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Muscle and joint sequelae in brachial plexus injury

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Chapter

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Local injection of autologous bone marrow cells to regenerate muscle in patients with traumatic brachial plexus injury: a pilot study

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ABSTRACT

Introduction: Traumatic brachial plexus injury causes severe functional impairment of the arm. Elbow flexion is often affected. Nerve surgery or tendon transfers provide the only means to obtain improved elbow flexion. Unfortunately, the functionality of the arm often remains insufficient. Stem cell therapy could potentially improve muscle strength and avoid muscle-tendon transfer. This pilot study assesses the safety and regenerative potential of autologous bone marrow-derived mononuclear cell injection in partially denervated biceps.

Methods: Nine brachial plexus patients with insufficient elbow flexion (i.e., partial denervation) received intramuscular escalating doses of autologous bone marrow-derived mononuclear cells, combined with tendon transfers. Effect parameters included biceps biopsies, motor unit analysis on needle electromyography and computerised muscle tomography, before and after cell therapy.

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Results: No adverse effects in vital signs, bone marrow aspiration sites, injection sites, or surgical wound were seen. After cell therapy there was a 52 % decrease in muscle fibrosis ($p = 0.01$), an 80 % increase in myofibre diameter ($p = 0.007$), a 50 % increase in satellite cells ($p = 0.045$) and an 83 % increase in capillary-to-myofibre ratio ($p < 0.001$) was shown. CT analysis demonstrated a 48 % decrease in mean muscle density ($p = 0.009$). Motor unit analysis showed a mean increase of 36 % in motor unit amplitude ($p = 0.045$), 22 % increase in duration ($p = 0.005$) and 29 % increase in number of phases ($p = 0.002$).

Conclusions: Mononuclear cell injection in partly denervated muscle of brachial plexus patients is safe. The results suggest enhanced muscle reinnervation and regeneration.

INTRODUCTION

Brachial plexus injury (BPI) is often a result of a high-energy road traffic accident in young adults^{1,2}. The upper brachial plexus (C5-C6) is commonly affected, resulting in paresis of shoulder function and elbow flexion¹⁻⁴. Nerve surgery is aimed at restoring innervation of the biceps muscle, but often a deficit in the functionality of the arm remains. In that case, secondary surgery can improve arm function by transfer of the flexor-pronator group of the forearm (Steindler procedure) for active elbow flexion⁵⁻¹⁰. However, neither nerve surgery nor tendon transfers come close to restoring normal elbow flexion⁶.

Any procedure that improves elbow flexion without sacrificing the function of transferred donor muscles will have advantages for the patient. One approach is to improve the regenerative capacity of the partially denervated muscles. Denervation causes loss of contractile force and muscle atrophy. If innervation is restored quickly, good restorative ability exists. Conversely, prolonged interruption of innervation results in irreversible muscle atrophy, interstitial fibrosis and muscle fattening¹¹⁻¹⁴. Since regeneration of denervated muscles not only depends on nerve supply, but also on its regenerative capability, the muscle can be a target organ for cell therapy.

Muscle satellite cells are responsible for repair and maintenance of skeletal muscles; they are the main source of new myofibres. Myogenic precursor cells proliferate, fuse and form new myofibres in response to muscle damage¹⁵. Exhaustion of the pool of available satellite cells may contribute to poor functional recovery of long-term denervated muscle^{16,17}. A progressive and rapid decrease in the capillary-to-muscle fibre ratio occurs, further limiting regeneration¹⁸. Transplantation of primary satellite cells has been shown to improve the properties of reinnervated skeletal muscles in rabbits¹⁹. However, poor cellular survival and limited cell dissemination hampers successful satellite cell transplantation. Furthermore, only few cells fuse with host fibres. This suggests that a subpopulation of myogenic cells (i.e. stem cells) may be optimally suited for transplantation^{20,21}. Bone marrow (BM)-derived cells migrate to the site of muscle injury and contribute to the satellite cell pool²²⁻²⁴. The injection of autologous BM-derived mononuclear cells (MNCs) has been applied in clinical studies focusing on the muscles of the heart and leg²⁵⁻²⁸.

The primary objective was to assess the safety of autologous BM-derived MNC injection in partially denervated muscles of BPI patients. The secondary objective was to obtain a first estimate on regenerative potential of injected BM-derived MNCs in partially denervated muscles.

METHODS

Study design

This was a prospective study on autologous BM-derived MNC therapy for partially denervated biceps muscles of BPI patients, with focus on safety and the effect on muscle. Three escalating doses were evaluated in three groups, each comprising three patients. The MNC dose was equivalent to 50 % (group A), 100 % (group B), and 200 % (group C) of the cell dose in a former study at our institution²⁵. The protocol was approved by the medical ethics committee of Leiden University Medical Center. The Declaration of Helsinki protocols were followed and patients gave written, informed consent. The clinical trial was registered under ClinicalTrials.gov identifier: NCT00755586.

Patients

8 Nine adult traumatic BPI patients with partial denervation of the biceps muscle (Medical Research Council (MRC) grades between 1 and 3) were included²⁹. All patients were at an end-stage of functional results (i.e. ≥ 2 years after trauma or nerve surgery). Patients with a complete paralysis of the biceps muscle were excluded as they were assumed to have no regenerative capacity (MRC grade 0). This was corroborated by the absence of motor unit potentials (MUPs) during needle electromyography (EMG). Other exclusion criteria were a history of central or other peripheral neurological disorders, humeral fractures, and contraindication for BM harvesting (such as bleeding diathesis or an international normalised ratio (INR) > 2).

BM aspiration and MNC separation

BM was aspirated from one location at the posterior iliac crest. The procedure was performed under local (group A) or general anaesthesia (groups B and C). Due to ethical considerations associated with the use of general anaesthesia, in groups B and C the MNC injection was combined with a Steindler procedure, which was considered the standard surgical procedure in patients with an elbow flexion deficiency⁵. BM was separated as previously described^{25,28}. The isolated MNCs were concentrated in a volume of 10 ml. The isolation and concentration procedure was performed in a certified clean-room facility according to good manufacturing practice (GMP)^{25,28}. Further details of the methods are provided as supplementary material.

MNC injection

BM-derived MNCs were injected at 20 sites at the maximum palpable thickness of the biceps and at a standard injection depth of 0.5 cm. A total volume of 10 mL was injected. Further details of the methods are provided as supplementary material.

Safety

In order to assure safety, the absence of adverse events and muscle fibrosis was documented before proceeding to the next dose level. Vital signs were checked and the BM aspiration, MNC injection and surgical wound site were examined during a 24-hour hospital admission and at three and six months after cell therapy. The safety of MNC injection at muscle level was assessed by CT analysis and histology.

Evaluation of patients

All patients were evaluated pre-operatively and at three and six months post-operatively using clinical scores, CT analysis and EMG. Histology of the biceps muscle was undertaken pre-operatively and at three months post-operatively. Before analysis, all images were blinded for date and patients characteristics.

Clinical functionality

Active and passive range of movement (ROM) of the injured elbow was measured using a handheld goniometer. Force measurement of the injured biceps muscle was assessed using the MRC motor scale. Pain at rest was scored using a visual analogue scale (VAS) (0 - no pain to 10 - extreme pain). Finally, the Disabilities of the Arm, Shoulder, And Hand (DASH) questionnaire and the Short-Form Health Survey (SF-) 36 were used to measure quality of life and functional outcome^{30, 31}. To compare the DASH score with the SF-36 we calibrated the score to a 100-point metric score from 0 (worst health) to 100 (best health). The SF-36 score ranges from 0 (worst health) to 100 (best health).

CT scan

Patients were scanned using the Aquilion 64-slice CT scanner (Toshiba Medical Systems, Otawara, Japan). A total of 50 consecutive 1.0-mm multiplane reconstruction (MPR) images were computed in the coronal plane perpendicular to the main axis of the humerus. The MPR images were analysed and the mean muscle density (MMD) was calculated³². The MMD of the injured (injected) biceps muscle was compared with control muscles (the injured (non-injected) triceps muscle and non-injured (non-injected) contralateral biceps muscle). Further details of the methods are provided as supplementary material.

Quantitative needle EMG

Quantitative needle EMG was performed using disposable EMG needle electrodes with a Medelec Synergy EMG system (Oxford Instruments, Oxford, United Kingdom). EMG of the injured biceps muscle was compared with that of control muscles (the injured (non-injected) brachialis muscle and non-injured (non-injected) contralateral

biceps muscle). Since the mean number of MUPs of the triceps muscle was too low (≤ 2), the brachialis muscle was selected as a control. The multi-MUP analysis was performed using Medelec Synergy software version 11 (Oxford Instruments)³³. Further details of the methods are provided as supplementary material.

Histochemistry and immunohistochemistry of muscle biopsy

A biopsy of 0.5 cm × 0.5 cm × 0.5 cm of the injured biceps muscle was taken at the site of the MNC injection³⁴. It was immediately fixed in 4 % buffered formalin and embedded in paraffin. Tissue sections of 5 µm thickness were cut, deparaffinised and rehydrated for Masson's trichrome staining, routine haematoxylin and eosin (H&E) staining and immunohistochemistry to evaluate muscle, vascular and neural regeneration. For immunohistochemistry Pax7, CD56 and von Willebrand Factor (vWF) were used. Further details of the methods are provided as supplementary material.

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Muscle analysis

Muscle sections were analysed using a Zeiss image analysis system (KS400; Zeiss, Sliedrecht, The Netherlands) coupled to a camera (DXC-950P; Sony, Amsterdam, The Netherlands) and ImageJ software v1.38X (National Institutes of Health, Bethesda, Maryland). From each stained section, random images were selected, analysing at least 200 fibres. Further details of the methods are provided as supplementary material.

Statistical analysis

All tests were two-tailed and p - values < 0.05 were considered significant. SPSS v20.0 (SPSS Inc., Chicago, Illinois) was used to perform the analyses. Baseline characteristics are presented as mean and range. Data from muscle biopsies, EMG, CT, and clinical function are presented as mean and standard deviation (sd). Elbow flexion strength using the MRC motor scale is displayed as median and range. A paired t -test or one-way analysis of variance (ANOVA) was used to analyse the muscle biopsy parameters. A mixed model analysis was used to analyse EMG, CT and clinical functionality parameters. Patients were included as random effects on each outcome parameter. In order to determine changes in outcome at follow-up, the variable 'time' was included as fixed effect. In order to determine any dose-effect response between group A, B and C, the variable 'group' was also included in the mixed model as a fixed effect.

RESULTS

Baseline patient and injected MNC characteristics are shown in table I. During hospital stay and follow-up, no adverse events in vital signs (blood pressure, pulse

rate, temperature), BM aspiration site, injection sites or surgical wound (haematoma, infection) were observed. Post-operatively, the mean decrease in haemoglobin (Hb) concentration was 0.2 mmol/l (group A), 1.0 mmol/l (group B), and 1.8 mmol/l (group C). Hb concentration was normalized in all patients six months after BM aspiration. In order to assess the safety of MNC injection at a local muscular level, quantitative CT analysis and histology were used. With quantitative CT analysis, the MMD of the injured biceps muscle was compared with injured and non-injured control muscles. A significant decrease in the MMD of the injured biceps muscles in the total patient group was observed ($p = 0.03$). The decrease was 32 % at three months ($p = 0.04$) and 48 % at six months follow-up ($p = 0.009$). No dose effect was observed. The non-injured contralateral biceps and the injured (non-injected) triceps muscle showed no significant change in MMD (figure 1).

A Masson's Trichrome staining was performed to analyse the possible formation of muscle fibrosis after cell injection. As compared with the pre-injection biopsy, a decrease of 52 % in mean areas of fibrosis of the injured biceps muscle was measured at three months after cell injection in the total patient group ($p = 0.01$). No dose effect was observed (figure 2).

Table I: Baseline patient and injected mononuclear cell characteristics

	Group A (n = 3)	Group B (n = 3)	Group C (n = 3)
Age * (years)	33 (20 to 45)	30 (25 to 33)	23 (20 to 28)
Male † (n)	3 (100)	3 (100)	3 (100)
Brachial plexus injury type † (n)			
C5 - C6	1 (33)	1 (33)	0
C5 - C7	0	2 (67)	3 (100)
C5 - Th1	2 (67)	0	0
Time after trauma * (years)	7.4 (2 to 17)	8.0 (3 to 17)	2.9 (2 to 3)
Neurosurgery † (n)	3 (100)	2 (67)	3 (100)
Time after neurosurgery * (years)	7.0 (2.0 to 16.5)	10.1 (4.0 to 16.3)	2.6 (2.2 to 2.9)
Elbow flexion strength # (MRC scale)	3 (3)	2 (2 to 3)	2 (2 to 3)
Active elbow flexion RoM * (°)	65 (25 to 100)	12 (0 to 35)	30 (0 to 90)
Injected mononuclear cells * (x 10 ⁸ cells)	0.89 (0.6 to 1.3)	4 ‡	8 ‡
CD34 ⁺ * (x 10 ⁶ cells)	2.5 (0.5 to 5)	10.8 (7 to 16)	23.6 (15 to 32)
CD34 ⁻ * (x 10 ⁶ cells)	86.5 (57 to 127)	389 (384 to 393)	776 (769 to 785)

*Values are given as mean with range. †Values are given as number of patients with percentage. #Values are given as median with range. ‡No range is given as the exact number of cells was injected. MRC: Medical Research Council, RoM: range of motion.

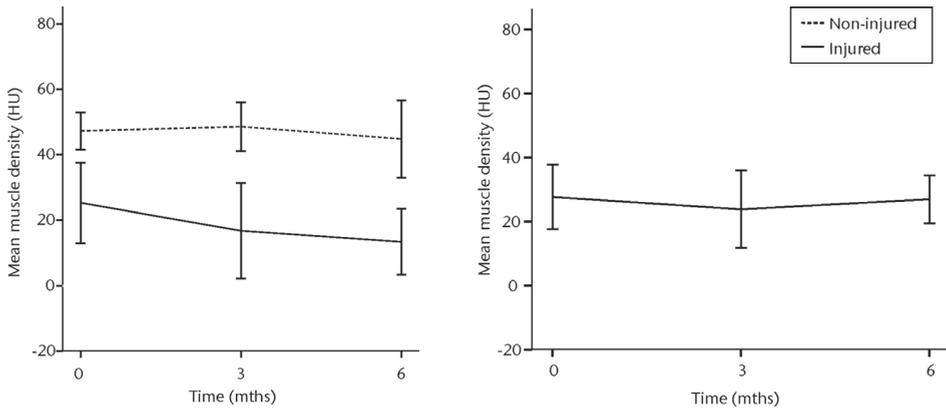


Figure 1: Muscle density

Graphs showing the mean muscle density (MMD) of the injured and non-injured biceps (A) and triceps (B) muscle pre-transplantation and at three and six months follow-up for the total patient group. Error bars denote 95 % confidence interval (HU, Hounsfield unit).

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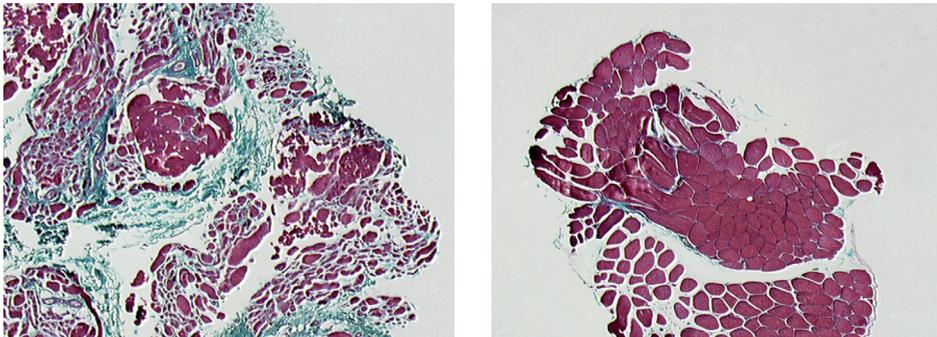


Figure 2: Masson's Trichrome staining

Histological images showing Masson's Trichrome staining of a representative muscle biopsy a) pre- and b) three months post-transplantation (magnification 5 ×).

In order to assess any regenerative potential of the injected MNCs, quantitative needle EMG and histology were performed. The mean amplitude, duration, and number of phases of the MUPs of the injured biceps muscle were compared with injured and non-injured control muscles. After three months, the increase was 36 % in mean amplitude ($p = 0.045$), 22 % in duration ($p = 0.005$) and 29 % in number of phases ($p = 0.002$). The increase in number of phases were sustained at six months follow-up ($p = 0.001$). A dose-effect relation between group A, B and C was observed only in the mean amplitude ($p = 0.03$). The non-injured contralateral biceps muscle, the injured (non-injected) brachialis muscle and non-

injured brachialis muscle showed no significant changes in amplitude, duration, or number of MUP phases. The mean number of analyzed MUPs was 5.3 (SD 2.2). This low number represents the severity of the nerve lesions in which patients were unable to recruit more motor units (figure 3).

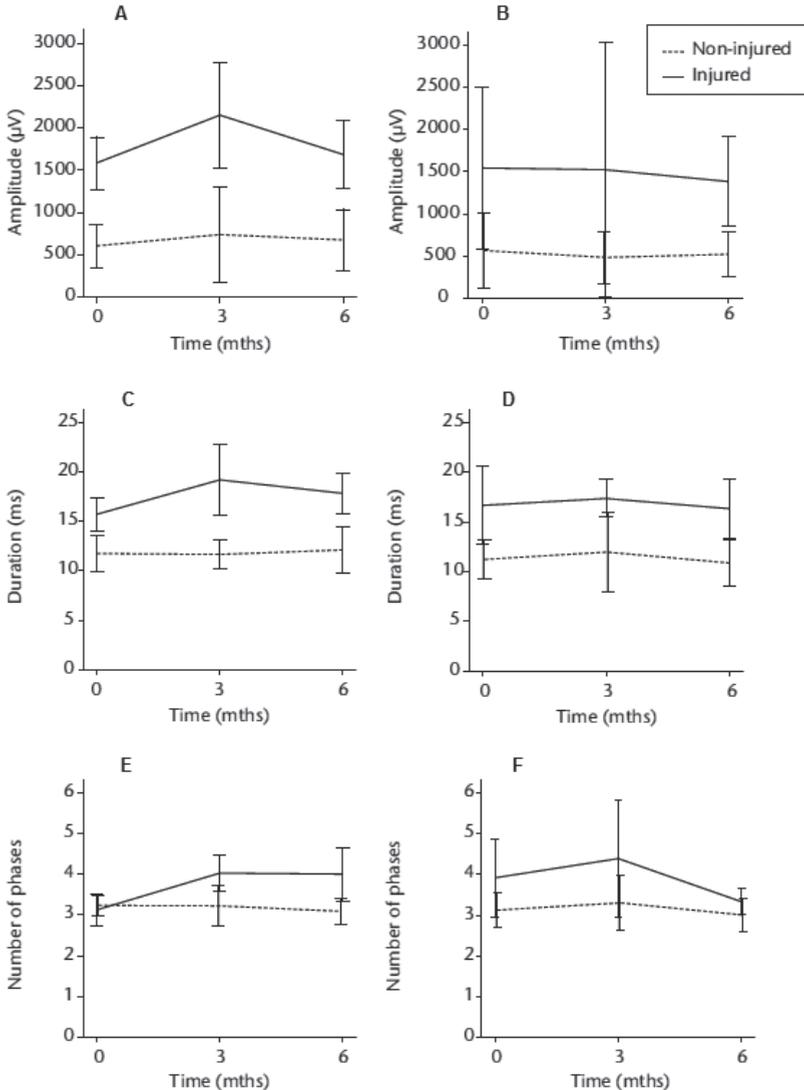


Figure 3: Quantitative needle electromyography

Graphs showing the mean amplitude (a and b), duration (c and d) and number of phases (e and f) of motor unit potentials of the injured and non-injured biceps (left column) and brachialis muscle (right column) pre-transplantation and at three and six months follow-up for the total patient group. Error bars denote 95 % confidence interval.

The myogenic repair potential of the injected MNC cells was also evaluated using the histology of the injured biceps muscle. Analysis of all patients showed a mean increase of 80 % in myofibre diameter three months after the cell injection compared with the pre-injection biopsy ($p = 0.007$). Pax7 staining demonstrated a 50 % increase in the number of satellite cells ($p = 0.045$). Von Willebrand Factor staining showed an increase of 83 % in the number of capillaries per myofibre ($p < 0.001$). No significant increase was demonstrated in the number of centronucleated myofibres or CD56 positive myofibres after cell therapy (table II).

Histological muscle regeneration was most apparent in group B, which observed a 126 % increase in myofibre diameter, a 186 % increase in percentage of centronucleated myofibres, a 117 % increase in number of satellite cells, a 100 % increase in capillaries per myofibre and a 70 % decrease in the area of fibrosis. No dose-effect relation was observed in any of the histological measurements. Figure 4 shows a representative example of H&E, Pax7, and vWF and CD56 staining of a muscle biopsy before and after cell therapy in group B. After cell therapy, the muscle biopsy of one patient in group A did not contain 200 muscle fibres and was therefore excluded from analysis. In group A, no analysis of the CD56 staining could be performed because one patient's muscle biopsy after cell therapy contained only 120 myofibres and was therefore excluded.

In order to observe function improvement after MNC injection, we measured the active flexion ROM and strength of the injured elbow before and after cell therapy compared with the injured and non-injured control muscles. In addition, quality of life questionnaires (SF-36 and DASH) and the VAS score for pain were used. Overall, all patients had a significant increase in active flexion ROM and flexion strength. The flexion ROM increase was 152 % at three months ($p < 0.001$) and 147 % at six months follow-up ($p < 0.001$). The flexion strength increase was 38 % at three months ($p < 0.001$) and 46 % at six months follow-up ($p < 0.001$). As expected, the increase in active elbow flexion ROM and flexion strength was most prominently significant in patient groups where cell therapy was combined with a modified Steindler procedure (groups B and C). No significant changes in SF-36, DASH or VAS score were observed comparing pre- and post-injection at three months (table III).

Table II: Histological results

Mean (sd) biceps histology	Group			p-value*	Total (n = 9)
	A (n = 3)	B (n = 3)	C (n = 3)		
Myofibre diameter (μm)					
0 months	254 (68)	173 (46)	171 (12)		193 (53)
3 months	392 (165)	391 (44)	274 (71)		347 (98)
Mean difference	+138	+218	+103	0.39	0.007 †
Centro-nuclear myofibres (%)					
0 months	5.1 (2.5)	2.1 (1.5)	2.6 (1.7)		3.1 (2.0)
3 months	2.7 (0.8)	6.0 (3.1)	3.4 (1.9)		4.2 (2.5)
Mean difference	-2.4	+3.9	+0.8	0.01	0.30 †
Fibrosis (%)					
0 months	18 (2)	31 (2)	23 (4)		25 (6)
3 months	17 (15)	9 (4)	12 (2)		12 (7)
Mean difference	-1	-22	-11	0.06	0.01 †
Pax7+ myofibres (%)					
0 months	17 (4)	6 (3)	14 (6)		12 (6)
3 months	18 (8)	13 (3)	23 (10)		18 (8)
Mean difference	+1	+7	+9	0.45	0.045 †
vWF+ per myofibre (%)					
0 months	0.8 (0.04)	0.5 (0.1)	0.5 (0.1)		0.6 (0.2)
3 months	1.1 (0.2)	1.0 (0.2)	1.1 (0.4)		1.1 (0.2)
Mean difference	+0.3	+0.5	+0.6	0.33	< 0.001 †
CD56+ myofibres (%)					
0 months	16 (n/a)‡	18 (6)	25 (3)		21 (5)§
3 months	16 (n/a)‡	18 (8)	21 (12)		19 (7)§
Mean difference	0	0	-4	0.94	0.60 †

Mean myofibre diameter, centro-nuclear myofibres, fibrosis, Pax7+ myofibres, vWF per myofibre and CD56+ myofibres of muscle biopsies of the injured biceps muscle before MNC injection and at three months follow-up * comparison of mean differences between time points between the groups (one-way analysis of variance (ANOVA)), † difference between time points for all patients (paired t-test), ‡ standard deviation not available as only one observation was made, § for total seven patients

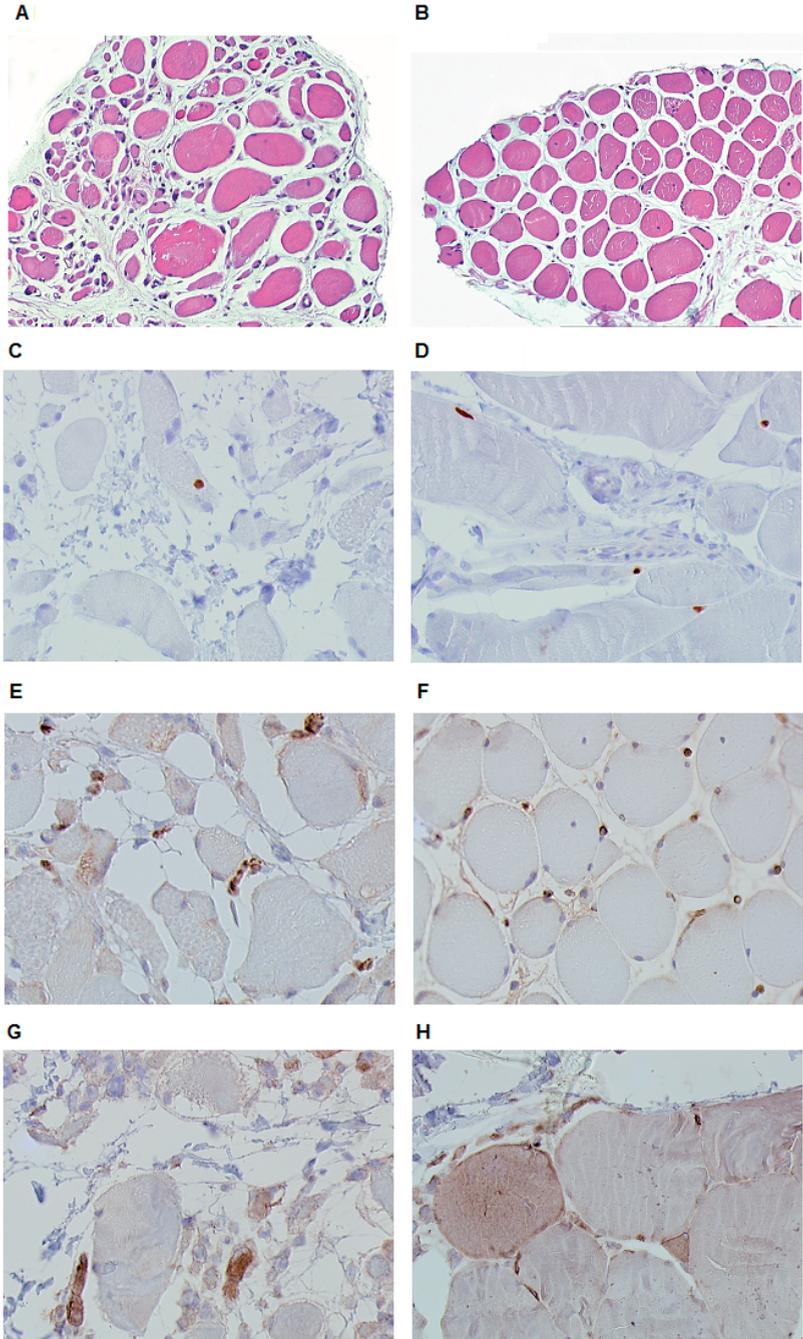


Figure 4: Histology

Histological images for specimens taken pre-transplantation (left column) and at three months follow-up (right column), showing staining of a representative muscle biopsy with haematoxylin and eosin (a and b: $\times 20$), Pax7 (c and d: $\times 40$), vWF (e and f: $\times 40$) and CD56 (g and h: $\times 40$).

Table III: Clinical results

Mean (sd) outcome*	Group			P - value†	Total (n = 9)
	A (n = 3)	B (n = 3)	C (n = 3)		
Flexion ROM (°)				0.22	
0 months	65 (38)	12 (20)	30 (52)		36 (41)
3 months	82 (28)	115 (5)	107 (15)		101 (22)
6 months	88 (20)	100 (20)	110 (22)		99 (20)
p-value‡					< 0.001‡
Median flexion strength (MRC grade) (range)				0.02	
0 months	3 (3 to 3)	2 (2 to 3)	2 (2 to 3)		2 (2 to 3)
3 months	3 (3 to 3)	4 (3 to 4)	4 (3 to 4)		3 (3 to 4)
6 monthss	3 (3 to 4)	4 (4 to 4)	4 (4 to 4)		4 (3 to 4)
p-value‡					< 0.001‡
SF-36				0.06	
0 months	86 (6)	57 (16)	69 (6)		78 (15)
3 months	91 (5)	71 (18)	74 (9)		78 (14)
6 months	82 (11)	72 (15)	75 (10)		76 (12)
p-value‡					0.06‡
DASH-DLV				0.15	
0 months	43 (19)	35 (25)	75 (16)		51 (25)
3 months	64 (39)	68 (19)	76 (12)		69 (23)
6 months	41 (16)	71 (17)	74 (16)		65 (20)
p-value‡					0.09‡
VAS for pain				0.02	
0 months	0.8 (0.8)	6.1 (2.1)	1.0 (1.1)		2.7 (2.9)
3 months	0.8 (0.6)	2.3 (2.0)	1.3 (1.6)		1.5 (1.5)
6 months	0.8 (0.6)	2.0 (0.5)	2.2 (3.0)		1.7 (1.7)
p-value‡					0.24‡

Elbow flexion range of movement (ROM) and strength of the injured elbow together with the Short-Form 36 and Disabilities of the Arm Shoulder and Hand (DASH; Dutch language version) questionnaires and visual analogue scale (VAS) for pain before mononuclear cell (MNC) injection and after three and six months * MRC, Medical Research Council; SF-36, Short-Form 36 (scored from 0 to 100); DASH-DLV, Disabilities of the Arm Shoulder and Hand – Dutch language version (scored from 0 to 100); VAS, visual analogue scale (scored from 0 (no pain) to 10 (extreme pain), † comparison of differences between pre-op and six months in the groups (mixed model analysis), ‡ difference between pre-op and six month values for all patients (mixed model analysis)

DISCUSSION

Autologous BM-derived MNC injection in a partially denervated biceps muscle is safe and shows no adverse events with respect to vital signs, BM aspiration sites, injection sites or surgical wounds. Only a mild anaemia was observed, which recovered fully. The interstitial fibrosis present at long-term denervation decreased by 52 % after MNC injection. These results run parallel to the results of CT scan analysis, which demonstrated a decrease of 48 % in MMD.

Our secondary aim was to estimate myogenic repair and reinnervation of partial denervated muscles after MNC injection. Histological analysis, quantitative needle EMG and CT scan analysis demonstrated muscle improvement after MNC injection, which was not present in the control muscles of the sound arm or in the non-injected injured muscles of the affected arm.

Our study has some limitations. While useful to quantify fatty degeneration of rotator cuff muscles, CT analysis only provides an indirect measure of the amount of muscle lipid or fibrosis³². We observed a significant decrease in MMD of the injured biceps muscles after cell injection, which could reflect decrease in muscle fibrosis and increase in muscle vascularisation, muscle fattening or oedema from the procedure³⁵. Histology of the muscle biopsies demonstrated a 52 % decrease in muscle fibrosis. In addition, an 83% increase in capillaries per myofibre was observed. Using routine H&E staining, no increase in muscle fattening was seen, thus the decrease in MMD reflects either a decrease in muscle fibrosis or an increase in muscle vascularisation. The presence of muscle denervation was assessed using quantitative needle EMG. Analysing a minimum of 20 MUPs per analysed muscle is generally accepted in the literature^{33, 36, 37}. Unfortunately, we could not obtain a minimum of 20 MUPs per analysed muscle (mean 5.3 MUPs, SD 2.2), due to the partial denervation. Nevertheless a significant difference in mean amplitude, duration and the number of phases was found.

Another limitation was that in groups B and C, the BM-derived MNC injection was combined with a flexor-pronator transfer, which improves elbow flexion. Since the flexor-pronator group has to be fixated to the distal humerus with a bicortical screw, it is possible that BM may leak underneath the screw, stimulating the muscle in its vicinity (biceps triceps). However, CT changes were not seen in the control triceps muscle, nor were EMG improvements in the injured but non-injected brachialis muscle. A final limitation was low number of patients and lack of a control patient group (i.e. modified Steindler in a partially denervated biceps muscle with no MNC injection).

Quantitative needle EMG was used to determine muscle innervation. After injection of BM-derived MNC cells, an increase of 36 % in amplitude, 22 % in duration, and 29 % in number of phases of MUPs was observed, suggestive for

muscle reinnervation. Both human and animal studies have shown that BM stromal cells can differentiate into neural cells and improve nerve regeneration³⁸⁻⁴¹.

Histology was used to evaluate the myogenic repair on a microscopic level. Upon muscle denervation, animal studies have shown a decrease from 2104 μm to 50 μm in myofibre diameter, a decrease from 1.6 % to 0.2 % in capillary-to-myofibre ratio and an increase from 11 % to 35 % in fibrosis^{14, 17, 18}. Our data are consistent with these studies, demonstrating a mean myofibre diameter of 193 μm , a mean capillary-to-myofibre ratio of 0.6 and a mean fibrosis of 25 % before cell injection.

The number of cells in the satellite pool within a muscle appears to be correlated to postnatal growth characteristics of the muscle (Pax7). In adult muscle, up to 10 % Pax7 positive myofibres have been reported^{16, 17, 42}. Before cell injection, we observed mean Pax7 positive myofibres of 11.9 % (SD 6.5). This higher percentage of Pax7 fibres is probably due to the partially denervated biceps muscle in the current study.

After MNC injection, we observed an increase of 50 % in Pax7 positive myofibres and an increase of 83 % in vWF positive capillaries per myofibre. This may demonstrate that MNC injection can result in restoration of the satellite cell pool and vascular bed, which can play an important role in functional recovery following long-term denervation. Indeed, the increase of 80 % in myofibre diameter suggested myofibre regeneration and thus restoration of muscle atrophy. Muscle regeneration was most prominently observed in patient group B, with an increase of 117 % in Pax7 positive myofibres, 100 % in capillary-to-myofibre ratio, 126 % in myofibre diameter, 186 % in centronuclear myofibres and a decrease of 70 % in fibrosis. These results are suggestive of an optimal dose in group B, although a dose-effect response was not significant.

Skeletal muscle is a regenerative tissue in which mononuclear precursor cells respond to injury by dividing and fusing with damaged fibres¹⁵. As such, it is an attractive target for cell-based therapy. Muscle fibre regeneration levels of up to 12 % have been achieved by using appropriate transplant variables, including cell dose and mode of cell delivery²⁴. The present study is the first in which BM transplantation in a human muscle denervation model was assessed with objective measurements (histology, CT muscle and EMG). In humans, transplantation of the MNC fraction of the adult BM enhanced cardiac- and skeletal muscle function in post-*ischaemic* heart failure and peripheral artery disease²⁵⁻²⁸. These effects could be attributed to the incorporation of stem cells and building muscle or by supplying the logistics required for efficient proliferation of innate cells via paracrine effects^{43, 44}. Heterochronic parabiosis was shown to restore the regenerative potential of satellite cells, which suggests that satellite cell activity can be modulated by their microenvironment⁴⁵. Chronic inflammation does not affect

the *in vitro* regenerative potential of human satellite cells, which underscores the *in vivo* influence of the microenvironment on muscle regeneration⁴⁶. Unravelling the molecular mechanism behind the observed regeneration in BPI patients is a challenge that still has to be met. In the future, this can be studied in newly developed animal models representative for BPI^{47, 48}.

In conclusion, BM-derived MNC injection is safe in partially denervated muscle of traumatic BPI patients. Significant muscle improvement has been observed in muscle biopsies, quantitative needle EMG and CT scan analysis. Although promising, the preliminary results of the present study require confirmation in a larger controlled clinical study.

SUPPLEMENTARY MATERIAL

8

Bone marrow aspiration and mononuclear cell (MNC) separation

Bone marrow (BM) was aspirated from one location at the posterior iliac crest under aseptic conditions in the operating theatre using a 14-gauge, 40 mm needle (SteryLab, Milan, Italy). The procedure was performed under local (group A) or under general anaesthesia (groups B and C). In group A, 60 ml of BM was aspirated, which was considered the maximum amount that can be aspirated under local anaesthesia. Following separation, a mean mononuclear cell (MNC) dose of 0.89×10^8 MNCs (SD 0.38) was obtained. In groups B and C, a MNC dose of 4.0×10^8 MNCs and 8.0×10^8 MNCs was anticipated, respectively. In order to get these cell concentrates, 350 ml and 650 ml of BM was aspirated under general anaesthesia for groups B and C, respectively. Due to ethical considerations, in these two patient groups BM aspiration was performed in combination with a modified Steindler procedure, which is our standard secondary surgical procedure with a flexion deficiency of the elbow. BM was collected in flasks containing Hanks balanced salt solution and heparin and was then separated over a density gradient (Ficoll isopaque specific density of 1.077 g/cm^3). The MNCs were harvested and washed in 0.9 % NaCl with 0.5 % human serum albumin (RVG 16910; Sanquin, Amsterdam, The Netherlands). The BM-derived MNC doses were concentrated in a volume of 10 ml. The Ficoll isolation procedure was performed in a clean-room facility according to good manufacturing practice (GMP) and took approximately three hours^{25, 28}.

MNC injection

BM-derived MNCs were injected in the biceps brachii muscle. The 20 injections were centred on the largest diameter of the muscle belly at a distance of approximately $0.5 \text{ cm} \times 0.5 \text{ cm}$ from each other. With each injection, a volume of 0.5 ml MNCs was injected at a standard injection depth in the muscle belly (which was palpated manually) of 0.5 cm using a 26-gauge needle (Becton Dickinson,

Breda, the Netherlands). The same surgeon, who also performed the tendon transfer, performed these injections (RN).

Clinical functionality

In order to maximise reliability and validity of the range of movement (ROM) measurements, the arm was kept in a standardised position (0 ° anteflexion, 0 ° abduction, 0 ° rotation of the shoulder). Force measurement of the injured biceps muscle was also performed in a standardised arm position (0 ° anteflexion, 0 ° abduction, 0 ° rotation of the shoulder and 90 ° elbow flexion). All measurements were performed by the same examiner (RN).

CT scan

The Aquilion 64-slice CT scanner was used (Toshiba Medical Systems, Otawara, Japan). The scanning parameters were set at 135 kVp and a detector pitch of 53 (pitch factor 0.828). A soft-tissue filter and boost three-dimensional (3D) artifact suppression was used, producing a 512 × 512 matrix of 1 mm thick slices (slice overlap: 0.2 mm). A total of 50 consecutive multiplane reconstruction (MPR) images were computed by reconstructing images in the coronal and sagittal plane so that in every patient axial, slices were used for analysis, thereby correcting for oblique slices. As observed, the largest transversal diameter of the biceps brachii muscle was shifted from the midpoint of the humerus to the distal 2/3 point of the humerus. Therefore, 50 consecutive images were selected from 25 mm superior and 25 mm inferior to this distal 2/3 point of the humerus. This was measured using the most superior part of the humeral head and the most superior part of the olecranon. Images were first recalibrated for air (-1000 HU) based on samples outside the patient. Subsequently, the triceps muscle and the biceps brachii muscle were manually outlined as separate regions of interest (ROI). All pixels containing bone tissue were automatically excluded from the segmentation by applying a threshold value of 200 HU. A histogram was constructed from all pixels within the outlined ROI to calculate the mean muscle density (MMD)³².

Quantitative needle electromyography

Quantitative needle electromyography (EMG) was performed using the Medelec Synergy EMG system (Oxford Instruments, Oxford, United Kingdom). The EMG signals were recorded with filter settings of 5 Hz for high pass and 10 kHz for low pass. For the biceps brachii muscle, the electrode was positioned in the middle of the muscle belly. For the brachialis muscle, the electrode was positioned medial to the biceps muscle at the level where the latter muscle adheres to the tendon. When the EMG signal was crisp, no further adjustment of the electrode position was made and motor unit potentials (MUPs) during voluntary contraction were recorded

for 30 seconds. Potentials that were regarded as belonging to unique motor units but were judged to represent a motor unit already collected were omitted, as well as those with a noisy baseline or other artifacts. Among the remaining MUPs, the automatic duration cursor settings were manually corrected if necessary. If the duration cursor was changed, the amplitude and number of phases of the MUPs were recalculated before analysis³³. All analyses and re-analyses were performed by the first author (SH) and an experienced neurophysiologist (JvD) blinded to the patient characteristics.

Histochemistry and immunohistochemistry of muscle biopsy

A biopsy of the injured biceps muscle was performed at the site of the MNC injection using a forceps with two sharp-edged jaws (Blakesley Conchotoma; DK Instruments, West Bengal, India)³⁴. The muscle biopsies before and after cell therapy in group A were taken under local anaesthesia at the outpatient clinic. The muscle biopsies before cell therapy in groups B and C were performed under general anaesthesia in combination with the modified Steindler procedure. The muscle biopsies after cell therapy in groups B and C were taken under local anaesthesia at the outpatient clinic.

For Masson's trichrome staining, sections were incubated subsequently for 60 minutes at 56 °C in Bouin's fluid (640960; Klinipath, Duiven, the Netherlands), 15 minutes in 1 % Biebrich Scarlet (03336; Brunswig Chemie, Amsterdam, the Netherlands), 15 minutes in 5 % Tungstophosphoric acid (1005830100; Merck, Darmstadt, Germany) and 10 minutes in 2 % light green SF yellowish (C.I. 42095, 115941; Merck) resulting in a green staining for fibrosis and red staining for muscle fibres.

For immunohistochemistry, the primary antibodies monoclonal mouse anti-human Pax7 (1:20, 352-523; Developmental Studies Hybridoma Bank, Iowa City, Iowa), monoclonal mouse anti-human CD56 (1:80, clone NCAM16.2, 345811; BD Biosciences, Erembodegem-Aalst, Belgium), and polyclonal rabbit anti-human von Willebrand Factor (vWF) (1:4000, A0082; Dakocytomation, Heverlee, Belgium) were used. Sections were pretreated by boiling for 20 minutes at pH 6.0 in 10 mM citric acid buffer (C0759-500G; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (Pax7 and vWF) or at pH 9.0 in 1 mM ethylenediamine-tetraacetic acid disodium salt buffer (1073; Baker, Deventer, the Netherlands) (CD56) for antigen retrieval. Next, sections were incubated for 60 min in 5 % normal goat serum (NGS) (X0907; DakoCytomation, Heverlee, Belgium) and 1 % bovine serum albumin (BSA) (A9647-50G; Sigma-Aldrich Chemie) in phosphate buffered saline (PBS) to block nonspecific binding. For Pax7, 0.3 % Triton X 100 (T8787; Sigma-Aldrich Chemie) and 0.01 % hydrochloric acid (1003171000; Merck) was

added to the blocking solution. Sections were incubated with the primary antibodies overnight and subsequently for 20 minutes in 0.3 % hydrogen peroxide (1072090250; Merck) in PBS to block endogenous peroxidase. Next, the sections were incubated for 60 minutes with the biotinylated secondary antibody rabbit anti-mouse (1:200, E0464; Dako-Cytomation) (Pax7, CD56) or goat anti-rabbit (1:200, E0432; DakoCytomation) (vWF). In order to visualise immunolabelling, sections were incubated for 30 minutes with horseradish peroxidase labeled avidinbiotin complex (Vectastain Elite ABC kit, PK6100; Brunschwig, Amsterdam, the Netherlands) and subsequently with diaminobenidine (DAB liquid, K3468; DakoCytomation) as chromogenic substrate resulting in a brown precipitate. Finally, sections were counterstained for one minute with Harris' haematoxilline solution (1092532500; Merck), dehydrated and mounted on Micromount (1731; Surgipath Europe, Peterborough, United Kingdom).

Muscle analysis

The area of fibrosis was analysed in the Masson-trichrome stained sections by dividing each pixel into three color components (hue, saturation, intensity). The threshold was defined and kept constant throughout the analysis. The percentage of green fibrotic area was measured. The haematoxylin and eosin (H&E) stained sections were used to measure the myofibre diameter and the percentage of centronucleated, regenerating myofibres. In order to measure the myofibre diameter and the percentage of centronucleated myofibres, only transversely cut myofibres were measured. In order to compensate for myofibres that were partially longitudinal the minimal diameter was calculated. The Pax7 and CD56 stained sections were used to calculate the percentage of myofibres containing a Pax7 positive cell and percentage of CD56 positive myofibres respectively. The vWF sections were used to assess the number of capillaries around each fibre. All transversely cut capillaries were counted. If a capillary was sectioned longitudinally, it was counted as one each time it crossed a junction between three or more muscle fibres.

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ICMJE CONFLICT OF INTEREST

None declared

AUTHOR CONTRIBUTIONS

S. Hogendoorn: Design of study, Data collection, Data analysis, Writing the paper

B. J. Duijnisveld: Data collection, Data analysis, Writing the paper

S. G. van Duinen: Data collection (histology), Data analysis, Writing a part of the paper

B. C. Stoel: Data collection (quantitative CT), Data analysis, Writing a part of the paper

J. G. van Dijk: Performing the EMGs, Data collection (EMG), Data analysis, Writing a part of the paper

W. E. Fibbe: Facilitating with the stem cell laboratory (BM-derived MNC separation), Design of the study, Writing a part of the paper

R. G. H. H. Nelissen: Design of the study, Performing the surgeries, Data collection (clinical studies), Data analysis, Writing the paper

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