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Can Decalcified Nacre Induce New Bone Formation?

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Abstract: Undecalcified nacre has been proved to have the potential to stimulate new bone formation but the decalcified nacre has not been demonstrated so far. Does the decalcified nacre have osteoinductive potential? We design an experiment to test this issue. In our study, a group of EDTA-decalcified nacre chips, which were previously immersed in an alpha modified of Eagle's medium, were cultured respectively with 0.2 and 0.4 million of osteoblasts. The second group of EDTA-decalcified nacre chips, which were previously immersed in a Dulbecco's phosphate buffered saline (without Ca^{2+} and Mg^{2+}), were cultured correspondingly with 0.3, 0.6, 0.9 and 2.0 million of osteoblasts. We demonstrated that new material was found at the nacre-osteoblast interface within 72 hours in the first group of 0.4 million of osteoblasts and in the second group of 0.9 and 2.0 million of osteoblasts. Therefore, we suggest that the decalcified nacre should have certain inductive potential for bone formation and that the pretreatment with alpha modification Eagle's medium might facilitate the formation of bone.

Key words: nacre, osteoblast, decalcification, osteoinduction, calvarium

Introduction

We show in this study our methods on the preparation of decalcified nacre and the possibility of using decalcified nacre as a bone substitute. There are several types of bone grafts used for clinical purpose, but problems remain to be solved. Ideal bone substitutes should be cheap, biocompatible, biodegradable, osteoinductive, free of transmission, and easy to be obtained. Autografts have good biocompatibility but append unwanted wounds in patients. Allografts and xenografts still have problems of transplantation rejection and limited sources of donors. Other substitutes of bone grafts, such as coralline, ceramics, and hydroxyapatite, may have acceptable biocompatibility but are neither biodegradable nor osteoinductive. Growth factors, such as bone morphogenetic proteins (BMPs), show the potential to induce new bone formation, but the clinical application of them is difficult at present. However, recent studies show that nacre seems to be an ideal bone substitute.

Nacre, the mother-of-pearl, is the inner layer of a shell. The inorganic component of nacre contains mainly calcium carbonate, which arranges in an aragonite form. Camprasse in Lopez's team first implanted the undecalcified nacre of a marine oyster (*Pinctada maxima*) into human bone in 1990. He found osteogenesis around the block of nacre and good welding of the bone to the nacre[4]. Lopez and her co-workers subsequently made block or powder of undecalcified nacre from the *Pinctada*

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maxima and use them for their later researches in vivo and in vitro[1,2,5-7,9,12]. Since all of the subsequent researches reinforced the initial findings, Lopez and her colleagues stated their hypothesis: the nacre has good biocompatibility, stimulates bone formation, and is suitable for clinical use[2]. Peter Westbroek and Frederic Marin[16] also described the possibility of nacre to be a good substitute for bone grafts from the viewpoint of evolution and scientific researches. Furthermore, Liao et al. found that the nacre of Margaritifera shell is a biocompatible, biodegradable, and osteoinductive material[8]. They implanted granules of undecalcified nacre of freshwater Margaritifera shell into the femoral sites and the back muscles of rats and find degradation of the undecalcified nacre and new bone formation around the nacre. The published papers revealed the successful use of undecalcified nacre, but the use of decalcified nacre has not been demonstrated so far. Here we report a possibility for inductive potential of bone formation by the decalcified nacre.

Materials and Methods

The shells of freshwater *Hyriopsis schlegeli* were collected from a pearl nursery near the Biwa Lake in Japan. We removed the outer prismatic layers of the shells with a handpiece (XL-150, Osada, Japan). To prevent overheating, we irrigated the shell with cool water. After air-drying, store the nacre at −80℃.

The nacre was pretreated in a solution of 5% acetic acid (Nacalai Tesque, Inc., Japan) for 24 hours at 4℃, and then washed in distilled water for 150 minutes at 4℃. Decalcify the nacre in a solution of pH 8 and 20% ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Inc., Japan) for 9 days at 4℃. Change the EDTA solution every 3 days. Take the decalcified nacreous membranes and wash the membranes with distilled water at 4 ℃. Change the distilled water hourly for three times. Freeze the nacreous membranes overnight at −80℃. Freeze-dry the nacreous membranes for two days by a freeze dryer (IWAKI™ Freeze Dryer FRD-50M, Asahi Techno Glass Inc., Japan). The decalcified nacreous membranes were then packed and sterilized by ethylene oxide gas. After sterilization, we cut a decalcified membrane into small chips of less than 1mm under aseptic manipulation. Prepare six plastic dishes (55㎠, coated with type one collagen; IWAKI™, Asahi Techno Glass Inc., Japan) and place one piece of the membranous chips to each dish.

Before culturing with osteoblasts, we immersed two of the six nacreous chips with one drop of culture medium, which contained an alpha modification of Eagle's medium (MEM; Cellgro®, Mediatech Inc., USA) and was for culturing osteoblasts in next steps. The other four nacreous chips were immersed in one drop of Dulbecco's phosphate buffered saline without calcium and magnesium (PBS; Nacalai Tesque, Inc., Japan). The procedure was facilitated by sterilized Pasteur pipettes (Chase Instrument Corp, USA). To retain the nacre chips at the bottom of dishes, we placed the dishes overnight in a drying oven (DS 44, Yamato Scientific Co., Japan) at 37℃.

The osteoblasts (MC3T3-E1, 1.5 million cells per vial) were taken from the calvaria of newborn mouse and were supplied by RIKEN Gene Bank. The culture medium was an MEM containing 10% fetal bovine serum (SBS Pty. Ltd, Australia) and 1% L-Glutamine (BioWhittaker Molecular Applications, USA). We cultured the osteoblasts for three generations to wash out the antifreezer (10% dimethyl sulfoxide) and to increase the amount of osteoblasts. We counted the fourth generation of osteoblasts with a Burker-Turk deep counter (Erma Tokyo, Japan) after three weeks. Transfer 0.2 and 0.4 million of osteoblasts into the two dishes with medium-immersed nacre and 0.3, 0.6, 0.9, and 2.0 million of osteoblasts into the other four dishes with PBS-immersed nacre, respectively. In each dish, 10ml of culture medium was

also added. The medium was administered slowly from the rim of each dish to prevent floating the nacre chips. The six cultures were then maintained in a humidified atmosphere of 95% air and 5% CO2 at 37℃. The culture medium was changed every three days. The outcome was observed by an inverted light microscope (ECLIPSE TE300, Nikon, Japan) and was recorded by photos at different intervals of time.

We chose the 72nd hour as a favorable time to determine whether the new material is formed or not. This was because our first time to change the medium was 72 hours later. The osteoblasts could attach the nacre chips more firmly at the time and we could change the medium without floating the nacre chips.

Results

This experiment revealed that whitish new material was formed by osteoblasts and was around the nacre (Fig. 1-4). The time, at which the osteoblasts started to produce the whitish material, was earlier in the dishes with more osteoblasts (Table 1). Table 1 also shows that the new material was formed within 72 hours in PBS-immersed-nacre group when the decalcified nacre was cultured with more than 0.9 million of osteoblasts. Furthermore, in medium-immersed-nacre group, as few as 0.4 million of osteoblasts could form whitish material in 72 hours.

Discussion

It is evident that the demineralized bone matrix can induce ectopic bone formation[13]. The purpose of demineralization is to present more osteoinductive matrix to the surrounding mesenchymal cells and to provide invading tissues with more space[3,13]. Therefore, we supposed that the decalcified nacre should have similar function.

Although the uses of undecalcified nacre of *Pinctada maxima in vivo or in vitro* are well published[1,2,4-7,9,12,16], however, one of the papers demonstrates that the undecalcified nacre stimulates the osteoblasts to form a material similar to nacre *in vitro*[9]. To eliminate the disturbance of this fact, we use decalcified nacre. The preparation and the application of decalcified nacre have not been reported yet. In this study, we demonstrated whether the decalcified nacre, which was made from freshwater *Hyriopsis schlegeli*, has the ability to stimulate osteoblasts to produce new material. We believe this to be the first report about the use of decalcified nacre *in vitro*.

The use of decalcified nacre also has several advantages. Firstly, if the matrix of nacre has osteoinductive potential, we can state that the osteoinductive material exists in the matrix. Secondly, the use of undecalcified nacre in vivo might

Table. 1. The decalcified nacre in the media of different amounts of osteoblasts induces new material formation at different intervals of time.

Million of Cells (Pretreated Solution)	Observation Intervals (Hours)	New Material Found (Hours)
2 (PBS)	0.5, 1, 2, 4, 8, 12, 18, 24, 36, 48, 60, 72, 96, 120, 156, 204	48
0.9 (PBS)	4, 24, 48, 72, 96, 144	72
0.6 (PBS)	4, 24, 48, 72, 96, 144	96
0.4 (Medium)	4, 24, 48, 72, 96, 144	72
0.3 (PBS)	4, 24, 48, 72, 96, 144	144
0.2 (Medium)	4, 24, 48, 72, 96, 144	Not observed*

PBS: Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+}.
Medium: alpha modification of Eagle's medium+10% fetal bovine serum+1% L-Glutamine
*In this group, our observation stopped at the 144th hours. The new material was not found within 144 hours.

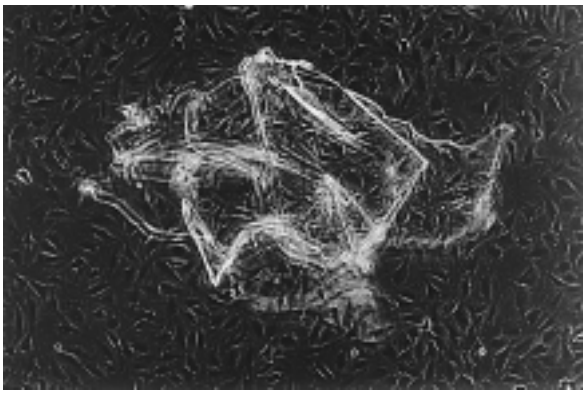

Fig. 1. Culture for four hours. The decalcified nacre is surrounded by 0.9 million of osteoblasts. No whitish material is found. (100x)

Fig. 3. Culture for two hours. The decalcified nacre is surrounded by two million of osteoblasts. Whitish material is not formed yet. (100x)

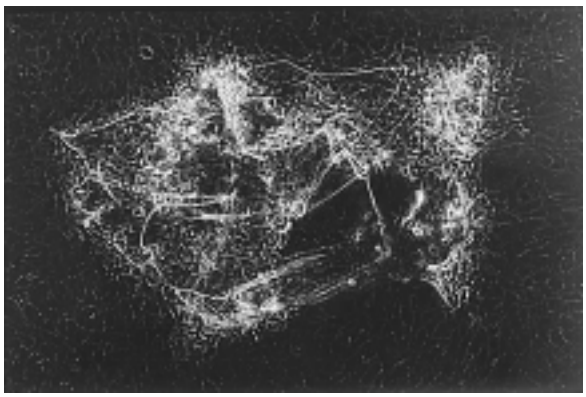

Fig. 2. Culture for 144 hours. In a culture of 0.9 million of osteoblasts, the whitish material is found around the nacre chip. (100x)

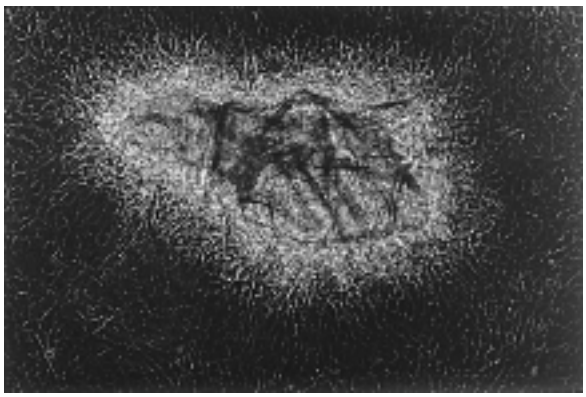

Fig. 4. Culture for 204 hours. In a culture of two million of osteoblasts, the whitish material is found around the nacre chip. (100x)

encounter a similar problem to the undecalcified bone. The remodeling phase would be prolonged due to the resorption of undecalcified part by osteoclasts. The decalcified nacre seems to be superior to decalcified one from this viewpoint. Thirdly, when the matrix of nacre is osteoinductive, the presentation of more matrixes to the osteoblasts would facilitate the osteoinductive process.

In our study, the pretreatment of MEM (alpha modification of Eagle's medium) or PBS (Dulbecco's phosphate buffered saline without calcium and magnesium) was used to hold the decalcified nacre at the bottom of dishes. Both MEM and PBS were used during the culturing process and, therefore, they might not be the additional interfering factors. The evidence was that the MEM-pretreatment of decalcified nacre could assist the formation of new material and was superior to the PBS pretreatment. One explanation might be that MEM occupies the air-filled space of decalcified nacre and the nutrient environment facilitates the ingrowth of osteoblasts. Another explanation might be that PBS would activate the endogenous enzymes to degrade the osteoinductive components in the matrix of nacre as in the matrix of bone[14]. However, the osteoinductive components of nacre have not been identified. It also needs further investigation to clarify these mysteries.

The decalcified method in our study was proved feasible. In an alpha modification of Eagle's medium, osteoblasts are confluent at the bottom of a

plastic dish but do not form any whitish material. Contrarily, when the decalcified nacre and the osteoblasts were cultured simultaneously in an MEM, the osteoblasts produced whitish material. This indicated that the decalcified nacre would induce osteoblasts to form new material. There was only one type of cells, osteoblasts, in the culture medium, so the new material should be produced by the osteoblasts. Although the whitish material was produced by the osteoblasts and was possible to be bone, however, we did not demonstrate that in this study. To demonstrate the whitish material, we should perform the energy dispersive X-ray microanalysis (EDX) for the inorganic components (such as calcium and phosphorus) and immunohistochemical stains for the organic components (such as collagen type I and osteonectin). We will reveal this unclear point in our proceeding papers.

Four components of matrix (Lustrin A, perlucin, perlustrin, and mucoperlin) have now been identified from different mollusks but their roles on the mechanism of mineralization are still unclear. Lustrin A is supposed to be a structure protein of the nacre of *Haliotis rufescens*[11]. The perlucin and perlustrin may participate in the biomineralization of sodium bicarbonate in the shell of *Haliotis laevigata*[15]. The nacreous mucoperlin of *Pinna nobilis* may play a role on the termination of mineralization and has similar molecular structures to mucins, which are secreted by epithelial cells in the gastrointestinal tract or the genitourinary tract[10]. According to these facts and the result of this study, it seems that the matrix of nacre have the ability to initiate mineralization and can be prepared in accordance with similar principles to the preparation of demineralized bone.

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脱灰真珠層は新生骨を誘導できるか

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無脱灰の真珠貝は新生骨の形成を活性する能力を持っていることが証明されている．しかし，脱灰した真珠貝についてはまだ検討がなされていない．脱灰した真珠貝は骨誘導能を有するか，われわれはこの点を検討するための実験を行った．

本実験において，第１グループでは alpha　Eagle 変法培養液に浸した，EDTA で脱灰済みの真珠片を0.2および0.4（10×10^5）個の骨芽細胞の中に入れ，各々培養した．第２グループにおいてはリン酸緩衝溶液（Ca イオンおよび Mg イオンを含まない）に浸した，EDTA で脱灰済みの真珠片を0.3，0.6，0.9，2.0（10×10^5）個の骨芽細胞の中に入れ，各々培養した．その結果，第１グループにおいては0.4（10×10^5）個の骨芽細胞を含む培地において，第２グループにおいては0.9（10×10^5）個および2.0（10×10^5）個の骨芽細胞を含む培地において72時間以内に真珠層と骨芽細胞の接触面に新生物質の形成が認められた．脱灰真珠貝は骨誘導能を持っておりまた，alpha Eagle 変法溶液は骨形成を促進することが示唆された．