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Increased vascularisation enhances axonal regeneration within an acellular nerve conduit

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Despite major advances in microsurgical techniques, the functional results of periphery nerve repair remain largely unsatisfactory. Furthermore, if a defect exists such that a nerve graft is required, the results are generally worse than those following a primary repair. The autologous nerve graft, the current 'gold standard', has inherent disadvantages and there has been a long quest to find a suitable alternative. The role of vascularisation in nerve regeneration is poorly defined and the aim of this work was to define and quantify the effects of increased vascularisation on nerve regeneration. Rat sciatic nerve defects (1 cm) were bridged with a silicone chamber containing vascular endothelial growth factor (VEGF; 500 or 700 ng/ml) in a laminin-based gel (Matrigel®) known to support axonal regeneration. Chambers were harvested between 5 and 180 days to follow the progression of neural and vascular elements. Following immunohistochemical staining, computerised image analysis demonstrated that VEGF significantly increased vascular, Schwann cell and axonal regeneration within the chamber up to 30 days post-insertion, and stimulated regeneration of up to 78% more myelinated axons at 180 days, compared to plain Matrigel® control. Furthermore, the non-linear vascular dose-response to VEGF was clearly reflected in the Schwann cell and axonal staining intensity, supporting the highly significant relationship between vascularisation and Schwann cell staining seen within the chamber ($P < 0.001$). Target-organ re-innervation at 180 days was similarly enhanced by VEGF in an identical dose-dependant manner. VEGF at 500 ng/ml increased recovery of gastrocnemius muscle weight by 17% and footpad innervation by 51% ($P < 0.05$) compared to control, indicating the long-term functional benefits of VEGF.

Key words: Vascular endothelial growth factor – Schwann cells – Matrigel® – Fibronectin – Silicone chamber

The results of peripheral nerve repair are still far from satisfactory. A permanent, sensory deficit in over 89% of digital and 99% of median nerve primary repairs has been reported,[1] with some authors going further stating 'no adult with a major peripheral nerve transection has ever attained normal sensibility'.[2] If a graft is required the results are generally worse than those following primary suture. The autologous nerve graft, the current 'gold standard', has the inherent disadvantages of limited availability, donor site morbidity and an internal architecture that may not favour the recipient nerve. There have been a long-standing quest to find a suitable alternative.[3]

Whilst the importance of the extracellular matrix, cell adhesion molecules, Schwann cell-axonal interactions and nerve growth factors has become increasingly understood, the role of vascularisation in nerve regeneration remains

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obscure. Increased rates of axonal regeneration in the vicinity of larger blood vessels[4] and changes in capillary number and permeability that are dependant upon successful axonal regeneration suggest an interaction between regenerating axons and blood vessels. However, whilst this raises the possibility that nerve regeneration may be enhanced by manipulating vascularisation, studies so far have produced conflicting results.[5] Possible reasons for these contradictory results are the variety of models used, a frequent failure to assess both vascularity and axonal regeneration and difficulties in the histological differentiation between perfused and non-perfused vessels.

The aim of this work was to describe qualitatively the morphological relationship between regenerating axons and blood vessels within an acellular conduit, unhindered by an existing vascular network. Although such descriptions may allude to the importance of vascularisation in nerve regeneration, it is only by manipulating individual elements specifically and assessing the consequences that the true nature of the relationship can be shown. A further aim, therefore, was to enhance specifically the vascularisation within the nerve conduit and to quantify the morphological effect of such vascular enhancement on Schwann cell and axonal regeneration within the chamber and to assess the long-term benefits of nerve regeneration.

Silicone tubes, a well-described model for the study of nerve regeneration,[3] were filled with Matrigel®, a basement membrane extract of the Engelbreth-Holm-Swan (EHS) tumour that gels at room temperature and is known to support blood vessel and nerve regeneration.[6–8] Furthermore, Matrigel® has been used as a matrix for the slow release of active growth factors *in vivo*.[9,10] By adding vascular endothelial growth factor (VEGF), which is mitogenic specifically for endothelial cells, it was possible to study in ideal conditions the effect of this growth factor on the enhancement of vascularisation and nerve regeneration.

Blood vessel, axon and Schwann cell regeneration within the chamber were studied using immunohistochemistry and computerised image analysis. Dual fluorescence staining within the same histological preparation enabled the demonstration of their true relative positions. Percentage recovery of gastrocnemius muscle weights and footpad reinnervation were used in the assessment of long-term recovery.

Materials and Methods

Regeneration chambers, Matrigel® and growth factors

Silicone chambers (length 13 mm, internal diameter 1.5 mm) (Altec Products Ltd, UK) were sterilised with 2 megarads gamma radiation. Growth factor-reduced Matrigel® (Universal Biologicals Ltd, UK), supplied frozen, forms an irreversible gel on warming to room temperature. Liquid Matrigel® was mixed with cooled test solution to give a 70% Matrigel® solution that was kept on ice until use. Test solutions consisted of sterile 10 mM phosphate-buffered 15 mM saline containing 0.1% bovine serum albumin (Sigma, UK) and human recombinant vascular endothelial growth factor (rhVEGF; R&D Systems Europe Ltd, UK). Final rhVEGF concentrations of 500 ng/ml or 700 ng/ml were used with a plain Matrigel® control.

Surgical procedure

Male in-bred Lewis rats, 7–8-weeks-old, were anaesthetised with an intramuscular solution of fentanyl citrate and fluanisone (Hypnorm) at 0.8 mg/kg and intraperitoneal diazepam (4 mg/kg). The left sciatic nerve was exposed through a lateral thigh incision and intermuscular approach under aseptic conditions. The nerve was divided 4 mm distal to a consistent superior vascular pedicle and a 10 mm gap followed the removal of a 2 mm distal segment and nerve end retraction. The proximal and distal nerve ends were drawn 1.5 mm into an empty silicone chamber and secured with two 10/0 epineurial nylon sutures. Cooled Matrigel® solution was then injected into the chamber by sliding a needle alongside the proximal nerve stump. Within 5 min the Matrigel® set, the wound was then closed, and the animal allowed to recover and fed standard meal and water freely. The nerve and tissue from the contralateral unoperated side were used as control.

Six animals from each experimental group (500 ng/ml or 700 ng/ml VEGF, and plain Matrigel®) were harvested at 10, 15 and 30 days for the quantification of blood vessel, Schwann cell and axonal regeneration within the nerve chamber. To assess long-term recovery, a further 6 animals from each group were sacrificed at 180 days to calculate the recovery of footpad skin innervation and gastrocnemius muscle weight.

Tissue processing and immunohistochemistry

Animals were sacrificed via enflurane inhalation and the nerve conduits with attached proximal and distal nerve removed immediately. The specimens were pinned to card to avoid shrinkage during fixation in Zamboni's solution prior to washing in PBS sucrose (15%) and storing at 4°C. The nerve ends were trimmed 2 mm from the point of entry into the conduit to give a fixed reference point for measurements.

Indirect immunofluorescence staining of longitudinal cryostat sections (15 μm) was used to visualise both vascular and neural elements according to a previously described technique.[11] To stain endothelial cells, mouse monoclonal antibodies to rat endothelial cell cytoplasmic

antigen (RECA-I, Serotec, UK) were used at a dilution of 1/20. Schwann cells were stained with rabbit polyclonal antibodies to S-100 (Dako, UK; diluted 1/1200) and regenerating axons stained with a mouse monoclonal antibody to pan neurofilament (Affiniti, UK; diluted 1/2000). The second layer antibodies used were either goat anti-mouse conjugated to cyanine-3 (Affiniti, UK; diluted 1/100) or goat anti-rabbit conjugated to FITC (TCS Biologicals, UK; diluted 1/100). All antibody dilutions were made in a PBS solution containing 0.02% sodium azide (BDH Chemicals, UK), 3% goat serum (TCS Biologicals) and 3% rat serum to reduce background staining.

Computerised image analysis (Seescan Analytical Services, Cambridge, UK) was used to assess blood vessel, Schwann cell and axonal regeneration within the conduit. The total area of staining was assessed across the full width of tissue at set distances (1, 2, 3, 5, 8 and 9 mm) within the regeneration chamber. For each animal and each stain calculations were made on 2 randomly chosen sections and the results averaged. Neural and vascular images were captured on Kodak Elite 400 film (Kodak, UK), double exposure photography demonstrating their true relative positions.

Footpad re-innervation

Footpad skin overlying the hind-foot fourth ray was removed, fixed in 4% paraformaldehyde and stored in PBS-sucrose. Histological sections were taken perpendicular to the skin surface plane and stained immunohistochemically in the manner described above. Regenerated cutaneous nerve fibres were visualised using a rabbit polyclonal antibody to protein gene product 9.5 (PGP, Vectraclone, UK: diluted 1/1200) and a second layer goat anti-rabbit conjugated to FITC. For each animal, computerised calculations of the total area of staining within 4 alternate fields (25x magnification) along the epidermal-subepidermal layer were made from 2 randomly chosen sections. The average staining area on the experimental side was expressed as a percentage of that on the contralateral control side.

Gastrocnemius muscle weights

The gastrocnemius muscles were exposed through a mid-calf incision and dissected cleanly from their origin and insertion. The muscles were weighed immediately and the weight of that on the experimental side expressed as a percentage of that on the contralateral control side.

Statistical analysis

Statistical analysis was performed using a STATA (STATA Corporation, TX, USA) software package. For the analysis of the immunostaining area of blood vessels, Schwann cells and axons within the chamber at set distances (1, 5 or 8 mm) an initial two-way analysis of variance for each variable was performed with group and time factors. The assumption of normality was checked with Shapiro and Francia's test, and the assumption of constant variance checked with Barlett's test of equality of variances. A one-way analysis of variance for each time point was performed separately for each distance.

Similarly, for the analysis of footpad re-innervation and recovery of gastrocnemius muscle weight, a one-way analysis of variance was used to compare group means. Data were assessed for normality and equal variance and, if both conditions were satisfied, a Tukey all pair-wise multiple comparison procedure performed.

All graphical representations throughout this work are of group means presented with standard error bars.

Results

Morphology of the plain Matrigel® control

Although the extent of Schwann cell and axonal regeneration was greater at 15 days than at 10 days, the patterns of regeneration and vascularisation were the same and, therefore, these two groups are considered together. The gap between the nerve ends was bridged by a sleeve of peripheral non-staining cells, possibly of perineurial origin,[12] that enclosed the core of Matrigel®.

A denser cellular infiltrate extended into the chamber from both proximal and distal nerve stumps, extending from the periphery of the nerves as well as their centres. There was a well-defined interface between the cellular infiltrate and the Matrigel®. Blood vessels, Schwann cells and axons extended into this cellular infiltrate with a variable orientation, although there was a clear relationship between the parallel orientations of the individual elements.

The main vascular front extended almost to the limit of the cellular infiltrate along with, or slightly in advance of, the main Schwann cell/axonal front, although occasionally single Schwann cells and axons extended slightly in advance of the blood vessels. Occasionally, blood vessels extending into the Matrigel®, beyond the front of the dense cellular infiltrate, were cut in transverse cross-section. These vessels were generally surrounded by a cuff of non-staining cells, which in turn were frequently surrounded by Schwann cells. Throughout the time course, Schwann cells and axons extended together, neither element significantly preceding the other. The penetration of the Schwann cells and axons into the chamber was maximal in those well vascularised regions containing longitudinally oriented vessels. Schwann cell

Table 1 Immunostaining area ($x10^{-3}$ mm^2 ± SEM) at 1 mm into the chamber from the proximal nerve stump at 10 days

	Control (no VEGF)	VEGF (500 ng/ml)	VEGF (700 ng/ml)
Blood vessels	11.45 ± 0.77	10.72 ± 1.38*	18.75 ± 2.25*
Axons	43.01 ± 9.00	33.73 ± 7.31**	83.77 ± 8.51**
Schwann cells	67.97 ± 13.38	57.09 ± 14.56*	125.24 ± 19.29*

Each figure represents the total area of immunostaining, within a one microscope frame-wide strip, across the full width of regenerated tissue. The values are the means from 2 sections per animal, $n = 6$ per group.
*P <0.05 rhVEGF 500 ng/ml *versus* rhVEGF 700 ng/ml.
**P <0.01 rhVEGF 500 ng/ml *versus* rhVEGF 700 ng/ml.

Table 2 Blood vessel immunostaining area ($x10^{-3}$ mm^2 ± SEM) within the chamber at 15 days

	Control (no VEGF)	VEGF (500 ng/ml)	VEGF (700 ng/ml)
1 mm	6.47 ± 1.92**	23.28 ± 3.78	17.67 ± 3.35
5 mm	1.70 ± 0.57*	3.12 ± 1.41	4.85 ± 1.05
8 mm	5.03 ± 0.57*	11.02 ± 2.38	7.78 ± 1.02

Values represent the total area of immunostaining across the full width of regenerated tissue at the given distances within the chamber from the proximal nerve stump. Calculations were made as in Table 1.
*P <0.05 plain Matrigel® control *versus* rhVEGF (groups mean).
**P <0.001 plain Matrigel® control *versus* rhVEGF (groups mean).

and blood vessel penetration was always greater from the proximal nerve end.

By 15 days, blood vessels and Schwann cells had bridged the chamber, typically extending maximally along the periphery of the regeneration cable enclosing a residual core of Matrigel®. Good penetration of Schwann cells and axons within the central region was associated with longitudinally oriented blood vessels, although orientation at the regeneration front was generally less organised indicating some remodelling of the regenerated tissue.

At 30 days, whilst some variability in the orientation of the blood vessels, Schwann cells and axons persisted at the sites of the original nerve ends, orientation within the regenerated tissue was otherwise longitudinal, indicating further re-organisation. Axons had entered the distal nerve stump at this stage.

Vascular and neuronal response to VEGF

Following an initial pilot study, doses of 500 ng/ml and 700 ng/ml rhVEGF were used to assess the effect of increased vascularisation on Schwann cell and axonal regeneration.

At 10 days, rhVEGF at 500 ng/ml had little effect on the amount of blood vessel staining throughout the chamber, whilst rhVEGF at 700 ng/ml increased vascularisation compared to control at all distances measured. The difference between the two rhVEGF dose groups was statistically significant at the 1 mm distance (Table 1). This pattern was clearly reflected in the staining of both Schwann cells and axons, rhVEGF at 500 ng/ml similarly failed to increase staining for either Schwann cells or axons, but 700 ng/ml rhVEGF increased staining for both. Again, the differences between the two rhVEGF dose groups was statistically significant at 1 mm for both axons and Schwann cells (Table 1).

At 15 days, blood vessels and Schwann cells had bridged the chamber in all cases. Both doses of rhVEGF

Table 3 Schwann cell immunostaining area ($x10^{-3}$ mm^2 ± SEM) within the chamber at 15 days

	Control (no VEGF)	VEGF (500 ng/ml)	VEGF (700 ng/ml)
1 mm	85.11 ± 11.55*	127.62 ± 32.16	110.90 ± 9.39
5 mm	0.16 ± 0.08*	0.49 ± 0.35	2.27 ± 0.89

Values represent the total area of immunostaining across the full width of regenerated tissue at the given distances within the chamber from the proximal nerve stump. Calculations were made as in Table 1.
*P <0.05 plain Matrigel® control *versus* rhVEGF (groups mean).

increased the area of immunostained blood vessels within the silicone chamber at all distances measured, compared to control. The differences between the means of the control group and the average of the two rhVEGF groups were significant at all distances measured (Table 2). At this stage, however, rhVEGF at 500 ng/ml generally stimulated a greater increase in vascularisation than rhVEGF at 700 ng/ml, although the differences between the two rhVEGF groups did not reach significance.

Schwann cell staining was similarly increased in an almost identical manner, again the lower dose of rhVEGF generally having the greater effect. Increases in Schwann cell staining were significant at 1 mm and 5 mm (Table 3). There were no significant differences in area of axonal staining between any of the groups.

At 30 days postoperatively, the values of blood vessel staining within the chamber (Fig. 1) were slightly lower than those seen at 15 days, suggesting some vascular re-organisation. VEGF increased vasculare staining throughout the chamber, the increases being significant at 1 mm and 8 mm. As at 15 days the lower 500 ng/ml dose of rhVEGF generally had the greater effect although the differences between the two rhVEGF groups were not significant.

The vascular staining pattern was clearly reflected in that of the axons with rhVEGF stimulating increases in

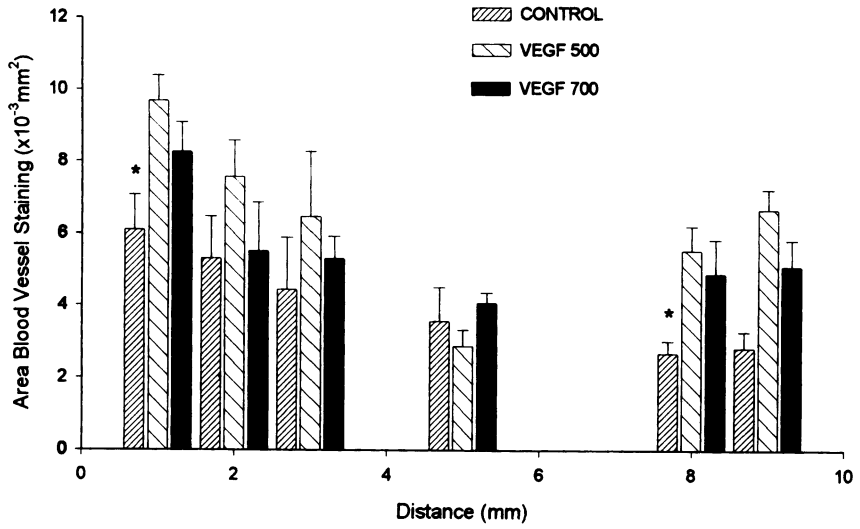

Figure 1 Total area of blood vessel staining for the 3 groups at given distances within the chamber at 30 days. Each bar represents the total area of immunostaining, within a single microscope field-strip, across the full width of regeneration tissue at that distance from the proximal nerve stump. The values are the means from 2 sections per animal, $n = 6$ per group. *P <0.05 plain Matrigel® control *versus* rhVEGF (groups mean).

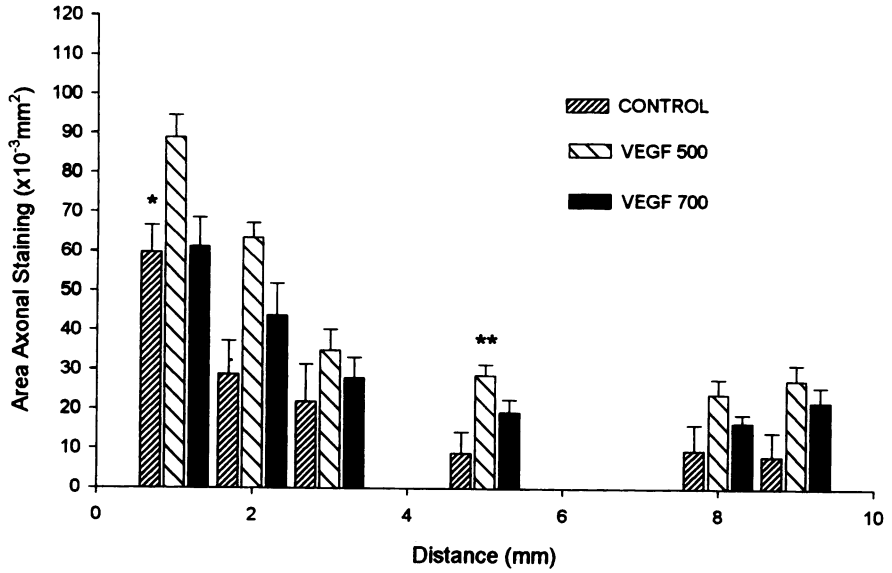

Figure 2 Total area of axonal staining for the 3 groups at given distances within the chamber at 30 days. Calculations were made as for Figure 1. *P <0.05 plain Matrigel® control *versus* rhVEGF (groups mean); **P <0.05 rhVEGF 500 ng/ml *versus* rhVEGF 700 ng/ml.

axonal staining throughout the chamber (Fig. 2). The dose response to rhVEGF closely resembled that of the blood vessels with rhVEGF at 500 ng/ml having the greater effect throughout the chamber compared to rhVEGF at 700 ng/ml. The Schwann cell staining pattern (results not shown) was similar to that of the axons, rhVEGF stimulating significant increases in staining at 1, 5 and 8 mm (P <0.01 control *versus* rhVEGF groups mean).

Footpad re-innervation

Both doses of rhVEGF enhanced the recovery of footpad innervation, increasing the percentage recovery from 16.9% in the control to 24.4% and 51.4% in the 700 ng/ml and 500 ng/ml dose groups, respectively. The difference between the control group and the 500 ng/ml rhVEGF group was statistically significant (P <0.05).

Gastrocnemius muscle weights

Both doses of rhVEGF increased the recovery of gastrocnemius muscle weight (rhVEGF 500 ng/ml 47.9% of the contralateral gastrocnemius weight, rhVEGF 700 ng/ml 34.3%) compared to the plain control (31.3%), although the increases failed to reach significance. However, the dose response followed that of the recovery of footpad innervation with rhVEGF 500 ng/ml having a greater effect than rhVEGF 700 ng/ml.

Discussion

Whilst vascularisation within a silicone nerve regeneration chamber has been variously described by a number of authors, most of these workers have used transverse nerve sections for assessment and a more detailed description of orientations and spatial relationships has not been given. The model used in the present study has allowed the clear visualisation of the spatial–temporal relationships between regenerating elements within the chamber, demonstrating a clear relationship between the orientations of the blood vessels, Schwann cells and axons throughout the chamber at all time points.

A dense non-staining cellular infiltrate extended in advance of the main blood vessel and nerve regeneration fronts. It has been suggested such an infiltrate may consist largely of macrophages and leukocytes,[13] whilst other workers claim fibroblasts and pericytes are the predominant cell types.[14–16] Vascularisation generally lagged slightly behind the limit of the infiltrate. Matrigel® consists of randomly orientated basement membrane components, principally laminin and type IV collagen, which are important determinants of cellular activity. The Matrigel® may, therefore, influence both blood vessel and neural regeneration either directly, or indirectly through the cellular infiltrate.

However, the fact that behind a more randomly orientated regeneration front the elements were longitudinally orientated, and that by 30 days all elements within the chamber attained this longitudinal direction, would suggest a remodelling of both neural and vascular elements. We have reported similar findings in a fibronectin conduit model,[11] highly suggestive of a close relationship between regenerating nerves and blood vessels.

This work also demonstrated that rhVEGF significantly increases vascularisation and this is associated with a similarly significant increase in nerve regeneration. The vascular dose-response to the added rhVEGF was not linear, with the higher dose having a significantly greater effect at early time periods, and subsequently the lower dose having the greater effect. Such non-linearity may be related to heparan sulphate proteoglycan within the Matrigel®,[17] which is known to bind VEGF.[18] Cell surface heparan proteoglycan interaction is required for VEGF binding to high affinity receptors[19] and matrix composition may, therefore, modify the availability of bioactive growth factor. Alternatively, the non-linearity may be associated with the activities of different VEGF receptors (at least two high-affinity receptors have been identified[20–22]), or due to a delayed indirect effect of VEGF. Although monocyte migration has been stimulated by VEGF,[23] monocyte receptor binding affinity is about 20 times lower than that of the high affinity endothelial cell receptors, making a direct endothelial cell effect at the lower dose of VEGF more likely.

The non-linear vascular dose-response to rhVEGF was also clearly reflected in the Schwann cell and axonal staining intensity. Although it has recently been suggested rhVEGF may have a direct effect on Schwann cell and axonal regeneration,[24] our studies have failed to demonstrate this and the strong similarity in staining patterns of all three elements is highly suggestive of a close relationship between blood vessels and regenerating nerves.

However, such increases in Schwann cell and axonal regeneration are of no value if they do not lead to improved functional recovery. Early increases in axonal staining may reflect excessive axonal sprouting, and since those axonal sprouts that do not make a functional connection are resorbed,[25] the initial histological appearance does not necessarily correlate with functional return.[26] Unfortunately, direct functional assessment in a rat is difficult. Therefore, an indirect assessment of functional recovery was made through the measurement of gastrocnemius muscle weights and footpad re-innervation. Again, the lower dose of VEGF had the greater effect, causing a significant increase in gastrocnemius muscle recovery. Although other increases failed to reach significance, the dose-response mirrored that seen at the later time points within the regeneration chamber.

Conclusions

This work has shown a convincing relationship between regenerating nerves and blood vessels. Furthermore, this relationship has been exploited *in vivo*, with a VEGF-induced enhancement in vascularisation being associated with an increase in nerve regeneration that was maintained long-term. Such results may prove useful in improving the clinical outcome of peripheral nerve repair.

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