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EXPERIMENTAL

The Impact of Different Degrees of Injured C7 Nerve Transfer: An Experimental Rat Study

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Background: Ipsilateral C7 nerve transfer is an available procedure in C5C6 2-root avulsion injury of the brachial plexus. However, concomitant injury of a normal-looking C7 cannot be ruled out. The efficiency of a concomitant injury of C7 transfer was investigated.

Methods: Forty-two Sprague-Dawley rats were randomly assigned to 5 groups. They all underwent a 2-stage procedure. In the first stage from dorsal spine approach, left C5 and C6 roots were avulsed and C7 was crushed with jeweler's forceps with different degrees: group A ($n = 6$), C7 not injured; group B ($n = 10$), C7 crushed for 10 seconds; group C ($n = 10$), C7 crushed for 30 seconds; group D ($n = 10$), C7 doubly crushed for 60 seconds; and group E ($n = 6$), C7 transected and not repaired. Four weeks later in the second stage, the C7 was reexplored via volar approach, transected, and coapted to the musculocutaneous nerve. At 12 weeks following the nerve transfer, functional outcomes were assessed.

Results: Grooming test, muscle weight, electromyography, and muscle tetanic contraction force all showed that the biceps muscles were significantly worse in group C (moderate crush) and group D (severe crush). Group B (mild crush) and group A (uninjured) showed no difference. Group E (C7 cut and not repaired) was the worst.

Conclusions: An injured but grossly normal-looking ipsilateral C7 can be used as a motor source but with variable results. The result is directly proportional to the severity of injury, potentially implying that better results will be achieved when longer regeneration time is allowed. *(Plast Reconstr Surg Glob Open 2014;2:e230; doi: 10.1097/GOX.0000000000000198; Published online 9 October 2014.)*

In brachial plexus injury, ipsilateral C7 is quite often used to transfer to the upper trunk in C5C6 2-root avulsion for shoulder and elbow restoration.[1–3] C7 transfer brings a vast number of myelinated nerve fibers. Its anterior division contains 29.8% of median and 23.7% of musculocutaneous nerves (MCNs), and the posterior division comprises 22% axillary, 45% radial, and 53% dorsal

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DOI: 10.1097/GOX.0000000000000198

Received for publication October 29, 2014; accepted August 14, 2014.

Presented at 7th World Congress of the World Society for Reconstructive Microsurgery, July 11–14, 2013, Chicago, Ill.; 51st Annual Meeting of the Austrian Society for Plastic, Aesthetic and Reconstructive Surgery (ÖGPÄRC), October 3–5, 2013, Velden am Wörthersee, Austria; 35th Annual Meeting of DAM [Deutschsprachige Arbeitsgemeinschaft für Mikrochirurgie der peripheren Nerven und Gefässe (German-speaking Microsurgery Association for the Peripheral Nerves and Vessels)], November 21–23, 2013, Deidesheim, Germany; 2014 Annual Meeting of the American Society for Peripheral Nerve, January 8–14, 2014, Kauai, Hawaii; 2014 Chang Gung Mayo Clinic Symposium in Reconstructive Surgery, May 7–10, 2014,

thoracodorsal nerves.[4] In addition, loss of a C7 spinal nerve is mostly compensable,[5–7] resulting in no impairment of original function. C7 spinal nerve can be used as a neurotizer (motor and sensory) for transfer contralaterally[4,8–11] or ipsilaterally.[1–3] However, ipsilateral C7 transfer does not always work well. The health of a C7 donor nerve is a critical determining factor for success of nerve transfer. Clinically, the C7 root is involved more often than any other root in brachial plexus injury, from stretching to avulsion injury.[12–14] It is difficult for a surgeon to decide if a macroscopically normal-looking C7 is suitable for transfer. The purpose of this study was to assess the impact of different degrees of injured C7 when used as a donor nerve for transfer.

PRELIMINARY STUDY

To see the phenomenon of the nerve regeneration after different crush injuries, and to determine when is the suitable time for nerve transfer and for nerve evaluation after crush injury, 20 Sprague-Dawley rats underwent the preliminary study. The rats, approximately 250 g each in weight, were randomly separated into 4 groups (A–D). From the back spine approach, the C5 and C7 were avulsed, and the C6 was crushed by microneedle holder (curved one, 8/150 mm with lock, Prima, Germany) and by the same person (T.N.-J. Chang) with different degrees: group A, crushed for 10 seconds; group B, for 30 seconds; group C, for 60 seconds; and group D, doubly crushed by 2 same needle holders for 60 seconds. Functional outcomes of the biceps muscle were evaluated with grooming test, electromyogram-muscle action potential (stimulated the MCN at its origin from the lateral cord and recorded the biceps muscle), muscle (biceps) tetanus contraction force, and muscle (biceps) weight at 1, 2, 4, 8, and 12 weeks postoperatively.

Results are shown in Figure 1. At 4 weeks after nerve crush, all 4 evaluation methods showed that all the crushed nerves seemed to reach a point to start to activate the denervated biceps muscle. At 12 weeks postoperatively, all 4 methods showed that the 4 groups have reached their individual plateau of functional recovery.

In conclusion, by this preliminary investigation, 4 weeks after nerve crush injury will be a suitable time to perform the nerve transfer. Functional evaluation of the upper extremity was usually performed at 12 weeks after nerve surgery in rats.[15–17]

MATERIALS AND METHODS

Forty-two Sprague-Dawley male rats, weighing approximately 250 g each, were randomly separated into 5 groups (A–E) and underwent a 2-stage procedure (Fig. 2). Left brachial plexus was used as the experimental site, C7 spinal nerve the motor neurotizer, and MCN and biceps muscle the targets to be neurotized. All animals were cared for in accordance with established principles for the care of research animals approved by Chang Gung Memorial Hospital Animal Care Committee. Postoperatively, the rats were housed in a light-controlled and temperature-controlled environment and given water and standard chow ad libitum until they were humanely killed for further investigation.

In the first stage, from the back cervical spine approach, the C5 and C6 were avulsed, and C7 was crushed by No. 5 jeweler's forceps[14] (Venus 5, Micro forceps, Regine, Switzerland SA) performed by the same surgeon (C.-H.J. Tzou) to exert different degrees of injuries simulating the clinical trauma: group A (n = 6, control), C7 was uninjured; group B (n = 10), C7 was crushed for 10 seconds (simulating Sunderland I injury)[15]; group C (n = 10), C7 was crushed for 30 seconds (simulating Sunderland II injury)[15]; group D (n = 10), C7 was doubly crushed by 2 forceps for 60 seconds (simulating Sunderland III injury)[15]; and group E (n = 6, negative control), C7 was completely transected (simulating Sunderland V injury).[15] The proximal end was sutured to the nearby ligament.

To change the crush tool from microneedle holder in the preliminary study to the No. 5 jeweler forceps in this study, the microneedle holder was considered not strong enough for nerve crush. In addition, to decrease the number of killed rats, group A (positive control) and group E (negative control) were all 6 rats in contrast to n = 10 in the study groups. The relationship of severity of the nerve crush injury and

Taipei, Linkou, Taiwan; 25th Annual Meeting of the European Association of Plastic Surgeons, May 29–31, 2014, Lacco Ameno, Isle of Ischia (Na), Italy; and 12th Congress of the European Society of Plastic, Reconstructive and Aesthetic Surgery, July 6–11, 2014, Edinburgh, United Kingdom.

Disclosure: *The authors have no financial interest to declare in relation to the content of this article. This study was supported by the grant of the Chang Gung Memorial Hospital, Linkou, Taiwan (CMRP-G3B1211), and the Erwin Schrödinger Fellowships scholarship (J3254-B09) of the Austrian Science Fund (FWF) awarded to the first author (C.-H.J. Tzou). The Article Processing Charge was paid for by the Austrian Science Fund.*

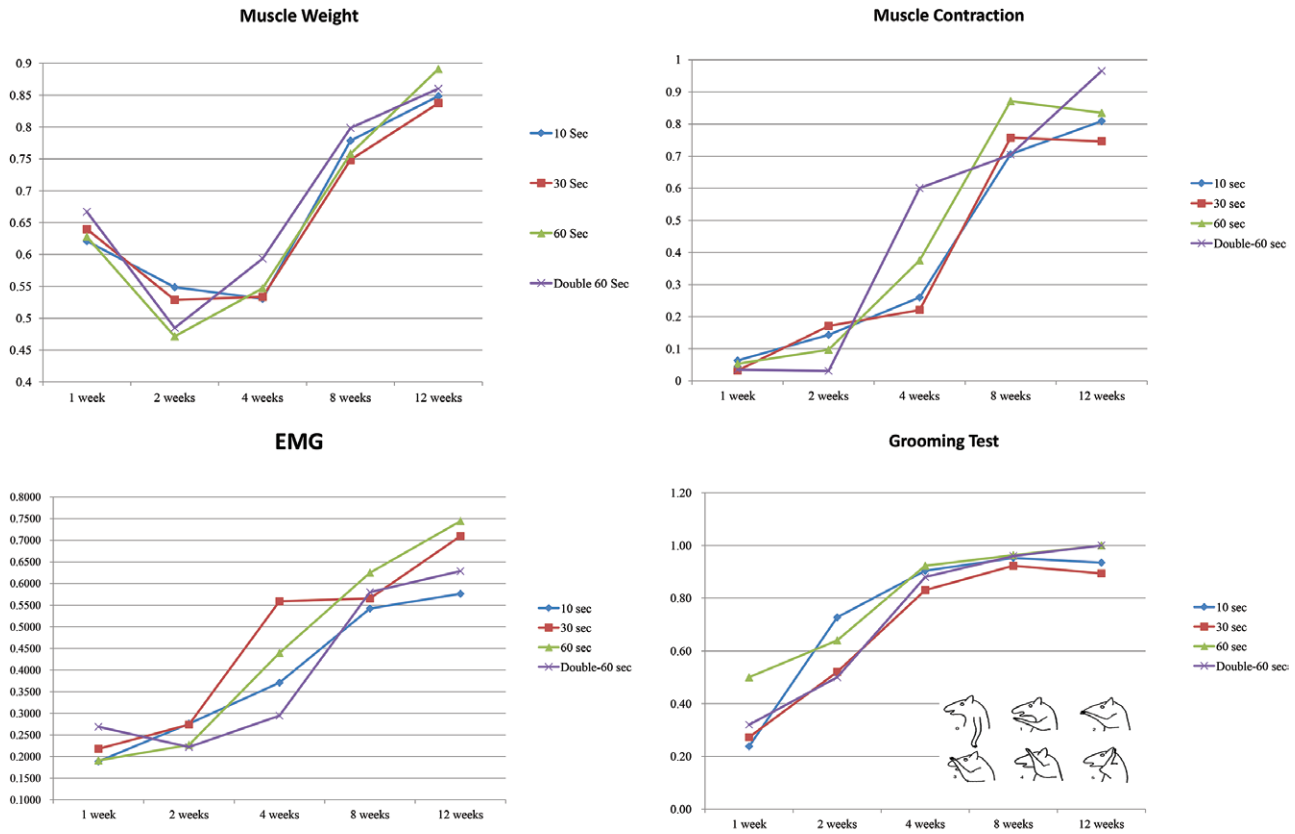

Fig. 1. Preliminary study: The 4 groups of rats' musculocutaneous nerve were crushed by jeweler's forceps with different duration: crushed 10 seconds, 30 seconds, 60 seconds, and double crushes 60 seconds. Functional outcomes of biceps muscle were evaluated with grooming test, muscle action potential, muscle tetanus contraction force, and muscle weight at 1, 2, 4, 8, and 12 weeks postoperatively (see text). The *y*-axis in the graphs: "mV" for electromyogram; "mg" for muscle weight; "g" for muscle contraction force; "an average score" for the grooming test (scores 0–5).

Sunderland classification was a hypothesis based on the preliminary study and clinical experience.

In the second stage, 4 weeks later, from the volar neck approach, left brachial plexus was explored. The distal C7, close to the MCN, was transected. The MCN was transected too. The proximal C7 stump was coapted to the nearby distal MCN stump, end-to-end directly without tension (Fig. 2).

Regeneration for 12 weeks following nerve transfer was allowed. Biceps muscle was evaluated with behavioral (grooming test), electromyogram-muscle action potential, muscle tetanus contraction force, muscle weight, and histomorphometry (histology and axon counts) of the normal C7 and MCN (distal to the nerve coaptation). All assessments were performed bilaterally; thus, the healthy side (group H, Table 2) served as each animal's own control.

Surgical Procedures

First Stage: Crush Injury

All surgical procedures were performed under sterile conditions and anesthesia by inhalation of isofluorane (Halocarbon Laboratories, River Edge, N.J.). The animals were placed in the prone position, hair was shaved, and skin was washed with antiseptic solution. Under an operating microscope, the left brachial plexus was exposed via a vertical incision in the paravertebral region, and the nerve trunks were identified using a nerve stimulator (Vari-Stim hand-held nerve locator/stimulator, Medtronic Xomed, Minneapolis, Minn.). The C5 and C6 roots were avulsed, and C7 was crushed for various durations (10 seconds, 30 seconds, and double crush with 2 jeweler's forceps 60 seconds) using jeweler's forceps No. 5. The crush site was marked with one 10-0 nylon suture. The wound was closed with nylon 4-0 sutures.

Second Stage: Nerve Transfer

After 4 weeks, the animal was put in supine position. The left brachial plexus was reexposed via an incision from the supraclavicular to the cubital region. The C7 was identified and transected at distal to the crush site but close to the MCN. The MCN was cut right after its bifurcation from the lateral cord. Coaptation of C7 and MCN was performed with 11-0 nylon sutures. The terminal MCN distal to the bi-

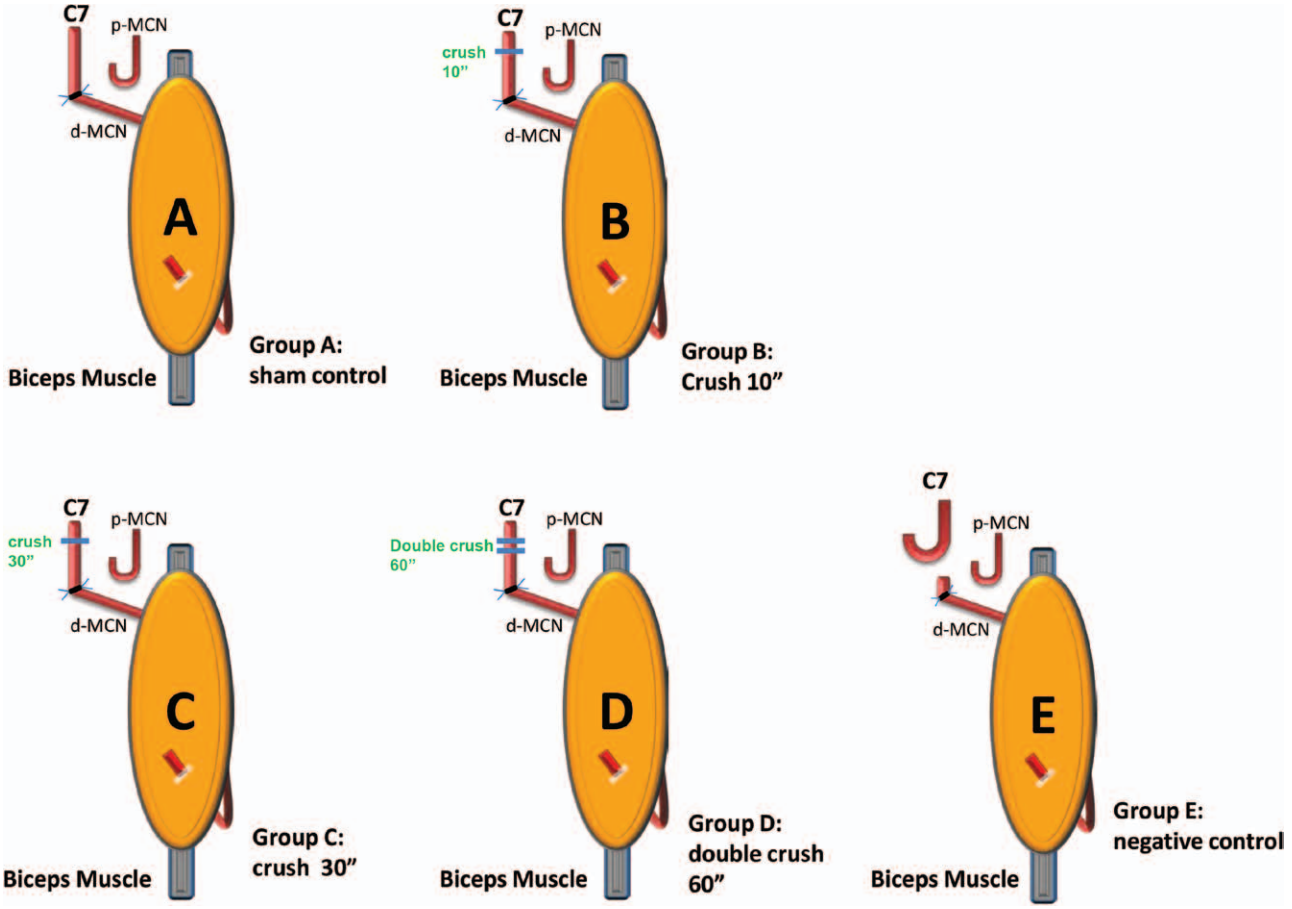

Fig. 2. The schematic drawing shows 5 groups of rats with different crushing injury of the C7 which was transferred to the musculocutaneous nerve at fourth week after the crushing injury. Group A: C7 was uninjured, transected. The musculocutaneous nerve distal to the biceps was cut in whole groups and transferred back into the biceps muscle to avoid loss of regenerated axons. C7 indicates C7 spinal nerve; p-MCN, proximal musculocutaneous nerve stump; d-MCN, distal musculocutaneous nerve stump.

ceps muscle was cut and transferred back into the biceps muscle to avoid loss of regenerated axons. The wound was closed with 4-0 nylon sutures. To obtain postoperative immobilization, the left arm was immobilized to the chest with adhesive bandage for 1 week. Animals were rewarmed and returned to their cage.

Third Stage: Outcomes Evaluation

Twelve weeks after the second stage of nerve transfer, all experimental limbs were checked with grooming test, muscle electrophysiological study, tetanus contraction force, and weight (Table 1). To avoid animals' weight-dependent result discrepancies, each animal's experimental side was also expressed in ratio (%) of experimental/healthy site (E/H) in parentheses (Table 2). Axon counts of the MCN distal to the nerve coaptation site were also performed (Table 3).

Behavioral Analysis

Function of the upper extremity was analyzed at 12 weeks after surgery using the grooming test developed by Bertelli[16] and Inciong,[17] which assessed shoulder abduction and elbow flexion. Water (1–3 ml) was applied over the animal's snout to provoke a reproducible grooming response: attempting to remove drops of water from their heads, animals raised and elevated their forelimbs behind the ears, then brought them down to the snout to be licked. Digital video recordings were assessed in slow motion to categorize the forelimb function on a 5-point scale: 5 points if the paw reached behind the ear, 3 points if the paw passed the snout but did not reach the eye, and 1 point if the paw moved but did not reach the snout. Multiple assessments were performed, and the best score was recorded.

Electromyogram

At 12 weeks, after the grooming test, animals were prepared again under general anesthesia. The left C7, MCN, and biceps muscle were exposed. A recording electrode was placed in the biceps muscle

Table 1. Comparison of Muscle Weight, Muscle Electromyography, and Muscle Contraction Force among Groups

Groups	Outcomes		
	Muscle Weight (g)	Biceps Muscle Action Potential (mV)	Biceps Muscle Tetanus Contraction Force (g)
A (C7 intact and transfer, $n = 6$)	0.30 ± 0.02	4.05 ± 0.37	36.40 ± 3.79
B (C7, 10″ crush and transfer, $n = 10$)	0.29 ± 0.03	3.81 ± 0.90	32.65 ± 12.40
C (C7, 30″ crush and transfer, $n = 10$)	0.25 ± 0.02	2.82 ± 0.58	30.72 ± 9.76
D (C7, 60″ double crush and transfer, $n = 10$)	0.26 ± 0.03	2.38 ± 0.89	17.88 ± 14.16
E (C7 and MCN cut without transfer, $n = 6$)			
H (control, right side of upper limb)	0.38 ± 0.04	5.56 ± 0.82	50.29 ± 12.65
P value (KW test, ABCD, and control)	<0.001	<0.001	<0.001
P value (KW test) (ABCD)	0.002	0.099	0.001
Significant difference by post hoc Dunn test	AB (1.000), **BC (0.020)**, **AC (0.012)**, BD (0.224), **AD (0.019)**, CD (1.000)	NA	AB (1.000), BC (0.192), **AC (0.012)**, **BD (0.012)**, AD (0.019), CD (1.000)

The values given in parentheses followed by mean ± SD are the valid sample sizes.
Dunn test (pairwise comparison) was performed if *P* value by Kruskal-Wallis test was <0.05 (significant).
Bold indicates signficant value.
KW, Kruskal-Wallis test; MCN, musculocutaneous nerve; NA, not applicable.

Table 2. Comparison of Experimental Side with Nonoperative Side among Groups with %

Groups	Outcomes		
	Muscle Weight Ratio (E/H) and %	Bimuscle Action Potential Ratio (E/H) and %	Bimuscle Tetanus Contraction Force Ratio (E/H) and %
A (C7 intact and transfer, $n = 6$)	0.88 ± 0.07 (88%)	0.83 ± 0.05 (83%)	0.72 ± 0.14 (72%)
B (C7, 10″ crush and transfer, $n = 10$)	0.76 ± 0.08 (76%)	0.64 ± 0.05 (64%)	0.70 ± 0.16 (70%)
C (C7, 30″ crush and transfer, $n = 10$)	0.69 ± 0.06 (69%)	0.50 ± 0.10 (50%)	0.55 ± 0.18 (55%)
D (C7, 60″ double crush and transfer, $n = 10$)	0.67 ± 0.05 (67%)	0.44 ± 0.03 (44%)	0.32 ± 0.25 (32%)
E (C7 and MCN cut without transfer, $n = 6$)			
P value (KW test)	$P < 0.001$	0.016	$P < 0.001$
Significant difference by post hoc Dunn test	AB (0.460), BC (0.408), **AC (0.005)**, BD (0.054), **AD (<0.001)**, CD (1.000)	AB (0.828), BC (0.531), **AC (0.017)**, **BD (0.035)**, **AD (0.001)**, CD (1.000)	AB (0.398), BC (1.000), **AC (0.032)**, BD (0.083), **AD (0.001)**, CD (1.000)

The values given in parentheses followed by mean ± SD are the valid sample sizes.
Dunn test (pairwise comparison) was performed if *P* value by Kruskal-Wallis test was <0.05 (significant).
Bold indicates signficant value.
E/H, ratio of experimental side/healthy side; KW, Kruskal-Wallis test; MCN, musculocutaneous nerve.

and a subcutaneous ground electrode positioned adjacently in the electromyogram setup. Two small hook-shaped stimulating electrodes (2 mm apart) held MCN gently. Stimulation was delivered for each trial by an electrical stimulator (Biopac System, BSL Software Installation Package, Windows, Goleta, Calif.), fixed at 1 millisecond at a constant current between 10 mA and 10 A. The muscle action potentials were recorded.

Table 3. Comparison of Axon Counts of Musculocutaneous Nerve Distal to the Nerve Coaptation among Groups

Group	Axon Count (in average)
A (C7 intact and transfer)	826.4 ± 163.4
B (C7, 10″ crush and transfer)	529.0 ± 169.2
C (C7, 30″ crush and transfer)	425.0 ± 284.5
D (C7, 60″ double crush and transfer)	603.6 ± 191.8
Normal C7	1413 ± 380.0

Muscle Tetanus Contraction Force Measurement

After electrophysiological study, the biceps muscle contraction force was assessed with a force displacement transducer and computerized recording software (FT03 Force Displacement Transducers, Grass Instruments, Quincy, Mass.). Force measurement followed a modified protocol based on Terzis'[18] and Shibata's[19] procedure. Resting muscle length of biceps was determined, and its insertion was detached from the cubital region and then reattached with the muscle resting length to the force transducer. The shoulder, elbow, and wrist joints were immobilized with fixation pins to avoid motion artifacts during muscle-contraction measurements. Stimulating current was applied with a bipolar platinum electrode distal to the repaired side. The threshold stimulus was defined as the stimulus that was required to produce an observable muscle twitch. Stimulation of the MCN was performed for activation of the biceps muscle at different thresholds (1 to 10 times

the threshold), different voltages (range, 0.6–1.2V), and different frequencies (range, 1.0–60 Hz). With various stimuli, the maximal tetanic strength was determined at 1V and 60 Hz and recorded as grams/weight. The mean maximal isometric muscle contraction of the repeated muscle contraction forces (5 times with a pulse duration of 1.0 millisecond) was recorded. All data were controlled, analyzed, and recorded by MacLab Systems (AD Instruments, Colorado Springs, Colo.).

Muscle Weights

After the above measurements, animals were euthanized by additional pentobarbital administration. Biceps muscles (operative and nonoperative sites) were harvested and weighed immediately.

Axon Counts

Nerve specimens (3–5 mm in length) were obtained from MCN before its entry into the biceps muscle and from the C7 proximal to the coaptation site. Samples were fixed in 2.5% glutaraldehyde and postfixed in 2% osmium tetroxide. Each nerve was embedded in 100% Epon. One-micrometer-thick transverse sections were made from the nerve to obtain successive sections in 1-mm intervals, which were stained with 2% toluidine blue. The axon count per se is not a reliable measurement. The selected section was photographed under a light microscope (from original magnification 400× enlarged to 1000×). Under further magnification, the axons were counted by hand in each specimen.

Statistical Analysis

Data were expressed as mean ± SD values in each group. The Kolmogorov-Smirnov test was used to check for normal distribution of the data, and the Kruskal-Wallis test was performed to compare performances of each group in grooming test, muscle force and weight, and electromyography. Dunn's test was used as a post hoc test when groups were significantly different ($P < 0.05$). A Mann-Whitney test was used to compare the axon counts between groups. A P value < 0.05 was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics software, version 20.0 (IBM, released 2011, Armonk, N.Y.) by the Biostatistical Center for Clinical Research, Chang Gung Memorial Hospital, Linkou, Taiwan.

RESULTS

Behavioral Analysis/Grooming Test

The rat's operative and nonoperative sides for all groups were evaluated carefully. The grooming test on the right (nonoperated side) showed that the paw could pass at least the eyes and scored on average 4.17±0.51 in all groups. However, the experimental side (left) all showed only elbow flexion with mean score of 1, except the group E (complete division without repair), which had no movement at all.

Electrophysiologic Testing

Electromyography of the biceps muscle after stimulation of the MCN on the operative and nonoperative sides was performed (Table 1). In the operative side, it showed low amplitudes in groups C and D, with an average of 2.82±0.58 mV (50% of healthy side, Table 2) and 2.38±0.89 mV (44% of healthy side, Table 2), respectively, but high amplitudes in group B (average, 3.81±0.90 mV, 64% of healthy side, Table 2) and group A (average, 4.05±0.37 mV, 83% of healthy side, Table 2). These results of those rats who had crush injury and nerve transfer (groups B, C, and D) were statistically significant (<0.05) compared with those of the healthy side (group H, average, 5.56±0.82 mV). Statistically significant differences between the experimental groups were observed between groups A and C, groups A and D, and groups B and D (Table 1).

Muscle Tetanus Contraction Force Measurement

Biceps muscle tetanus contraction force decreased with more injury of the C7 nerve: group A with an average elbow flexion strength 36.40±3.79 g (72% of healthy side, Table 2), group B 32.65±12.40 g (70% of healthy side, Table 2), group C 30.72±9.76 g (55% of healthy side, Table 2), and group D 17.88±14.16 g (Table 1) (32% of healthy side, Table 2). Statistically significant differences ($P < 0.001$) could be seen when compared to the healthy side (average, 50.29±12.65 g). Statistically significant differences between the experimental groups were seen between groups A and C, groups A and D, and groups B and D.

Muscle Weight

Muscle weight evaluation presented the heaviest muscle weight in the healthy group (average, 0.38±0.04 g), followed by group A (average, 0.30±0.02 g, 88% of healthy side), group B (average, 0.29±0.03 g, 76% of healthy side), group C (average, 0.25±0.02 g, 69% of healthy side), and then group D with the lowest (average, 0.26±0.03 g, 67% of healthy side) (Tables 1 and 2). Statistically significant differences ($P < 0.001$) were observed between the experimental and healthy groups. Statistically significant differences between the experimental groups were seen between groups A and C, groups A and D, and groups B and C.

Axon Counts

Axon counts of healthy C7 spinal nerves (Table 3) were 1413±380.0 on average. Axon counts of the MCN distal of the coaptation site showed average in group A 826±163.4, group B 529±169.2, group C 425±284.5, and group D 603±191.8. Group D showed the highest. Perhaps a double crush injury serves a double stimulation of the donor nerve, but only resulted in a greater number of dysfunctional axons. Although substantial differences existed between the groups, they are not statistically significant ($P = 0.055$).

Statistically significant correlation was only observed between axon counts and muscle contraction ($P = 0.024$).

DISCUSSION

Classification of Nerve Injuries

Seddon,[20] in 1943, classified the nerve injures into 3 different types: neurapraxia, axonotmesis, and neurotmesis. Sunderland,[15] in 1951, expanded the Seddon classification of nerve injuries into 5 degrees: Sunderland I, segmental demyelination; Sunderland II, disrupted axon with intact endoneurium; Sunderland III, disrupted axon and endoneurium; Sunderland IV, disrupted endoneurium and perineurium, neuroma in continuity; and Sunderland V, complete transection of nerve. When C5 and C6 are avulsed, the nearby C7 has high tendency to have concomitant injuries, although it may show macroscopically normal appearance with positive nerve stimulation (Sunderland I to III injury). This might be the main reason why reconstruction with ipsilateral C7 transfer results in different outcomes clinically.

Crushed by Jeweler's Forceps

In our preliminary study, we used microneedle holder. In this study, we used jeweler forceps. People may wonder how to standardize the pressure control. The whole nerve crush process was performed by the same surgeon and used the same jeweler's forceps to avoid potential difference of the pressure applied.

Timing of the Ipsilateral C7 Nerve Transfer

Muscle tetanus contraction force in this study shows strong force in group A, 72% of the healthy limb (Table 2), and group B, 70%. However, unacceptable functional recovery is noted in group C, 55%, and group D, 32%, showing significantly lower than group A ($P < 0.035$). These findings are also confirmed by electromyography of the biceps muscle action potentials and muscle weights (Table 2). Statistically significant correlation of axon counts with data of muscle contraction force is observed (Table 3) in groups B and C, but not in group D. Axon counts of group D show around 12% more axons than group B and 42% more than group C.

Perhaps a double nerve crush serves as a double stimulation with more axon sprouting, but most of them are immature and functionless. The time point for nerve transfer in group D was just not optimal.

The more severely the nerve is crushed, the more axon sprouting with an immature pattern will be observed, which needs more time for maturation. At a period of 4 weeks following a more severe C7 crush injury, the C7 donor nerve might not have reached an optimum time point either of nerve regeneration or for nerve transfer. Early nerve transfer in more severe crushing injured nerve might downgrade the final functional results. Further investigation is necessary to determine whether the timing of a partially injured donor nerve for transfer has any effect on the outcome. Statistical analysis on the data from the preliminary study is valuable, which can give some clue. What will be the results at 16 weeks to conclude the optimal time for final evaluation? What will be the results if groups B–D are all allowed a 12 weeks regeneration period and then transferred? All of these questions need to be answered for us to determine whether a late transfer of a partially injured donor nerve has an advantage in terms of the outcome.

CLINICAL IMPLICATIONS

For a closed injury of the brachial plexus, the general recommendation for timing of exploration is still under debate. Some authors recommend an urgent exploration and nerve repair simultaneously to avoid a difficult dissection at a later stage.[21,22] However, judgment of the degree and extent of a normal-looking donor nerve in a traction injury in the acute stage is difficult and doubtful. Selection of a healthy stump is very crucial for a successful repair. A close supervision of the patient for a period of 3–6 months[14,23,24] seems logical and practical based on this experimental study. The benefits of such waiting will outweigh the advantage of early surgery. How early can a transfer downgrade the final outcome? How late can a transfer upgrade the final outcome? Further investigations are necessary too.

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ACKNOWLEDGMENTS

We thank Pei-Ju Chen and Ruby Yih-Ru Chang for their professional and continuous technical assistance throughout this study and Ms. Hsiao-Jung Tseng, M.P.H., from the Department of Biostatistical Center for Clinical Research, Chang Gung Memorial Hospital, Taoyuan, Taiwan, for her statistical support and analysis of the data.

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