MICROCRACKS DESIGN AND BONE INDUCTION OF SKULL BONE MODIFIED BY ULTRASONIC TREATMENT USING ACIDIC ELECTROLYZED WATER

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Abstract (use 9pt): Healthy bone has many physiological microcracks, which may be involved in the release of bone matrix-derived factors and act to accelerate bone-remodeling process. In this study, the mouse parietal bone fragments (5x5x1mm³) were demineralized by acidic electrolyzed water (AEW: pH2.7) or distilled water (DW: pH5.2) at 120W and 38KHz for 20 min. Each bone was implanted into subcutaneous tissue of 10 week-old male nude mouse, and explanted at 4 and 6 weeks. AEW-bone showed clear enlargement and union of cracks on SEM. AEW-bone revealed active bone induction over wide areas at 6 weeks, while DW-bone induced new bone in limited area. We concluded that the AEW-bone had better performance in bone induction than the DW-bone. Our micro-damage technique combined with AEW and the ultrasonic irradiation will contribute to improve surface area and 3D structure of the dense bone and promote bone formation in the initial stage for bone remodeling.

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INTRODUCTION

Surface of bone consists of physiological microcracks and trabecular fractures¹. The microcracks and fractures on the surface of the bone are believed to be sites of active remodeling². It is well known that bone induction occurred predominantly in demineralized bone matrix (DBM)³⁴⁵. We believe surface area and 3D interconnected porous structure are supreme factors for better cellular performance in bone induction and conduction.

It was reported that a three chambers-double in-type electrolytic system can easily produce electrolyzed water (acidic and alkaline) that has excellent sterilizing and cleaning effects and it has been applied for dental, medical and food-processing fields⁶⁷. Acidic electrolyzed water (AEW) was effective for dissolution-precipitation of bone hydroxyapatite crystals⁸. Dissolution by AEW can be assisted by ultrasonic irradiation using high frequency sound waves. Ultrasonic waves can bring bubble cavitation and make hot spots. Ultrasonic treatment may be an effective wet synthesis technique for surface modification by biomimetic characterization of biomaterials.

We assume that the physiological cracks (so called micro damages) should be involved in the release of bone matrix-derived factors and accelerations of initial bone formation. The aim of this study is to estimate the bone-inductive capability of skull bone treated with ultrasonic irradiation in the AEW (pH 2.7) or DW (pH5.2) in subcutaneous tissue of nude mice.

MATERIALS AND METHODS

Preparation of Acidic Electrolyzed Water (AEW)

Saturated sodium chloride (NaCl) solution was continuously and effectively electrolyzed at 9.1 V and 9.0 A under a flow rate of 4200 cm³ min⁻¹ by the 3 chambers-double in-type electrolytic system⁶⁷. AEW (pH 2.7) was collected from the anode region in the system. [Figure 1]

Preparation of Experimental Samples

Male nude mouse of 10 weeks old were used in this study. Nude mouse were purchased from Hokudo Co. Ltd, Japan. Experimental animals were sacrificed
by inhalation of excessive dose of ether. Full thickness skin flap including periosteum was excised over the parietal region. Parietal bone was exposed and harvested avoiding suture area with adjacent bones. Harvested bone was prepared into 5x5x1 mm^3 size. The bone fragments were treated with ultrasonic irradiation at 120W, 38 kHz for 20 min at pH 2.7 (AEW-bone group) or 5.2 (DW-bone group). Each bone was implanted into subcutaneous tissues of posterior abdominal wall in male nude-mouse of 10 weeks old. Implantation was carried out under general anesthesia (Nembutal® 50mg/kg). Explantation of the specimen was done at 4 and 6 weeks by sacrificing the mouse with excessive inhalation of Ether. The tissues were fixed, demineralized, embedded in paraffin, sectioned and stained with Hematoxylin and eosin (HE). The specimens were observed by scanning electron microscope (SEM) and optical microscope before and after implantation. Animal experiments were approved by Animal Research center of Health Sciences University of Hokkaido, Japan (Authorized No 109). All surgical procedures were performed in sterile conditions.

RESULTS

Scanning Electron Microscope (SEM) Findings

AEW-bone showed many, deeper and distinct microcracks, with evidence of collagen fibers arranged inside the cracks about 50-100 µm in length. The surface irregularities of superficial acid soluble collagen and inorganic minerals were cleared. In contrary, DW-bone exhibited microcracks that were long but shallow with size ranging from 10 to 20 µm. Roughened surface was observed as a result of ultrasonic treatment. Furthermore, the specimens that received no treatment exhibited micro cracks of about 5 µm in length. [Figure 2]

Histological Findings

Histological picture of treated bone of both groups showed increased number and size of marrow space and newly formed osteoblast. Marrow space and outer surface of the implant material were covered by newly formed osteoblasts at 4 weeks [Figure 3].

AEW bone showed uneven thickness, numerous bony protuberances on the outer surface, and

FIGURE 1. Diagrammatic representation of three chamber double in electrolytic system. Electrolysis of sodium chloride (26.5% NaCl) solution was continuously and effectively carried out at 9.1V and 9.0A under the flow rate of 4200cm^3.min^-1. AEW of pH 2.5 to 6.5 in anode region and alkaline electrolyzed water of pH 8.0 to 11.0 in cathode region was separately collected from this device.
osteoblast lining on marrow space and cortical surface at 4 weeks after implantation [Figure 4].

Numerous irregular branching of newly formed bone extending into the connective tissue at 6 weeks were seen. Anastomosing trabeculae of newly formed bone with abundant and haphazardly placed osteocytes along with blood vessels and osteoblast were observed. These newly formed bones can be seen with new marrow space filled with osteoblastic differentiation [Figure 5].

On the other hand, DW-bone showed empty lacunae in the old bone with presence of new osteocytes in limited area representing localized new bone formation. In other areas, where active regeneration was not present, marrow space was filled with fibrous or fatty tissue. [Figure 6]
FIGURE 4. AEW bone at 4 weeks after implantation (HE)

◆: osteoblast lining; ★: bony protuberance

FIGURE 5. AEW bone at 6 weeks (HE)

★: new bone; ◆: osteoblastic differentiation; m: muscle

FIGURE 6. DW-bone at 6 weeks (HE)

★: new osteocytes; ◆: empty lacunae
DISCUSSION

The maintenance of normal bone mass depends on a dynamic balance between bone resorption by osteoclasts and bone formation by osteoblasts. Among many factors involved in new bone formation, release of proteins and growth factors have been gaining attention recently. Growth factors such as insulin like factors, fibroblast growth factors, and platelets derived growth factors are involved in driving osteoprogenetor proliferation and differentiation. Because of their ability to induce de-novo bone formation at ectopic sites, bone morphogenetic proteins (BMP), have been extensively studied in vivo and in vitro. It was suggested that the release of BMP was accelerated in partial demineralized implant material. Our result is consistent to the previous one. We found a number of areas of new bone formation in AEW group increased as compared to DW-group and untreated group.

Hydroxyapatite (Hap) component of bone may hinder the bioavailability of growth and differentiation factor. In studies examining the relationship between residual calcium and osteoinductivity of DBM, Zhang and colleagues determined that samples containing approximately 2% residual calcium had the highest osteoinductivity of DBM, thereby highlighting the importance of partial dissolution of HAp. In this study, we were able to create partially dissolved bone graft material by the use of 3 chambers-double in-type electrolytic system. SEM result of the treated bone clearly showed the partial dissolution of the HAp crystals with visible collagen fibers in the AEW group. Histological results supported the SEM results. SEM evidence of less and shallow microcracks coincided with histological findings of lower osteogenic activity in untreated bone. SEM of AEW-bone showing clear enlargement and union of cracks were supplemented by HE findings of change in bone size, suggestive of higher osteogenic activity. The evidence of new bone with newly formed narrow space with presence of osteoblastic differentiation may lead to new bone formation. From these findings, it can be clearly stated that, ultrasonic irradiation with AEW, can effectively bring about partial demineralization of skull bone and microcracks, as a result there is increased surface area as compared to DW-treated and untreated normal bones.

Supersonic irradiation with AEW can bring about surface modification of hard tissues such as bone and dentin. It is recently shown that the surface area, tube diameter and length increase in dentin treated by ultrasonic irradiation with AEW in time dependent manner. Result from our experiment was consistent to this finding. We could observe the increase in the microcracks size in the bone treated with ultrasonic irradiation as compared to untreated normal skull bone. The microcracks or porosity developed after this procedure may help to permeate the body fluids and aid in the process of mobilization of BMPs and other growth-related proteins.

CONCLUSION

Microcracks were created on the skull bone modified by ultrasonic treatment using acidic electrolyzed water. Partial dissolution of cortical bone might be an effective procedure for mobilization and release of BMPs and growth factors. We concluded that the AEW-bone had better performance in bone induction than the DW-bone. Our micro-damage technique with the combination of the AEW and the ultrasonic irradiation will contribute to improve surface area and 3D structure of the dense bone and promote bone formation in the initial stage for bone remodeling.

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