right distal counts, the mean for the CS group is greater than

Figure 3. Right proximal nerve section of rat #1 in the collagen splint group. Note the oblique growing fibers.

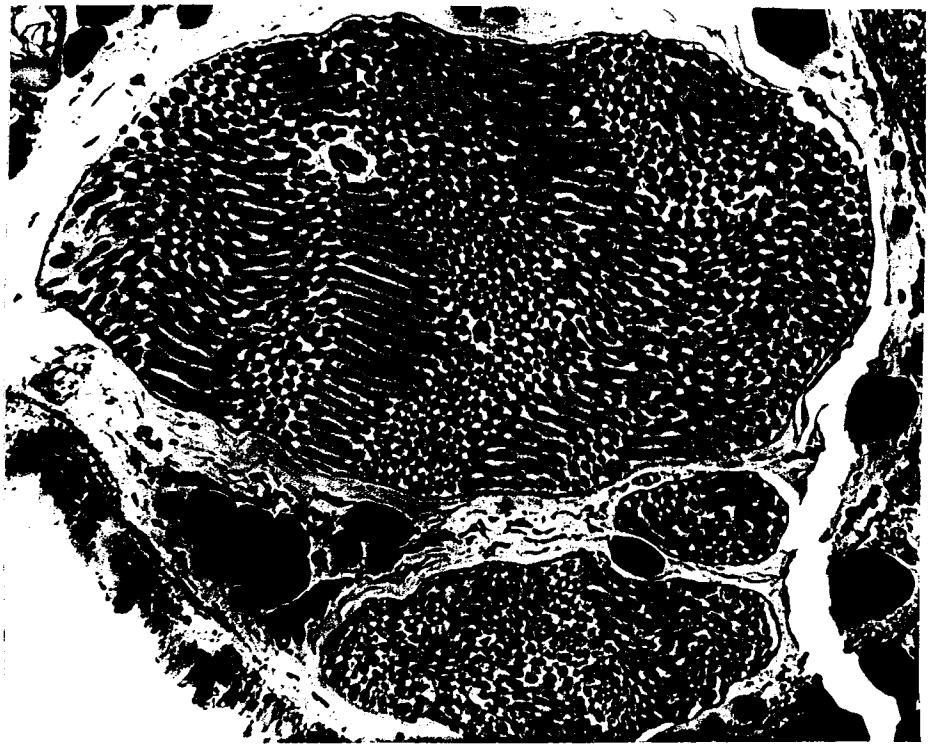


Figure 4. Right distal nerve section of rat #1 in the collagen splint group.

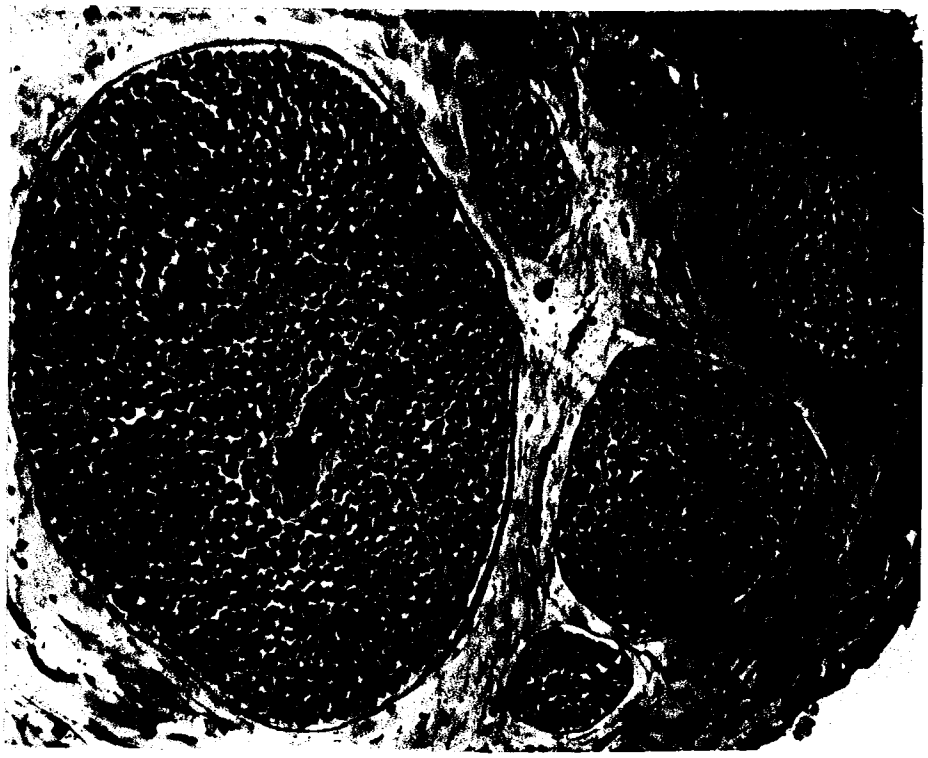


Figure 5. Left nerve section of rat #1 in the collagen splint group.

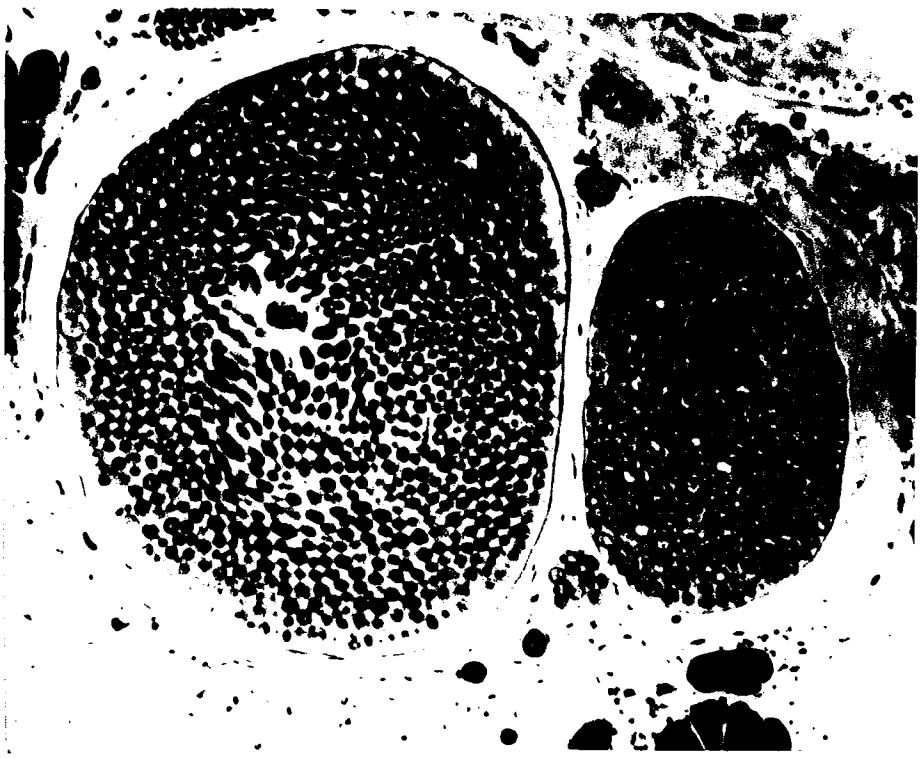


Figure 6. Right proximal nerve section of rat #3 in the suture only group.

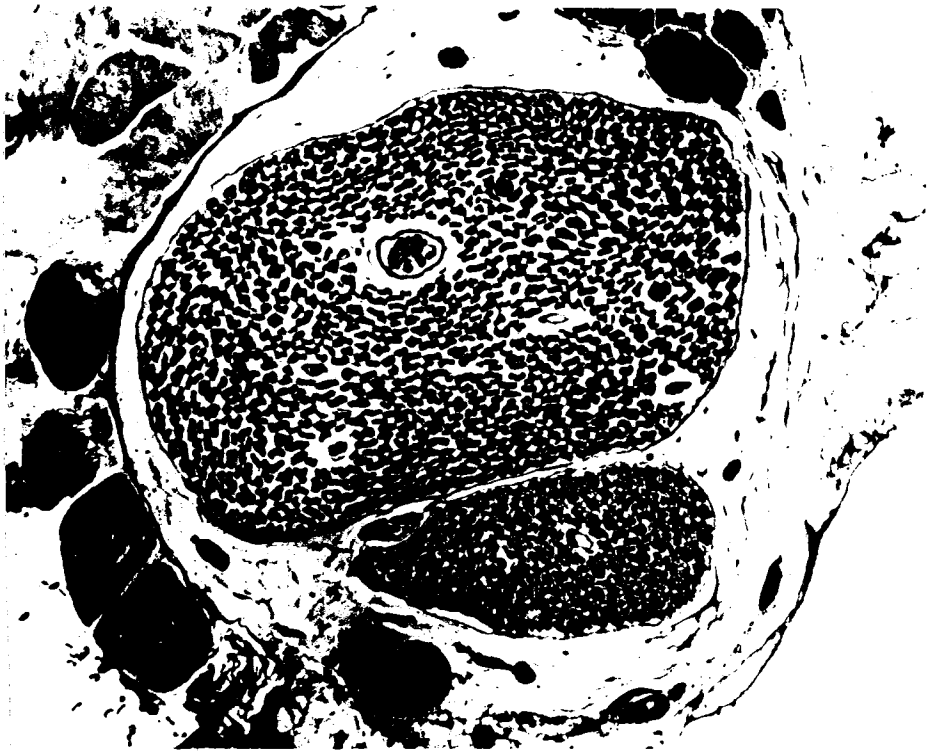
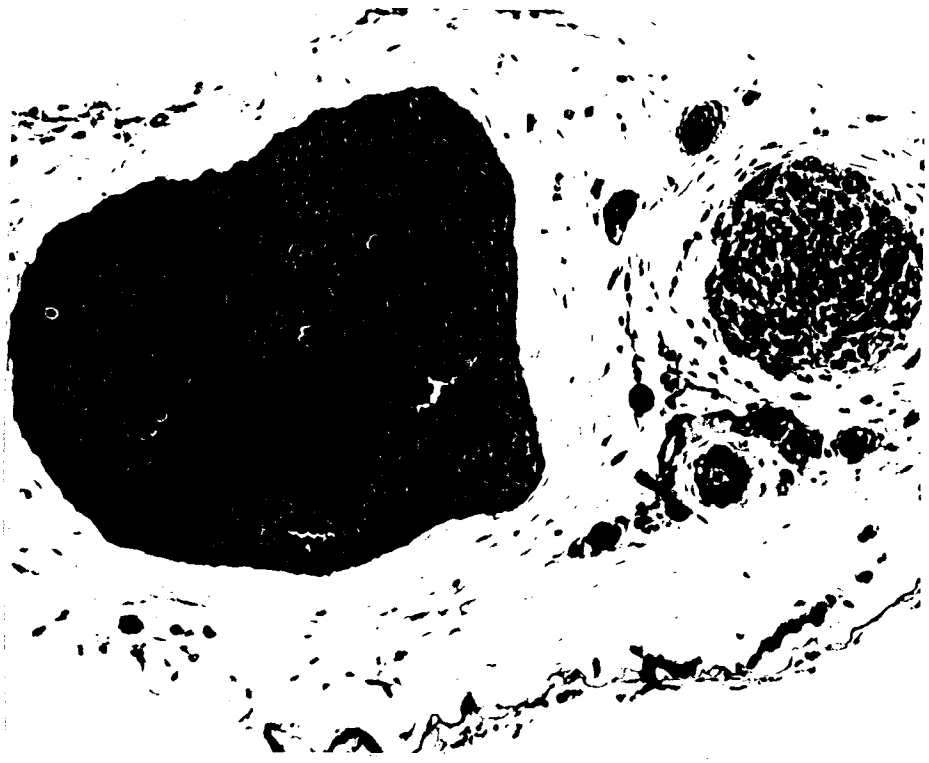


Figure 7. Right distal nerve section of rat #3 in the suture only group.



the mean for the SO group, indicating that regeneration was improved with the collagen splint. However, the difference was not statistically significant between the means for the right distal nerves of either group (p=.65). There was no significant difference shown between the means of the left and right proximal (p=.54) or between the left and right distal nerves (p=.97) in the suture only group.

	FIBER COUNTS - CS GROUP		
Rat	Left	Right Proximal	Right Distal
1	1298	1777	4549
2	1442	2280	4778
3	1579	1316	1361
4	1903	1621	929
5	955	1336	1197
6	1134	864	1471
Mean	**1385**	**1532**	**2381**
Standard Deviation	336	481	1779

Table 1. Axon counts for the collagen splint group.

Table 2. Axon counts for the suture only group. Right proximal count for rat #6 was lost during computer storage.

	FIBER COUNTS - S0 GROUP		
Rat	Left	Right Proximal	Right Distal
1	2061	1666	2016
2	2870	2144	1173
3	1312	1457	1112
4	2534	1632	1469
5	1386	2211	2722
6	1636	- - -	3194
Mean	1967	1822	1948
Standard Deviation	581	335	858

Electrophysiological Recordings

Collagen Splint (CS) Group Nerve and Muscle Recordings

The recorded compound nerve action potentials were averaged for at least 200 records for the right and left sides in each group. The mean for the right side of the CS group was 131 mV with a standard deviation of 68 mV. The mean for the left side was 171 mV $\pm$ 94 mV. The difference between the right and left sides was not statistically significant (p=.50).

The muscle potential average for the right side was 116 mV with a standard deviation of 27 mV. Left side averages were 163 mV ± 62 mV. The difference between the right and left sides was not statistically significant (p=.14).

Suture Only Group (SO) Nerve and Muscle Recordings

The nerve action potential mean for the right side was 104 mV with a standard deviation of 84 mV. The left side mean was 98 ± 62 mV. These can be considered to be identical.

The average muscle potential, for the right side, was 183 ± 24 mV. For the left muscle potential, the mean was 127 ± 55 mV. The difference between the sides was significant (p=.053)

Comparison of CS and SO Groups

Paired t-Test measures indicated that there was no significant difference between the means for the right nerves of either CS or SO groups (p=.58). Table 3 and 4 contain the raw data for both groups. There was no significant difference shown between the means of the left nerves of either CS or SO groups (p=.74) as well. For muscle potential measures, paired t-Tests indicated that there was a significant difference between the means for the right CS and right SO muscle groups (p=.049). There was no significant difference between the means for the left CS or SO muscle groups (p=.08).

Table 3. Electrophysiological data for collagen splint group. Data for rat #2 was lost during computer storage.

COMPOUND ACTION POTENTIALS (mV) - CS GROUP				
Rat	Right Nerve	Left Nerve	Right Muscle	Left Muscle
1	201.3	106.1	117.1	186.7
2	- - -	- - -	- - -	- - -
3	51.2	58.6	146.4	197.6
4	172	201.3	87.8	203.1
5	165	186.7	139.1	172
6	64.1	303.8	91.5	54.9
Mean	**131**	**171**	**116**	**163**
Standard Deviation	68	94	27	62

Table 4. Electrophysiological data for suture only group. Data not included was obtained, but lost during computer storage.

COMPOUND ACTION POTENTIALS (mV) - SO GROUP				
Rat	Right Nerve	Left Nerve	Right Muscle	Left Muscle
1	51.2	168.4	150.1	60.4
2	- - -	- - -	- - -	- - -
3	- - -	- - -	199.5	194
4	60.4	73.2	203.1	131.8
5	- - -	- - -	- - -	- - -
6	201.3	51.2	179.3	120.8
Mean	**104**	**98**	**183**	**127**
Standard Deviation	84	62	24	55

DISCUSSION

An important technical consideration in this study is how the axon counts compare to those previously reported in the literature. A comparison of counts in control nerves provides an indication of relative counting efficiency. This then will argue for or against the validity of the counts in the experimental nerves. Mattox and Felix [7] found an average of 1955 ± 279 buccal fibers in a population of 6 rats. The left (control) nerves studied in our experiment averaged 1676 ± 572 axons (CS mean = 1385, SO mean = 1967). However, in the CS group, one rat was found to have only 955 fibers. Because this count appeared to be so different from the others, the mean was again calculated excluding this rat. A revised average for the 5 CS rats then is 1471 ± 293 and a revised two group average is 1741 ± 551. Our normal fiber counts appear to be consistent with those reported in the literature. Therefore, it is reasonable to assume that the counts from the experimental nerves are valid.

The data obtained in this study provide several points of analysis relating to the effectiveness of suture versus collagen splint repair of the rat facial nerve. Because the collagen splint is a less traumatic procedure and presumably offers greater guidance in regeneration, it is reasonable to assume that this procedure might afford improved regeneration over the suture technique. There does appear to be a tendency for improved regeneration with the collagen splint (averaging 400 more axons in the distal segment), although this is not statistically proven (see Figures 8 and 9). Considering the large

standard deviations and small group sizes, this tendency may prove to be significant if a greater number of rats could be studied.

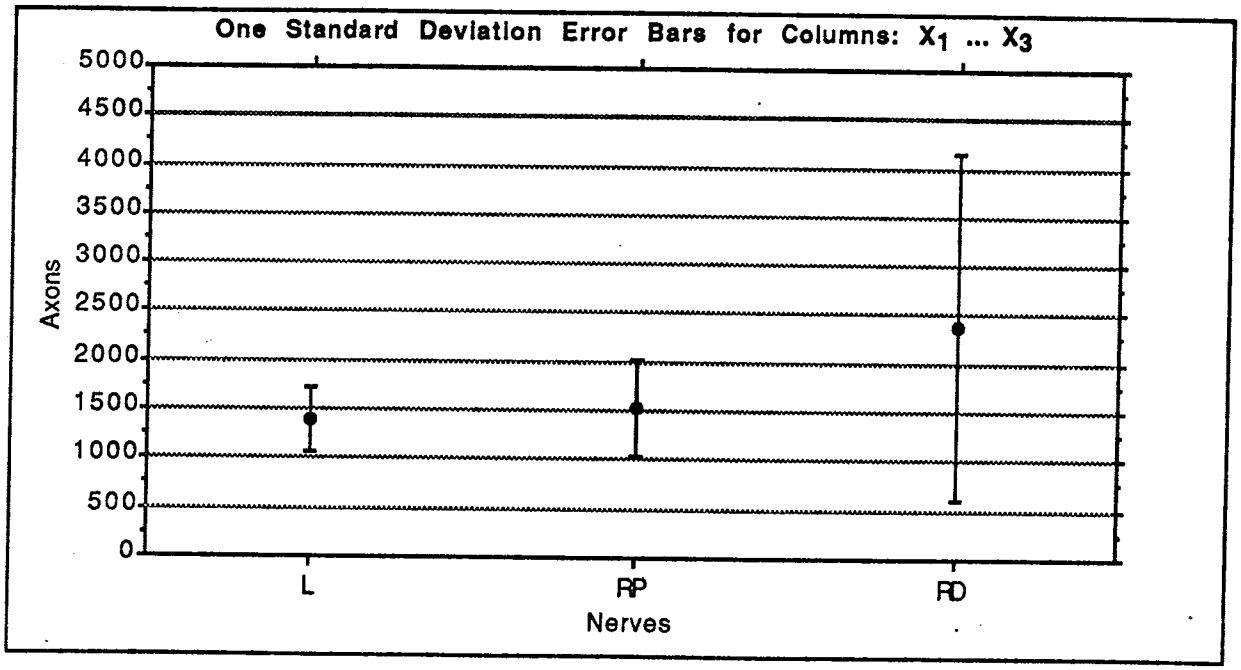
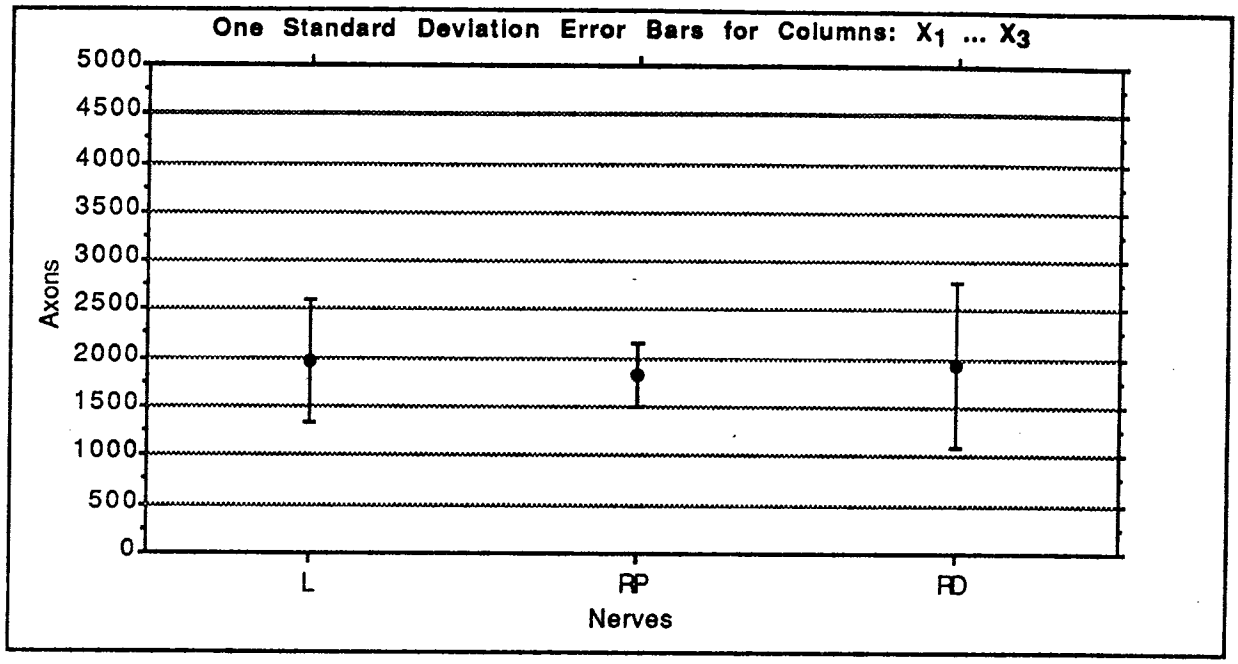


Figure 8. Mean fiber count values plus one standard deviation for the collagen splint group (L=left, RP=right proximal, RD=right distal nerve).

Figure 9. Mean fiber count values plus one standard deviation for the suture only group (L=left, RP=right proximal, RD=right distal).



The effectiveness of the collagen splint technique may also be analyzed by noticing that the right proximal and right distal fiber counts individually exceed the number of fibers counted for the left nerve (see Table 1). Presumably because the splint offered greater guidance, the sprouting of new nerve fibers, which occurred after transection, was channeled to the distal side. The right fiber counts reflect this non-traumatic repair procedure. This is not seen for the

SO group, presumably because such a traumatic repair method creates scar tissue which does not facilitate as much sprouting, and forms barriers to the sprouts which do arise. The sprouting may be present, but the axons may be escaping from the point of anastomosis.

Nerve and muscle potential recordings were highly variable, therefore conclusions are difficult to draw. This is similar to the situation seen in other published reports which have attempted electrophysiological recordings [26, 28-33]. Because of this, the need for further study into alternative methods of assessing regeneration is emphasized. Again, studying a larger population may decrease the variability.

SUMMARY

There appears to be a tendency for improved regeneration with the collagen splint technique over the suture only technique. However, further studies should be done on larger groups to facilitate statistical analysis of the results. Evaluation of potential clinical application will be better served by expanding this study.

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