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Research Article

Effect of Gelatin Hydrogel Sponge Loaded with Bone Marrow Mesenchymal Stem Cells and Hepatocyte Growth Factor on Peripheral Nerve Regeneration

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Abstract

Hepatocyte growth factor (HGF) can promote nerve cell proliferation. Gelatin hydrogel sponge can act as a scaffold for drug controlled release. . This study aimed to evaluate the beneficial effects of a gelatin hydrogel scaffold (sponge) loaded with BM-MSCs and HGF on radial nerve regeneration in a defect model. Twelve healthy calves (weighing 48.7 ± 5.54kg, mean ± S.D.) were used in this study. Stem cells were derived from bone marrow of ilium and cultured into culture dishes for two weeks. 1cm of the left radial nerve was defected under inhalation anesthesia. A sponge incorporating BM-MSCs and HGF (2 μg) (BM-MSCs-HGF/gelatin sponge) or a sponge incorporating saline (saline/gelatin sponge, control) was implanted into the nerve defect site. Postoperative changes in were evaluated by nerve function examination (assessment of standing gait and examination of proprioception) for 4 weeks after surgery. Histological examination was also carried out at 4 weeks after surgery. In standing gait and proprioception scores in the BM-MSCs-HGF/sponge group were significantly higher than those in the saline/sponge group ($P<0.05$). In histological examination, the regenerated nerve in the BM-MSCs-HGF/sponge group was significantly longer than that in the saline/sponge group. The number of Schwann cells in the BM-MSCs-HGF/sponge group was also greater than that in the saline/sponge group. Our results show that gelatin sponge incorporating BM-MSCs and HGF accelerates peripheral nerve regeneration in calves.

Keywords: Calf; Gelatin Hydrogel Sponge; Hepatocyte Growth Factor; Mesenchymal Stem Cell; Peripheral Nerve Regeneration

Abbreviations

HGF: Hepatocyte Growth Factor;

BM: Bone Marrow;

DDS: Drug Delivery System;

MSC: Mesenchymal Stem Cell;

TCP: Tri-Calcium Phosphate

Introduction

Fracture of the humerus in cattle can lead to radial nerve injury or rupture. Such radial neuropathy commonly impairs control of a number of extensor muscles in the upper limb including, the triceps, anconeus muscle, tensor latae muscle, extensor carpi radialis muscle, and extensor carpi ulnaris muscle. Animals with injured radial nerve are unable to extend the affected forelimb and have poor prognosis for recovery. In human medicine, a transected nerve completely, tissue engineering was introduced. Therefore, large animals expected to introduce tissue engineering, and recover completely.

Mesenchymal stem cells have been shown to be distributed in a number of tissues, including the umbilical cord, peripheral blood, adipose tissue, and bone marrow) and to possess multilineage differentiation capacity (Schauwer and Meyer 2011). Bone marrow mesenchymal stem cells (BM-MSCs) have multilineage capacity including osteogenic, adipogenic, and chondrogenic lineages. In vitro studies have shown that BM-MSCs can induce neural cells, including schwann cells, which are known to play a main role in peripheral nerve regeneration [1,2]. Although nerve growth factor and hepatocyte growth factor (HGF) are also known as regenerating factor [3,4]. these growth factors have a short biological half-life (ten minutes in the case of HGF) and therefore limited effect [5]. Drug Delivery Systems (DDS) containing gelatine hydrogel have been used to control the release of drugs with short biological half-life and improve their sustainability and efficacy. . For successful cells-induced tissue regeneration, it is indispensible to create a local environment that promotes cells proliferation and differentiation. Gelatin hydrogel can function as a DDS scaffold in nerve regeneration and promote axon outgrowth.

This study aimed to evaluate the beneficial effects of a gelatin hydrogel scaffold (sponge) loaded with BM-MSCs and HGF on radial nerve regeneration in a defect model. Postoperative recovery was assessed by nerve histological examination as well as its function.

Materials and Methods

Twelve healthy calves (male: 2, freemartin: 10) aged 40.9 ± 12.4 days (mean ± S.D.) and weighing 48.7 ± 5.54 Kg were used in this study. This study was approved by the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine.

Animals bone marrow was aspirated as described previously. In brief, the animals were sedated with 0.02 mg/kg bwt Xylazine hydrochloride (Emasasu®, 2%, Sumitomo Dainippon Pharma Seiyaku Co., Ltd., Japan), and an area over the ilium was clipped and scrubbed. The ilium was examined ultrasonographically (SonoSite 180 II ®, SonoSite, USA) and scrubbed clean with surgical scrub and alcohol. One millilitre of lidocaine hydrochloride (Xylocaine®, %2, AstraZeneca Co., Ltd., Japan) was subcutaneously infiltrated over the tuber coxae, and a biopsy needle (Jamshidi biopsy needle 11G×10cm, Cardinal Health, USA) was advanced approximately 2-1 cm into the ilium bone. One millilitre aliquots of ilium bone marrow were then aspirated into syringes coated with 100 IU heparin sodium (Novo-Heparin for injection, Mochida Pharmaceutical Co., Ltd., Japan). The aspirated bone marrow was transferred into a vacuum blood collection tube (Venoject® II heparin sodium, TERUMO, Japan).

BM-MSCs cultures were prepared as described previously by Takahashi and Yamamoto (2005). In brief, the collected bone marrow was stirred, and 0.1 ml was transferred into each culture dish (Dishes Nunclon™Δ 90 mm, Thermo Fisher Scientific Inc, USA) containing 8.5 ml of medium (Dulbecco's Modified Eagles Medium - high glucose, Sigma-Aldrich Japan, Japan) supplemented with 1.5 ml fetal bovine serum (Serum, Fetal Bovine, BioWest, France) and 0.1 ml antibiotic (Penicillin-Streptomycin solution, Sigma-Aldrich Japan, Japan). The bone marrow was then uniformly blended with the medium, and the culture dish was incubated in a humidified atmosphere of 5% CO_2 at 37°C until the 14th day after aspiration. To remove non-adherent cells and foreign particles, the culture was rinsed twice with phosphate buffered saline (NaCl: 137mmol/ l, KCl: 2.7 mmol/ l, Na_2HPO_4: 10 mmol/ l, KH_2PO_4: 1.76 mmol/ l, pH 7.4; PBS) 3 days after culture, and the medium was changed every two days thereafter.

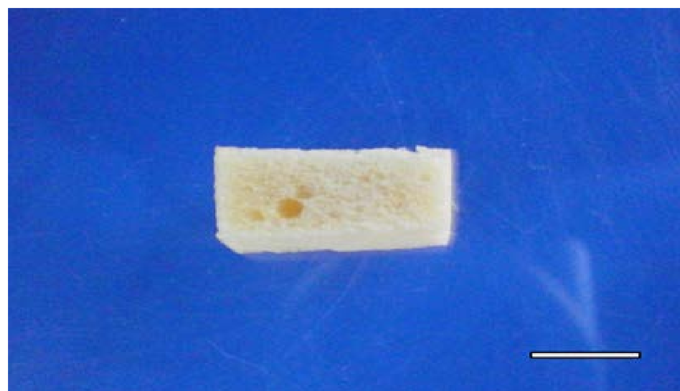

Figure 1. Dried gelatin hydrogel sponge (IEP: 5.0, 97.8% water content, porosity: 95.9%, pore size: 179.1±27.8 μm; mean ± S.D.)

5 mm×10 mm×5 mm. Bar:5mm.

Gelatin hydrogel sponges were prepared from gelatin isolated from bovine bone by the acid process (IEP: 5.0, Nitta Gelatin Co., Japan, Figure 1). At first, a 3 wt% gelatin solution was prepared by dissolving the gelatin in double distilled water (DDW) while mixing the solution over heat until it reached 37°C. Next, a 0.16 wt% glutaraldehyde (GA) aqueous solution was added, and the gelatin solution was further mixed for 15 seconds. The resulting solution was cast into a polypropylene dish and left at 4°C for 12 hours for gelatin crosslinking. The cross linked sponges were then placed in a 100 mM aqueous glycine solution at 37°C for 1 hour to block the residual aldehyde groups of GA, thoroughly washed with DDW, and then freeze-dried. This process allowed the preparation of dried gelatin hydorogel sponge (IEP: 5.0, 97.8% water content, porosity: 95.9%, pore size: 179.1±27.8 μm, 5 mm×10 mm×5 mm). A 2 μg of HGF (Re-

combinant humanHGF, HUMANEZYME, USA) solution (100 µl) was next impregnated into each sponge, which was kept at room temperature for over an hour to allow HGF complete incorporation.. The cell suspensions were loaded with the prepared sponge just before implant into the nerve defect.

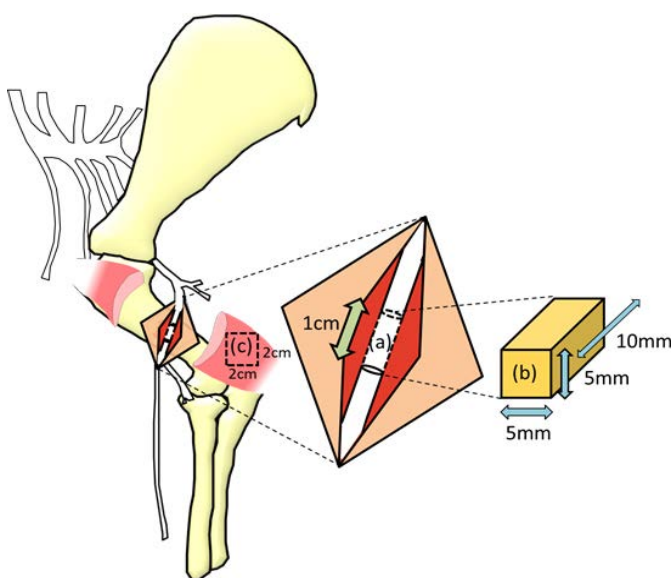

Figure 2. Radial nerve defect model. The 1 cm radial nerve defect (a) was created at the cranial lateral head of the triceps brachii muscle on the humerus. Implantation of the BM-MSCs-HGF/sponge or the saline/sponge into the nerve defect (b, 5mm×5mm×10mm). The implanted nerve was wrapped by the fasciae (c, 20mm×20mm).

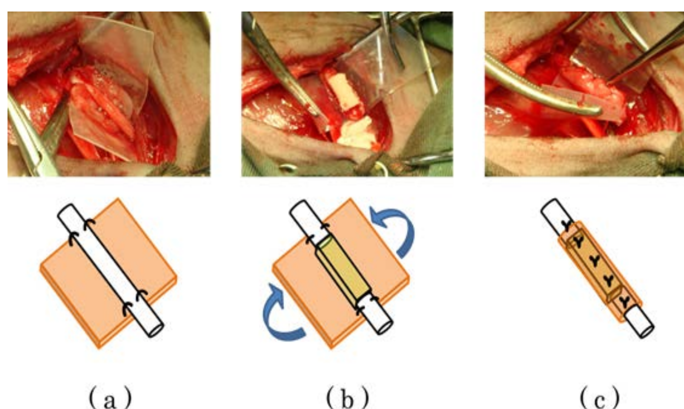

Figure 3. Implanted gelatin hydrogel sponge. The upper section:under operation. The middle section:operative procedure and illustration. The radial nerve (black arrow) was sutured to the fasciae (a), ,The sponge (white arrow) was implanted (b) and sutured (yellow arrow) with 6-0 unabsorption suture to the fasciae (c).

All calves were intravenously premeditated with 0.02 mg/kg Xylazine hydrochloride (Emasasu®, 2%, Sumitomo Dainippon Pharma Co., Ltd., Japan) and anesthesia was intravenously induced 5 minutes later with 0.02mg/kg diazepam (Horizon®, 0.5%, Astellas Inc., Japan) and 4 mg/kg thiamylal (Isozole®, Nichiiko Co., Ltd., Japan). Each calf was placed in lateral right recumbency, and the area over the humerus was shaved, disinfected and draped. A skin incision (10 cm) was made at the cranial lateral head of the triceps brachii muscle on the left side, and a blunt dissection was performed between the triceps brachii muscle and the brachial muscle. The radial nerve was then exposed and stimulated with an electric current to confirm contraction of the extensor muscle. The fasciae of the triceps brachii muscle (20×20 mm) were then separated and harvested. The radial nerve to be damaged was sutured with 6.0 unabsorption suture to the fasciae at the 4 point of the distal and proximal radial nerve (Figure 2). After the radial nerve was damaged and loss of muscle contraction was confirmed, a sponge loaded with BM-MSCs-HGF (BM-MSCs-HGF/sponge) and a sponge incorporating saline (saline/sponge) were implanted into the nerve defect. The sponge wrapped with the fasciae was sutured with 6-0 unabsorption suture (Figure 3). Finally, the muscle and skin were sutured with absorption suture (2-0 Vicryl®, Johnson and Johnson, Japan). After surgery, each animal received 1.0 mg/kg flunixin meglumine intravenously and 20,000 unit/kg penicillin intramuscularly for 3 days.

Animal standing gait was evaluated before and every day after surgery for four weeks. The standing gait was scored as 1: astasia, 2: possible standing (frequent knuckling), 3: possible standing (sometimes knuckling), 4: possible standing (rarely knuckling), 5: normal. When the left limb of the calf was lifted and allowed to drop to the ground, muscle contraction (proprioception) was evaluated and scored as 1: loss (no muscle contraction on the ground), 2: mild (little muscle contraction on the ground), 3: middle (bare muscle contraction on the ground), 4: normal (normal muscle contraction on the ground).

All calves were euthanized at 4 weeks after surgery and their regenerative nerves were harvested. Specimens of the nerves were fixed in 15% formalin, decalcified in 99% formic acid and embedded in paraffin wax. The specimens were then cut into 4-µm sections in the sagittal plane, stained with hematoxylin eosin (HE staining), and immunohistochemically examined (s-100). Additionally, the specimens were cut into 1 µm sections in the sagittal plane, stained with Methylene blue, and observed under a light microscope.

Standing gait and proprioception scores are given as median values (minimum, Maximum). Differences in standing gait and proprioception scores between the BM-MSCs-HGF/sponge group and the saline/sponge group were evaluated by Mann-Whitney's U test. Significance level was set at $P<0.05$.

Results

Although most calves got on their feet with a shoulder flex 1 to 2 days after surgery, it was difficult for them to walk due to severe knuckling in lower limb. This knuckling was gradually reduced from day 3 to day 13 after surgery with no significant difference between the treatment groups. Calves transplanted the saline/sponge showed persistent knuckling after day 13, calves transplanted the BM-MSCs-HGF/

sponge exhibited no knuckling at all. Evaluation of animals standing gait 16 and 19 days after surgery revealed significantly higher scores in the MSCs-HGF /sponge group than in the saline/sponge group (P<0.05, Figure 4).

No proprioception (muscle contract) was confirmed in any animal until day 18 after surgery. Proprioception in both treatment groups was clearly confirmed on day 19 and thereafter. . Proprioception score in the BM-MSCs-HGF/ sponge group: score2 (1, 3) was significantly higher than that in the saline/sponge group: score1 (1, 1) 21 days after surgery (P<0.05) (Figure 5).

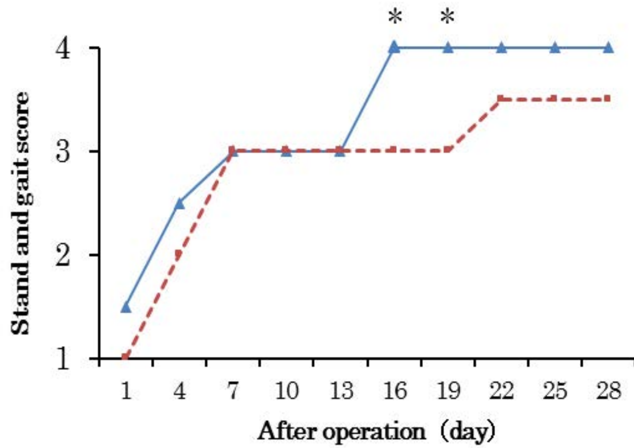

Figure 4. Time-dependent change in stand gait score. Stand gait median score in the BM-MSCs-HGF /sponge group (triangle) was significantly greater than that in the saline/sponge group (square) at 16 and 19 days. *: P<0.05.

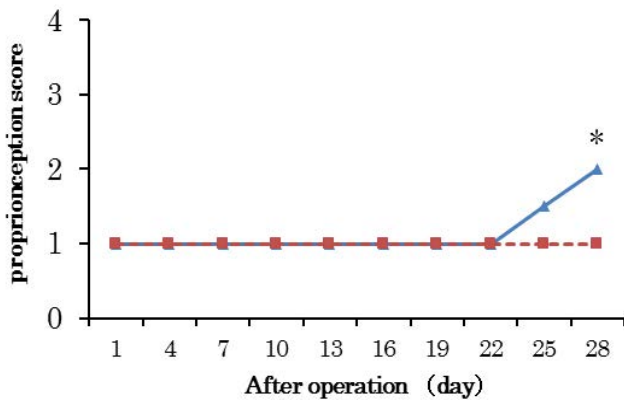

Figure 5. Time-dependent change of proprioception score. Proprioception median score in the BM-MSCs-HGF /sponge group (triangle) was significantly greater than that in the saline/sponge group (square) at 16 and 19 days. *: P<0.05.

Immunohistochemical (s100) examination of regenerated radial nerves specimens prepared harvested 4 weeks after surgery revealed significantly longer nerves in the BM-MSCs-HGF /sponge group than in the saline/sponge group (Figure 6). Transverse section of the regenerated nerve stained with Methylene blue is show in Figure 7. These transverse sections showed the presence of macrophages, schwann cells, and axon in both groups. Many young regenerated nerves could be confirmed in the BM-MSCs-HGF /spong group, but not in the saline/sponge group. The number of Schwann cells in the BM-MSCs-HGF /sponge group was greater than that in the saline/sponge group.

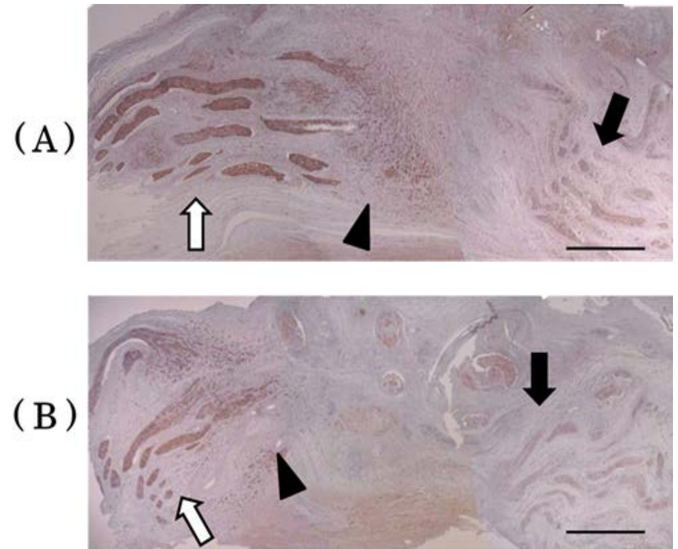

Figure 6. Nerve sections stained and examined immunohistochemically (s-100) in the sagittal plane at 4 weeks after surgery. (A): BM-MSCs-HGF /sponge group, (B): saline/sponge group. S-100 protein in stained brown. In the BM-MSCs-HGF /sponge group, noticeable regenerated nerve can be confirmed proximal. Bar:3mm. White arrowproximal nerve stump. Black arrow head:regenerated nerve. Black arrow:distal nerve stump.

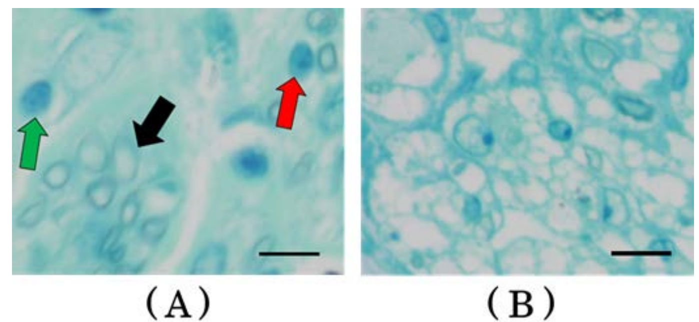

Figure 7. Extended image of nerve section stained with Methylene blue in the tranverse section at 4 weeks after surgery. (A): BM-MSCs-HGF/sponge group, (B): saline/sponge group. Bar: 10μm. Black arrow: Regenerating axon clusters. Red arrow: myelinating Schwann cell nucleus. Green arrow: unmyelinating Schwann cell nucleus. Clusters can be confirmed in the BM-MSCs-HGF/sponge group.

Discussion

We have shown in this study that BM-MSCs have the ability to differentiate into Schwann cells as reported by Wang et al. [2]. Differentiated Schwann cells are known to induce my-

elin secrete neurotrophic factor, such as nerve growth factor, brain-derived neuro trophic factor, ciliary neurotrophic factor, glial derived neurotrophic factor, and transforming growth factor [4]. Also, BM-MSCs co-cultivated Schwann cells are known to promote tissue proliferation [2]. It has been shown that neuroma is not formed following transplantation of MSCs derived Schwann cells. That is to say, BM-MSCs may be used as a substitute for Schwann cells to promote nerve regeneration promotion.

HGF can help various cells proliferate, including nerve cells by inhibiting cells death and preserving the slow development of amyotrophic lateral sclerosis. HGF has attracted a great deal of attention in central nervous system amyotrophic lateral sclerosis [6]. Disease-dependent reciprocal phosphorylation of serine and tyrosine residues of c-Met/ HGF receptor has been shown to contribute to disease retardation in transgenic mouse model of ALS [6]. HGF can improve nerve cells viability by stimulating c-Met and inhibiting activation of caspase, which cause death of nerve cells death [5,6]. Another way is to absorb glutamic acid by EAAT2 transporter [6]. Furthermore, HGF promotes peripheral nerve regeneration as neurotrophic factor [5,7], Based on this evidence we selected to use HGF in this study. The dose of HGF (2 μg) used in this study was selected based on the results of a previous study by [8,9].

Because gelatin hydrogel sponge is biodegradable and can control isoelectric point, it can be used for various tissues engineering [9-11]. This study used a specific gelatin hydrogel sponge (IEP: 5.0, water content: 97.8%) which strongly bind to HGF. HGF bound gelatin hydrogel sponge can achieve sustained release over a 2-week period [8,9] and therefore extend the effect of HGF. Nerve conduit is required to have a structure that does not inhibit development of axon as well as continuous porous structure to allow easy infiltration by nerve cells and microvessels. Gelatin hydrogel sponge has these features and can be used with various tissues in clinical practice [9-11]. In this study the gelatin hydrogel sponge was expected to help regenerate the axon and form nerve fascicle. Using our methods, it was possible to get the required amount of BM-MSCs after 14 days and to complete the implant in a short time. Therefore, the method used in this study is appropriate for nerve tissue engineering.

Most of the current nerve conduits have a tubular structure to allow l axon extension [12]. They are made of artificial biodegradable materials including silicon, polylactic acid, and polyglycolicacid [13], and can block the invasion of surrounding tissue and provide a foothold for axonal extension. However, these conduits are expensive and do not freely control length for nerve defect. On the other hand, nerve conduits made of autologous materials (vein, muscle, and or fasciae) does not have these disadvantages. Fasciae are superior material because they can easily be harvest in large amounts and do not require treatment with certain chemicals in advance. Moreover, fasciae have a little muscle fiber. Laminin with muscle fiber encourages migration, adhesion of Schwann cell and vascular supply and secretes neurotrophic factors, such as neuregulin [14]. Besides, fasciae prevent adhesion of surrounding tissue, because of their high fiber density and tolerance of macrophages invasion and vascular supply [14]. Therefore, fasciae were selected in this study as autologous material for nerve regeneration.

Immunohistochemical staining revealed Schwann cells lining up the distal nerve. Therefore, nerve regeneration using gelatin hydrogel sponge and fasciae seems to be appropriate for lead axonal extension. As Schwann cells line up in the BM-MSCs-HGF /sponge was significantly longer than that in the saline/sponge group, it is believed that the regenerated nerve in the BM-MSCs-HGF /sponge group was significantly longer than that in the saline/sponge group.

In a study reporting the effects of a similar implant in rat sciatic nerve defect model, the regenerated nerve reached 4 mm in four weeks after implanted nerve conduit as fascia[10]. In this study, the regenerated nerve bridged the gaps of about 70% of the nerve defect in four weeks after operation. Although we must consider difference in animal species and site of the nerve harvested, this study can be considered as a promising method for nerve regeneration. As confirmed by the results of this study, BM-MSCs and HGF can promote nerve regeneration. . Also, However, a longer time was required to cover on the whole nerve defect, because the regenerated nerve did not completely cover the nerve defect in both BM-MSCs-HGF /sponge and saline/sponge groups. With more time allowed the recovery would have been even better.

The number of Schwann cells in BM-MSCs-HGF /sponge was greater than that in the saline/sponge stained with Methylene blue. Many young regenerated nerves (cluster) were observed in BM-MSCs-HGF /spong stained with Methylene blue. The cluster was regarded as index of nerve regeneration in various study, because the cluster indicate development of new axon during the process of nerve regeneration [15,16]. The regenerated nerve in BM-MSCs-HGF /sponge is therefore more significant that in saline/sponge, since the presence of Schwann cells indicate nerve regeneration. Interestingly, there was no difference in the diameter of axon and the thickness of myelin between the BM-MSCs-HGF / sponge group and the saline/sponge group.

No proprioception (muscle contract) was confirmed until eighteen days after surgery in both groups. Proprioception has been clearly confirmed in the BM-MSCs-HGF /sponge group on Day 19 and thereafter. . Proprioception score in the BM-MSCs-HGF /sponge group (score 2) was significantly higher than that in the saline/sponge group (score 1) on day 28 after surgery. Though the results of histological examination showed that the repair was not complete in both groups, the results of evaluation of nerve function revealed a better recovery in the BM-MSCs-HGF /sponge group. It was previously reported that electrophysiologic examination in rat sciatic nerve defect model revealed little action potential in spite of no complete repair in histological observation [2]. A recovery of motor function (max 30% of normal function) accompanied with a little action potential was also observed [12]. In the result of histological examination, extension of

the axon and the presence of a number of Schwann cells indicate recovery of electric signal, resulting in recovery of motor function. Proprioception score in the BM-MSCs-HGF /sponge group was significantly higher than that in the saline/sponge group, because of fast nerve regeneration and good recovery of electric signal.

In conclusion, we have clearly shown here that implantation of gelatin hydrogel sponge incorporating BM-MSCs and HGF accelerates peripheral nerve regeneration. We expect this technique to be used in the treatment to nerve injury in cattle.

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