Hydroxyapatite coated magnesium alloy for peripheral nerve regeneration

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Magnesium (Mg) has been used as a neuroprotective agent and for orthopedic and cardiovascular stent applications for a long time. Mg is a promising material for neural regeneration own to its metallic nature, biodegradability, and biocompatibility. To evaluate the benefit of hydroxyapatite (HA) coated Mg alloy (WE43) nerve conduit in peripheral nerve injury regeneration. In vitro cell adhesion and proliferation of PC12 cells and Schwann cells over pure Mg, WE43, HA coated pure Mg (HA-Mg), and HA coated WE43 (HA-WE43) disks were assessed using scanning electron microscope and EZ-CYTOX assay. In vivo study was also performed for sham, silicone nerve conduits, and HA-WE43 nerve conduits to evaluate rat sciatic nerve regeneration over 10 mm gap. These rats were observed for three months with weekly functional assessment using sciatic functional index. Regeneration was evaluated through retrograde neuron labeling and histomorphometric analysis at the end of the experiment. Non-coated pure Mg and WE43 conduits showed fast resorption rate and high gas formation. Higher adhesion and proliferation of PC12 cells were found in HA-WE43 disks. At the end of three months, HA-WE43 nerve conduit showed mild resorption without detectable gas formation in the surrounding tissue. However, only scanty regenerated neural tissue was found within the conduit. HA-WE43 nerve conduit showed controlled degradation and absence of gas formation with scanty regenerated neural tissue.

Key Words: Hydroxyapatite, Magnesium, Nerve conduit, Peripheral nerve regeneration

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INTRODUCTION

In the clinical field of oral and maxillofacial surgery practice, injury to the facial nerve and peripheral branches of the trigeminal nerve is frequently reported following avulsive trauma, tumor ablative surgeries, and routine dental procedures. Subsequently, disturbance of facial expression, communication, symmetric smiling, and eye

and mouth closure associated the facial nerve injuries, while loss of sensation (hypoesthesia, anesthesia) to the upper and lower lips, maxilla, mandible, tongue, and chin associated injuries of the trigeminal nerve [1,2]. The nerve injury can be neurapaxia, axontmesis, or neurotmesis. In neurotmesis with loss of nerve segment, bridging the gap with autologous nerve graft is the gold standard approach. However, this measure is associated with short comings

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like donor site morbidity (traumatic injury, infection & neuropathic pain), the need for specialized skills and prolonged operation time [3]. Recently, nerve conduits, either synthetic or biological derived scaffolds, have grown immensely. Their main advantages are availability and absence of donor site morbidity [3].

Magnesium (Mg) has been used for orthopedic and cardiovascular stent applications and as a neuroprotective agent for long time. It is a promising material for neural regeneration own to its metallic nature, biodegradability and biocompatibility. Though, the fast degradation nature of Mg limits its use as a biodegradable material. Furthermore, its degradation results in alkaline byproducts and hydrogen gas. Therefore, Mg degradation rate must be controlled and the harmful degradation byproducts must be eliminated, neutralized, or removed from the system to permit its neural applications. The present study was conducted to evaluate the benefit of hydroxyapatite (HA) coated Mg alloy (WE43) nerve conduit in peripheral nerve injury regeneration.

MATERIALS AND METHODS

Preliminary evaluation of pure Mg and WE43 conduits

Six-week-old male healthy Sprague-Dawley rats (SD) of 200–250 g (n=2) were used for implanting two nerve conduits of pure Mg and WE43 over 10 mm gap of sciatic nerve. The conduits were re-explored 4 weeks later to evaluate the resorption status and gas formation. The inner and surrounded grown tissue of the conduit was submitted for histological evaluation with hematoxylin and eosin staining (Fig. 1A, B).

In vitro evaluation of PC12 cells and SCs adhesion and viability when co-cultured with Mg disks

HA-coated Mg disks preparation

The WE43 fabrication and HA coating were made

Fig. 1. Preliminary evaluation of bare Mg and WE43. (A, B) Clinical photos showing implant of bare pure Mg and WE43 conduits. (C, D) Large encapsulated gas surrounding both conduits. (E, F) Histological photomicrographs of inner conduit tissue structure with inflammatory cells invasion and necrotic areas ($\times 40$ and $\times 200$ magnification). Mg, magnesium; WE43, Mg alloy.

as described previously by Lim et al. [4]. An WE43 (Mg, 3.78 wt% Y, 2.13 wt% Nd, 0.46wt% Zr) (Daeryun Co., Shanxi, China) was purchased and formed into disk shape with a milling machine (Genoss Co., Suwon, Korea). For HA coating, disks were immersed into 0.5 M ethylenediaminetetraacetic acid calcium disodium salt hydrate (Ca-EDTA) and 0.05 M potassium dihydrogenphosphate (KH_2PO_4) solution, then heat-processed at 363 K for 2 hours. The pH was maintained at 8.9 by adding sodium hydroxide (NaOH) solution. Finally, the HA-coated disks were washed with distilled water (DW) and dried with air.

Cells seeding

For in vitro evaluation, 4 disks of pure Mg, WE43, HA coated pure Mg (HA-Mg), HA coated WE43 (HA-WE43) were sterilized under ultraviolet irradiation on a clean bench for 1 day. Subsequently, rat pheocromocytoma (PC-12) cells and Schwann cells (SCs) were seeded onto the disks at a density of 3×10^4 cells using Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum containing 1% penicillin/streptomycin (Gibco Life Technologies, Grand island, NY, USA).

Cells adhesion

PC-12 cells and SCs were seeded at on the disks and allowed to adhere in standard cell culture for 4 days. Thereafter, disks were rinsed three times using phosphate buffered saline (PBS) to remove non-adherent cells. The adherent cells on disks were fixed with 2.5% glutaraldehyde at room temperature for 2 hours. Cells were rinsed 2 times with PBS and post-fixed for 1 hour in 1% osmium tetroxide. They were rinsed again with PBS and dehydrated by processing over solutions of ethanol (50%-100%). Finally they were dried using hexamethyldisilazane and imaged using scanning electron microscope (SEM) (JSM-7401; JEOL, Peabody, MA, USA).

Cells viability and proliferation assay

Cells viability and proliferation were assessed using Enhanced Cell Viability Assay Kit EZ-CYTOX (Daeil Lab, Seoul, Korea) which based on WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disul-

fonate) to react with the cell mitochondrial succinate-tetrazolium reductase to form a water-soluble formazan dye [5]. Briefly, Cells were cultured for 4 days on the previously mentioned disks. Thereafter, adhered cells were detached using 0.05% trypsin-EDTA solution and collected in standard culture medium. Cells were resuspended in 100 μL of standard culture medium per well of 96-well plate. Four wells without cells acted as a control. A total of 10 μL of EZ-CYTOX reagent was added to each well and the plate was incubated for 4 hours. Absorbance was measure at 450 nm wavelength using a microplate reader.

In vivo evaluation of HA-WE43 nerve conduit

The selection of the Mg nerve conduit type was based on the preliminary in vivo observation and the in vitro evaluation.

Conduit fabrication

Nerve conduits (HA-WE43) were made of WE43 (Mg-Y-Nd-Zr) and coated by HA with dimensions of 14 mm in length, 0.2 mm in thickness, 1.6 mm in inner diameter and 20 μm sized- porosity (Genoss Co., Suwon, Korea) (Fig. 2).

Animals and groups

Using six-week-old male healthy SD rats, weighting 200–250 g, nerve conduits were implanted over 10 mm sciatic nerve gap for evaluating nerve regeneration in each group of Sham control (n=10), Silicone conduit (n=12), HA-WE43 conduit (n=12). Care and treatment of the animals were conducted in accordance with guidelines established by Seoul National University Institutional Animal Care and Use Committee (approval No. SNU-160426-4-1).

Surgical technique

Rats were anesthetized with intraperitoneal injection of chloropent (1 mL/100 g). The left sciatic nerve was explored and 5 mm segment was resected at 5 mm proximally to the sciatic nerve trifurcation. Either silicone or HA-WE43 nerve conduit was implanted to bridge the gap. The nerve ends were pulled 2 mm inside the conduits and fixed with one epineural stitch in each end using

Fig. 2. HA-WE43 nerve conduits fabrication. (A, B) SEM image showing the outer surface with 20 μm sized pores. (C) Optic image showing bare WE43 and HA-WE nerve conduits. (D) SEM cross-sectional image of HA-WE43 nerve conduit. HA-WE43, hydroxyapatite coated magnesium alloy; SEM, scanning electron microscope.

Fig. 3. Clinical photographs of nerve conduits implantation. (A) HA-WE43 nerve conduit. (B) Silicone nerve conduit. HA-WE43, hydroxyapatite coated magnesium alloy.

9-0 Nylon (Ethicon, Livingston, UK) under a surgical microscope (Carl Zeiss, Oberkochen, Germany). Conduits were flushed with heparinized saline before wound closure. Muscle layer was approximated with 4/0 Vicryl (Ethicon) and skin was closed using 4/0 Dafilon (B. Braun, Barcelona, Spain). Nerve regeneration was assessed over a 12-week interval (Fig. 3).

Gait analysis with sciatic functional index (SFI)

Pre- and postoperative footprints were recorded weekly until the end of the experiment interval as mentioned in a

previous report [6]. Briefly, for each footprint, print length (PL, or the longitudinal distance between the tip of the longest toe and the heel), toe spread (TS, or the distance between the first and fifth toes), and the intermediate toe spread (IT, or the distance between the second and fourth toes), both in the normal (N) and the experimental (E) paws were all measured. Based on these parameters, the SFI was calculated according the formula modified by Bain et al. [7].

$$\text{SFI} = -38.3 \left(\frac{\text{EPL} - \text{NPL}}{\text{NPL}} \right) + 109.5 \left(\frac{\text{ETS} - \text{NTS}}{\text{NTS}} \right) + 13.3 \left(\frac{\text{EIT} - \text{NIT}}{\text{NIT}} \right) - 8.8.$$

SFI values around -100 indicate total loss of function whereas values around 0 indicate normal function.

Retrograde labeling and quantification of neurons

After 12 weeks, 6 rats of each group were used for retrograde labeling and counting of back-labeled sensory neurons as described by Geremia et al. [8] and Alrashdan et al. [9]. Briefly, five sciatic nerves in each group were labeled with 4% Fluorogold (FG) (Fluorochrome, LLC., Denver, CO, USA), while one rat served as a negative control using DW. Sciatic nerves were sharply cut 5 mm distal to the distal end of the conduit and soaked in 4% FG for 20 minutes in a baseline well. The wound was closed and rats were placed back in their cages.

5 days later, the animals were anesthetized; transcardially perfused with 1% heparinized saline and fixed with 4% paraformaldehyde solution. L4, L5, and L6 dorsal root ganglions (DRG) were harvested and serially frozen sectioned into 20- μ m-thick sections using a Cryo-Cut microtome (Leica CM3050S Cryostat; Leica Microsystems, Wetzlar, Germany). A laser scanning confocal microscope (CLSM, LSM700; Carl Zeiss) was used to capture images of DRG sections. For quantification the back-labeled neurons, the largest three area sections from DRG were selected and the labeled neurons were counted, averaged and compared across the groups [10].

Histomorphometric analysis

After 12 weeks, 6 rats from each group were used for histomorphometric analysis. Sciatic nerves were re-exposed and conduits were harvested along with the inner regenerated nerve. The regenerated nerve segment was freed and immediately immersed into a fixation solution containing 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C for 24 hours. The regenerated nerve sample was transversely cut at the center and post-fixed with 2% osmium tetroxide. Subsequently, it was routinely processed and embedded with Epon 812 (Nisshin EM, Tokyo, Japan). Serial sections of 1 μ m thickness were cut with microtome and stained with 1% toluidine blue for light microscopy examination. Images were captured using a specialized system, SPOT RT-KE color mosaic, and digitized by SPOT software ver. 4.6 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

For simplifying axon counting, the total cross-sectional area of the nerve was measured at $\times 40$ magnification and three sampling fields were randomly selected at $\times 200$ magnification. Mean fiber density was calculated by dividing the total number of nerve fibers within the sampling field by its area (N/mm^2). Total fibers number was estimated by multiplying the mean fiber density by the total cross-sectional area of the whole nerve cross section assuming a uniform distribution of nerve fibers across the entire section.

Statistics

Data analysis was carried out using IBM SPSS statistics ver. 23 software (IBM Co., Armonk, NY, USA). All data were presented as mean with standard deviation of the mean. For nonparametric, data comparisons were performed with One-way ANOVA on ranks (Kruskal-Wallis) test. For parameter data, ANOVA test followed by hoc test was used to compare data between different groups. *p*-values less than 0.05 were considered statistically significant.

RESULTS

High gas formation in the preliminary pure Mg and WE43 conduits

When the preliminary implanted pure Mg and WE43 conduits were re-explored 4 weeks after implantation, they were partially degraded and a large encapsulated gas was found surrounding each conduit (Fig. 1C, D). The histologically examined inner and surrounded tissue revealed a fibrous tissue with invasive inflammatory cells and absence of any inner regenerated neural tissue (Fig. 1E, F).

In vitro findings

Cells adhesion as revealed by SEM

PC12 cells were found well adhered on HA-WE43 and HA-Mg disks (Fig. 4A, B). Other than that, neither PC12 cells nor SCs could be visualized on the remaining disks.

High proliferation of PC12 cells on HA-WE43 disks

As assessed by EZ-CYTOX assay, modest SCs proliferation was detected as compared to the control group with no difference across the various disks. In contrast, PC12 cells proliferation was higher particularly on HA-WE43 disks where it was significantly higher than Mg and WE43 disks (Fig. 4C).

In vivo evaluation of HA-WE43 nerve conduit

Gait analysis with SFI

Preoperative SFI showed normal gait in all groups' rats. While no change in sham group, a drop in SFI to around -90 to -80 in the first postoperative week was noticed and remained highly unchangeable till the 12th week postoperatively. No actual motor function recovery could be observed in all rats except in two rats in the silicone group where one recovered partially and the other recovered almost completely. SFI mean was -78.62 ± 21.97 for silicone group and 72.37 ± 18.74 for HA-WE43 group (Table 1).

Conduits degradation and gas formation

At 12 weeks after implantation, conduits showed a mild degradation and became thin but maintained a well-integrated structure. No gas formation could be observed in the surrounding tissue of the conduit. Grossly, silicone conduits showed a well regenerated nerve within the conduit, but a scanty neural tissue was found within the HA-WE43 conduit (Fig. 5).

Retrograde labeling and quantification of neurons

The retrograde labeled neurons were significantly lower in HA-WE43 group in comparison with the sham and silicone groups. The mean of retrograde labeled neurons was 39.09 ± 22.14 , 33.91 ± 17.86 , and 11.80 ± 6.59 for sham, silicone and HA-WE43 groups, respectively (Fig. 6).

Histomorphometric analysis

Sham and silicone groups showed a higher axon density with significant difference against HA-WE43 group. Sham group revealed a higher axon density, while silicone

Fig. 4. SEM images of PC12 cells adhesion on Mg disks. (A) HA-Mg disk. (B) HA-WE43 disk. (C) Neural cells viability and proliferation on Mg disks. Higher PC12 proliferation on HA-WE43 and HA-Mg disks. SEM, scanning electron microscope; Mg, magnesium; HA, hydroxyapatite; SCs, Schwann cells. * p -values < 0.05 vs. Mg and WE.

Table 1. Results of SFI, axon density and retrograded labeled neurons

Group	SFI	Axon density (fibers/mm ²)	Total fibers	Retrograde labeled neurons
Sham	-6.99±2.86	8,795.66±2,034.28	10,485.05±7,369.65	39.09±22.14
Silicone	-78.62±21.97	6,975.05±347.90	13,021.32±1,718.08	33.91±17.86
HA-WE43	-72.37 ±18.74	1,184.16±1,825.20	1,198.19±1,795.06	11.80±6.59

Values are presented as mean±standard deviation.

SFI, sciatic functional index; HA-WE43, hydroxyapatite coated magnesium alloy.

Fig. 5. Clinical photos of nerve re-exploration. (A) HA-WE43 with no gas formation and mild resorption. (B) Removal of regenerated nerve within HA-WE43 conduit. (C) Regenerated nerve of 10 mm with well-formed wall but defective lumen. (D) Silicone conduit with well regenerated lumen. (E) Removal of regenerated nerve within silicone conduit. (F) Well-formed nerve of 10 mm. HA-WE43, hydroxyapatite coated magnesium alloy.

Fig. 6. Fluorescent photomicrographs for retrograde labeled neurons within L4-L6 dorsal root ganglions which were significantly lower in HA-WE43 group in comparison with the sham and silicone groups. (A) Sham group. (B) Silicone group. (C) HA-WE43 group. HA-WE43, hydroxyapatite coated magnesium alloy. Scale bare=200 μm.

had a higher total fibers number. For HA-WE43, three conduits out of six showed no fibers at all, while in the remaining three conduits the axons were few and narrow

in diameter. The mean of axon density was $8,795.66 \pm 2,034.28$, $6,975.05 \pm 347.90$ and $1,184.16 \pm 1,825.20$ fiber/mm² for sham, silicone and HA-WE43, respectively. While

Fig. 7. Histological microphotographs for regenerated axons where Sham and silicone groups showed a higher axon density with significant difference against HA-WE43 group. (A, B) Sham group (C, D) Silicone group. (E, F) HA-WE43 group. HA-WE43, hydroxyapatite coated magnesium alloy. Scale bare=200 μ m.

total fiber number was $10,485.05 \pm 7,369.65$, $13,021.32 \pm 1,718.08$, and $1,198.19 \pm 1,795.06$ for sham, silicone and HA-WE43, respectively (Fig. 7).

DISCUSSION

As shown in the preliminary study, bare Mg or WE43 were susceptible to rapid degradation and severe gas formation which countered any neural regeneration. HA-WE43 was reported to be biocompatible with no toxicity as revealed by hematological test following placement of HA-WE43 screws in rabbit's tibia [4]. The present study's use of HA-WE43 indicated its benefit in providing a well-controlled degraded conduit without any observable accumulated gas. Such findings go in accordance with Lim et al. [11] study of plates which were inserted above the frontal bone and proved the absence of any gas formation, inflammation, infection, wound dehiscence, and/or plate exposure over 12 weeks interval, and such period is the minimum required time for neural regeneration following neurotmesis [12]. The in vitro evaluation showed

a favorable growth of PC12 cells on HA-WE43 disk. Unfortunately, the same high growth was not observed in case of SCs. The in vivo results revealed the formation of scanty neural tissue that made of integrated tissue wall but of partially hollow and defected lumen. In presumption to explain this pitfall of HA-WE43 nerve conduit to regenerate neural tissue, is the presence of rough surface that could prevent a proper adhesion of the cells, particularly SCs. Another possible explanation is the lack of flexibility in HA-WE43 nerve conduits which is unfavorable in the body areas of motion as it could apply a pulling tension on both regenerating nerve stumps. Surface modification of WE43 can be achieved using various materials. Recently, a micro-textured HA and poly (l-lactic)-acid polymer composite coated layer on Mg implant was introduced. Such material may provide the required biocompatible surface for SCs and flexibility for the conduits [13].

In conclusion, HA-WE43 nerve conduit showed a very slow controlled degradation and absence of gas formation in the surrounding tissues with scanty neural tissue regeneration.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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