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Effects of Calcium Phosphate Cement on the Peripheral Nerve Fibers

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Calcium phosphate cement (CPC) is a bioactive ceramic substance. To clarify the effects of CPC on the peripheral nerve, we applied CPC on the peripheral nerve fibers of experimental animals and investigated the nerve fibers by electron microscopy and by immunoblotting analysis using an anti-myelin-associated glycoprotein (anti-MAG) antibody. The results showed that there was neither axonal sprouting at the nodes of Ranvier nor down-regulation of MAG beyond the normal level in the nerve fibers. These findings suggest that there is no harm in using CPC near the peripheral nerve fibers.

INTRODUCTION

Surgical treatments of the jaw because of a cyst, tumor, or fracture may sometimes result in large bone defects postoperatively. For the treatment of such bone defects, bone filling is frequently performed with various materials such as autogenous bone, hydroxyapatite, or polymethylmethacrylate bone cement (PBC). Recently, calcium phosphate cement (CPC), a biomaterial that is highly biocompatible, has been widely adopted as a bone substitute by many orthopedists and dentists. CPC, consisting of liquid and powder components, is able to fit the contours of a bone defect easily because it retains its fluid quality for a few minutes before hardening. The usefulness of CPC is therefore well known to medical and dental clinicians. However, it has not been clarified how CPC affects the peripheral nerves when they in direct contact with each other. In order to clarified this, in our study, we kept CPC in direct contact with the sciatic nerve. The effect of CPC was assessed by electron microscopy and immunoblotting analysis using an anti-myelin-associated glycoprotein (anti-MAG) antibody. Although MAG, a transmembrane protein of the immunoglobulin superfamily, is a minor constituent of the myelin sheath and represents approximately 0.1% of the total myelin protein in the peripheral nervous system (PNS) (1), it plays a key role in the early stages of myelination as well as in the maintenance of stable axon–myelin interactions (2). After nerve injury, MAG expression is down-regulated and the axon is loosened from the Schwann cell, a process called Wallerian degeneration. Down-regulation of MAG is also seen in chronic nerve compression (3), which is thought to lead to axonal sprouting at the nodes of Ranvier. In our study, an electron microscopy examination revealed neither loosening of the axon from the Schwann cell nor axonal sprouting at the nodes of Ranvier, while slight downregulation of MAG, in comparison with normal nerves, was demonstrated by western blotting. Thus, we conclude that there is little effect of CPC

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on peripheral nerve fibers; nonetheless, CPC should be used with care near the nerve, because all risks of neurogenic symptoms could not be eliminated during this study.

MATERIALS AND METHODS

1. Bone substitutes

(1) CPC

CPC is composed of powder and liquid components. The powder component consists of α-tricalcium phosphate (75%), tetracalcium phosphate (18%), calcium hydrogen phosphate (5%), and hydroxyapatite (2%). The liquid component consists of sodium chondroitin sulfate (5%), sodium succinate (12%), and water (83%). Both these components were mixed for 1 min at a powder-to-liquid ratio of 3.3:1 (wt/wt) before the experiments.

(2) PBC

PBC is likewise composed of powder and liquid components: the powder component consists of methyl methacrylate-styrene copolymer (75%), polymethyl methacrylate (PMMA) (15%), and barium sulfate (10%), and the liquid component consists of methyl methacrylate monomer (97.4%), N,N-dimethyl-p-toluidine (2.6%), and hydroquinone microdose (15–20 ppm). Both these components were mixed for 1 min at a powder-to-liquid ratio of 2.2:1 (wt/wt) before the experiments.

2. Animals

New Zealand white rabbits (female, 2.0 kg body weight) were used for the electron microscopy examination, and white Wister rats (female, 42–70 days old) were used for the immunoblotting analysis. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of the Kobe University School of Medicine.

3. Electron microscopy examination

After the New Zealand white rabbits had been anesthetized with an intravenous injection of Nembutal (sodium pentobarbital, 0.5 ml/kg body weight), they were fixed in the supine position. The submandibular area was then locally anesthetized with 1% lidocaine added to a 100,000 dilution of epinephrine. The mental foramen was exposed with a submandibular incision and the surrounding bone was scraped using a dental engine under continuous physiologic saline rinsing; care was taken to avoid any damage to the mental nerve (Fig.1.a). After hemostasis, the area where the bone had been scraped was filled with either CPC or PBC that directly touched the mental nerve (Fig.1.b). Once the bone substitutes had hardened, the wound was closed with 3-0 silk thread. As a control group, the area where the bone had been scraped was not filled with any substitute, and the wound was closed with 3-0 silk thread.

A total of 27 rabbits were used in this experiment; there were 9 in each of the groups described above, and 3 from each group were sacrificed at 1 week, 1 month, or 3 months after the bone filling, respectively. All animals were fixed by means of transcardiac perfusion with Karnovsky's fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. The mental area was then exposed, and the bone substitute carefully removed without damaging the nerve. The nerve segment in direct contact with the cement was excised.

All samples were immersed in Karnovsky's fixative as above for 4 h at 4°C, and postfixed at 4°C in 2% OsO4 in 0.1 M sodium cacodylate buffer for 2 h. The sections were dehydrated in a graded series of ethanol. They were then immersed in n-butyl glycidyl ether (QY-1) and embedded in Epon 812. Ultra-thin sections were cut on a LKB Ultrotome and then double-stained with uranyl acetate and lead citrate and finally examined with a Hitachi H-7100 electron microscope.

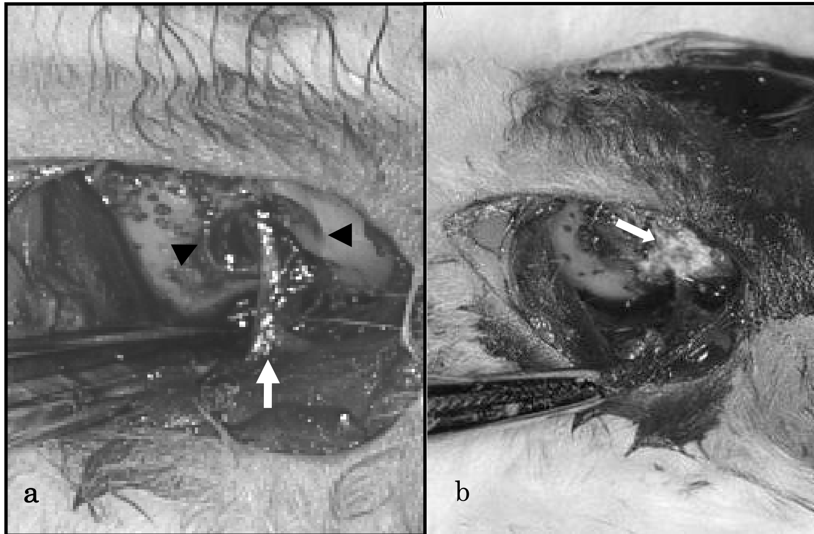

Figure 1. Animal experiment.
a: Scraping the surrounding bone (arrowheads) of the mental nerve (arrow).
b: Filling of the wound with CPC (arrow).

4. Immunoblotting analysis

The white Wister rats were anesthetized by an intraperitoneal injection of Nembutal (sodium pentobarbiturate 50 mg/kg body weight). The right sciatic nerve was exposed at mid-thigh and wrapped atraumatically in a sterile 2-mm plastic tube (internal diameter 0.7 mm) (Fig.2). The tube was filled with CPC and the wound was closed with 3-0 silk thread after the CPC had hardened. In the other group, the sciatic nerve was ligated tightly with 1-0 silk thread for 3 min instead of being wrapped in a tube. Subsequently, the thread was removed and the wound was closed with 3-0 silk thread. All rats were sacrificed at 10 days after this operation. The sciatic nerve of the operated side (right) was removed carefully to avoid damage and that of the non-operated side (left) was also removed as a control. Immunoblotting analyses were performed in a similar manner as previously reported (4,5). Fresh nerve segments dissected from the sciatic nerve were homogenized with an ultrasonic disruptor and solubilized with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) NP-40, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). The cell lysates were prepared by centrifugation at 12,000 rpm for 15 min to remove the insoluble material. The supernatants were eluted with Laemmli sample buffer. The whole cell lysates were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane filters (Immobilon, Millipore). The membranes were immunoblotted with rabbit

anti-MAG polyclonal antibodies (catalogue no.sc-15324) using a chemiluminescence reagent (Renaissance, NEN) and anti-β-actin antibody was used as a loading control.

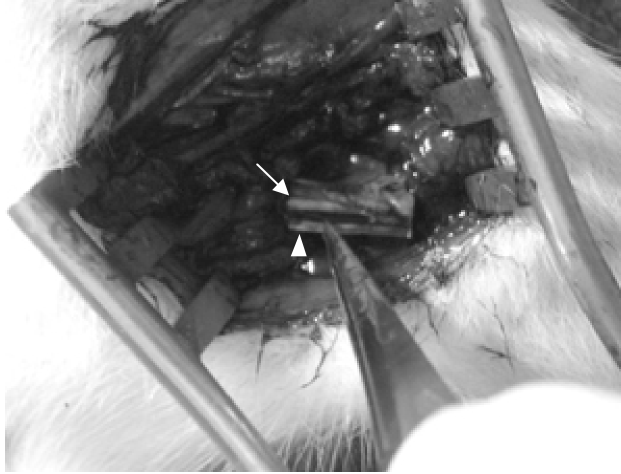

Figure 2. Animal experiment. Exposing the right sciatic nerve (arrow)and wrapping it in a sterile plastic tube (arrowhead).

RESULTS

Electron microscopy examination

It was observed that all the axons were surrounded by normal Schwann cells with tight contacts. There was no demyelination or Wallerian degeneration. The neurofilaments and microtubules in the axoplasm were ultrastructurally preserved, and axonal sprouting was not observed at the nodes of Ranvier. These findings were the same among the CPC, PBC, and control groups, and remained unchanged 1 week, 1 month, and 3 months after the bone filling (Figs.3.a–h).

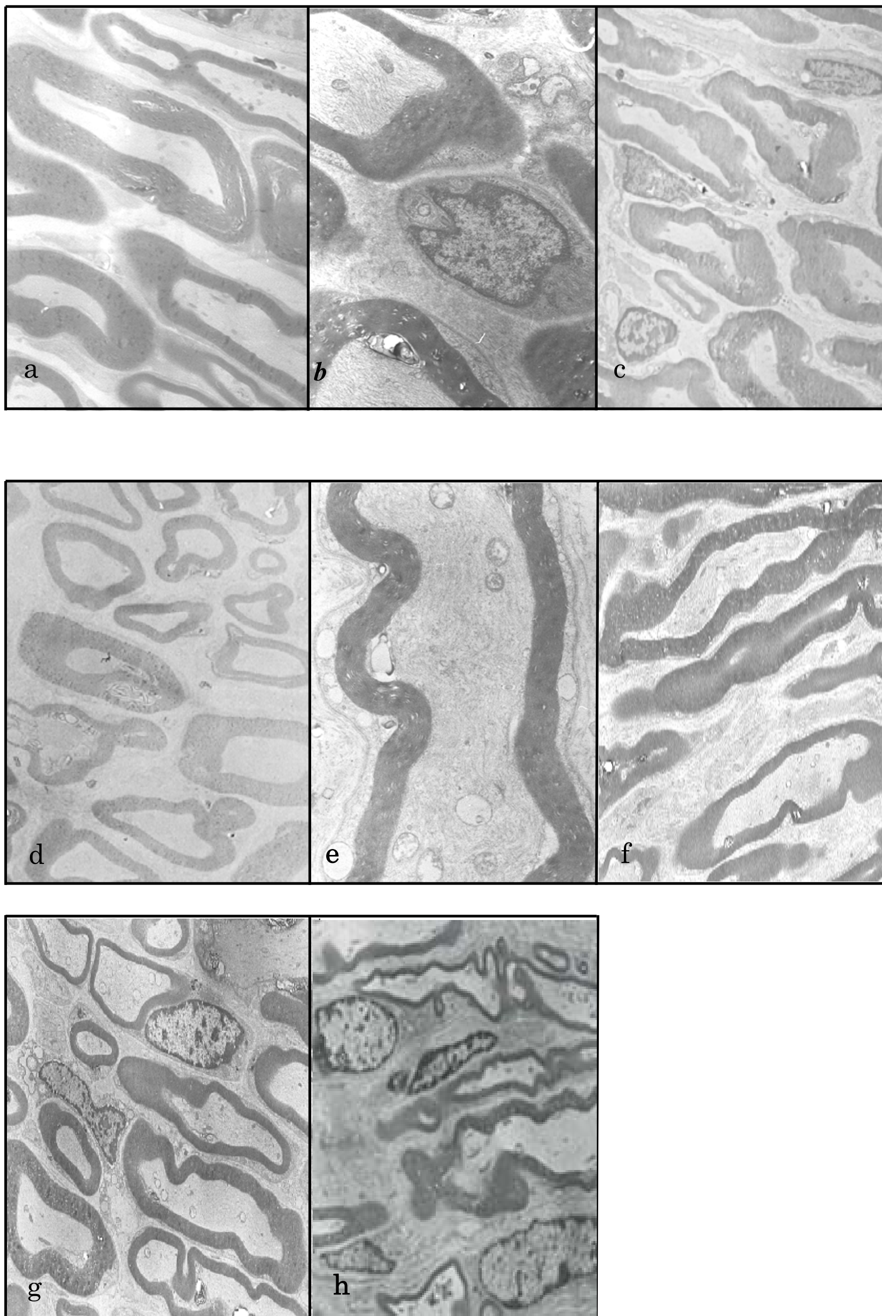

Figure 3. Findings of electron microscopy.
a: CPC group after 1 week (×4000). b: CPC group after 1 month (×7500). c: CPC group after 3 months (×4000). d: PBC group after 1 week (×5000). e: PBC group after 1 month (×12000). f: PBC group after 3 months (×6000). g: Control group after 1 week (×4000). h: Control group after 1 month (×4000).

Immunoblotting analysis

On the immunoblots of the homogenates from the rat sciatic nerve, anti-MAG polyclonal antibodies detected a band of 100 kD (Fig.4). In the lane of the ligated nerve, MAG protein level was considerably decreased in comparison with that of the normal nerve. MAG expression in the nerve that was in contact with CPC was a bit weaker than in the normal nerve.

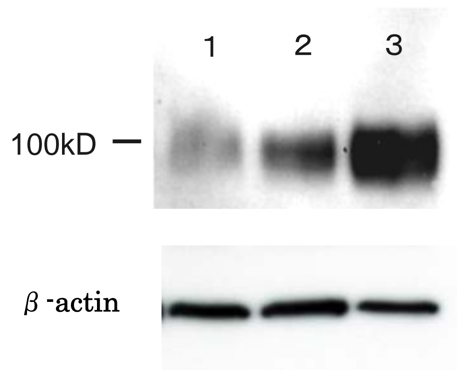

Figure 4. MAG immunoreactivity on western blots of homogenates of the rat sciatic nerves (lane 1: ligated nerve; lane 2: nerve in contact with CPC; lane 3: normal nerve). Anti-MAG polyclonal antibodies detect a band of 100 kD. MAG protein level in lane 1 is considerably decreased in comparison with that in lane 3. MAG expression in lane 2 is a bit weaker than in lane 3. Anti- β -actin antibody was used as a loading control.

DISCUSSION

Bone filling materials currently in clinical use are classified into 3 types: biotolerant, bioinert, and bioactive. PBC is a biotolerant material widely used in orthopedics because it combines mechanically with the surrounding bone by entwining with cancellous bone tissue (6). However, PBC causes some problems such as sudden hypotension by the remnant monomer entering the blood circulation, and local osteonecrosis due to its hardened heating (7). Hydroxyapatite (HA) and CPC are bioactive materials that are characterized by good biocompatibility and extensive bone conductivity; moreover, they are gradually absorbed and replaced by bone tissues (8–12). HA is only available in block or granular form, while CPC is a paste that can be fitted easily to any contour of the bone defect. However, if a nerve is located near the bone defect, CPC may be pushed into the nerve space, which may potentially damage the nerve fibers. In this study, we undertook animal experiments to clarify whether CPC is harmful to the nerve by electron microscopy and immunoblotting analysis.

The characteristics of CPC that may affect the nerve are 1) expansion on hardening, 2) heat on hardening, and 3) pH. Although PBC usually contracts on setting, CPC increases in length by less than 0.5% and in volume by less than 1% on setting (13). Polymerization of

PMMA often generates heat (14), whereas CPC becomes a hard mass through hydration without heating. The old types of α-tricalcium phosphate cement need added acid to solidify, whereas CPC solidifies without any acid, therefore, CPC has a pH of 7.1–7.6, which is the stable pH range. Consequently, it is possible that only hardened expansion of CPC affects the nerve fibers. However, in our study, there was no degeneration of nerve fibers in the ultrastructural findings of a CPC group. Therefore, we consider that such minor hardened expansion of CPC confers little damage to the nerve fibers. Moreover, hardened heat of PBC is also thought to be safe for the nerve fibers based on the ultrastructural findings of the PBC group in our study. Other possible effects on the nerve fibers are stretch stress or microangiopathy caused by perineural ablation in the experimental procedures; however, this study demonstrated that the operation itself could not damage the ultrastructure of the nerve fibers.

MAG is a key myelin specific protein as well as myelin basic protein constituents in the peripheral nervous system (3). This protein has 2 isoforms, L-MAG and S-MAG, which differ only by the c-terminal portion of their cytoplasmic domains (15,16). When the nerve has a crush injury, the expression of both isoform proteins decreases rapidly below 10% of the the normal nerve. L-MAG expression recovers to 100% of the control level by 13 d ay after injury, and S-MAG expression recovers to 100% by 20 day after injury. It has also been shown that the local down-regulation of MAG is a critical signal for the axonal sprouting response because MAG inhibits axonal outgrowth (17). In our study, MAG expression in the nerve surrounded with CPC was a bit weaker than that in the normal nerve, but was much more intense than in the ligated nerve. Since we found no axonal sprouting at the nodes of Ranvier by electron microscopy, down-regulation of MAG in the CPC group was considered to be within the normal limits.

Seddon (18) classified nerve injuries into 3 types: neurapraxia, axonotmesis, and neurotmesis. Nerve injuries diagnosed as axonotmesis or neurotmesis are the result of continuous pressing or complete cutting and can be detected histologically, whereas neurapraxia, which is characterized by a slight detrition or temporary blood-supply disorder of the nerve fibers, cannot usually be detected by electron microscopy. Consequently, our study cannot eliminate all risk causing neurogenic symptoms despite no findings of any degeneration of the peripheral nerve by electron microscopy. Further studies are needed before any definite conclusions are drawn regarding the risks of using CPC near the peripheral nerve.

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