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Effects of Decalcified and Undecalcified Nacre of Freshwater Mollusk *Hyriopsis schlegeli* on Mouse Osteoblasts (MC3T3-E1) to Induce Osteoid *in vitro*

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Abstract: We tested the nacre of the freshwater mollusk *Hyriopsis schlegeli* for its ability to induce the osteogenesis in the present study. Powdered EDTA-decalcified (0.3-1mm) and undecalcified nacre (300-425μm) were cultured with mouse osteoblasts (MC3T3-E1) for four weeks. Three experimental groups were compared in the present study. Medium for the culture was a combination of alpha-modified Eagle's medium, 10 % fetal bovine serum, and 1 % L-glutamine. The group A (n=10) applied undecalcified nacreous powder alone, whereas the group B (n=10) applied only decalcified nacreous powder. In group A and B, one grain of nacreous powder was placed in each dish. The group C (n=20) applied one decalcified and one undecalcified nacreous grains in each dish. These two grains were placed at a distance of less than 1mm. The samples were stained with alizarin red S, von Kossa, van Gieson, Mallory, and alcian blue at the end of the 4-week culture. The results showed that the newly formed meshed tissue around the decalcified nacre was less prominent than that around the undecalcified nacre. The meshed tissue around the decalcified nacre was positively stained with van Gieson and Mallory, which are considered to be specific for collagen. The meshed tissue around the undecalcified nacre was presumably osteoid tissue because it was positively stained with all stains except alcian blue. Thus, the undecalcified nacre of freshwater mollusk *Hyriopsis schlegeli* may induce osteogenesis, while the decalcified nacre is less potent to induce calcified osteoid.

Key words: Nacre, Osteoblasts, Osteoid, Osteogenesis

INTRODUCTION

Bone defect has big negative impact, and the importance of bone reconstruction is therefore obvious. Nevertheless, clinically applicable bone is not obtainable from *in vitro* cultures.

The traditional method of applying bone tissue to a patient is bone grafting with autografts, allografts, or xenografts. The scarcity of donors limits the use of these grafts. Other complications of allografting and xenografting are rejection and disease transmission[6,22], although autolyzed, antigen-extracted, allogeneic bone is claimed to be a safe allograft[10].

Several synthetic materials, which include lactate[8], ceramics[27-28], and hydroxyapatite[23], are being tested as bone substitutes. Although they have favorable biocompatibility, these materials cannot prompt osteogenesis *in vivo* and *in vitro*. Therefore, they are usually used in combination with chemical inducers (e.g., steroids and beta-glycerol phosphate[16]) or growth factors (e.g., bone morphogenetic proteins[20,23]) to initiate biomineralization.

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However, it is difficult to maintain an effective concentration of growth factors or chemical inducers at the implanted sites. Safety issues in using chemical inducers *in vivo* have not yet been addressed. An ideal bone graft or bone substitute is still not available.

Nacre, also known as mother-of-pearl, is being investigated as a bone substitute. Lopez et al. have shown that the nacre of seawater mollusk *Pinctada maxima* is able to maintain the activity of subcutaneous fibroblasts[1,14]. Others have also demonstrated that it induces osteoblasts and bone morrow cells to form osteoid *in vivo* and *in vitro*[2,11-13,21]. Although the main component of nacre is calcium carbonate, the mechanism of nacreous mineralization, which is dominated by mantle-secreted proteins, is reported to be similar to that of mammalian ossification.[17-18,25-26] From this similar mechanism of biomineralization, nacre may be a better bone substitute than other synthetic materials.[1,4,18,26] However, whether the nacre of freshwater mollusk will also induce osteogenesis is still obscure.

In this paper, therefore, we tested the nacre of freshwater shell *Hyriopsis schlegeli* for osteogenesis using a series of histological studies.

MATERIALS AND METHODS

Decalcified and undecalcified nacre were prepared for culturing with mouse osteoblasts (MC3T 3-E1 cell line) *in vitro*. The freshwater mollusk *Hyriopsis schlegeli* was taken from the Biwa Lake of Japan. We removed the outer prismatic layer of shells with a grinder (XL-150, Osada), washed the remaining nacreous parts with 5% acetic acid (Nacalai Tesque) for 24 hours at 4°C, removed the debris on the nacreous surfaces, and then washed them with deionized water for two hours at 4°C. Decalcified and undecalcified nacre were prepared by the following steps.

Preparation of the undecalcified nacreous powder

After being washed with deionized water, the nacre was air-dried and then powdered using a blender (Osaka Chemical Co.). The size of the powdered nacre was determined with sieves of different apertures. The sieved powder (300-425μm) was then packed and sterilized with ethylene oxide gas.

Preparation of the decalcified nacreous powder

Nacre was decalcified in 20% ethylenediaminetetraacetic acid (EDTA; pH 8) (Nacalai Tesque) at 4°C for nine days. The EDTA solution was renewed every three days. The decalcified nacreous membranes were separated and washed with distilled water at 4°C for four hours. The distilled water was changed hourly. The nacreous membranes were frozen overnight at -80°C. Then, they were freeze-dried for two days (IWAKI™ Freeze Dryer FRD-50M, Asahi Techno Glass). The decalcified nacreous membranes were ground to a powder using a blender and the powder was sieved. The decalcified nacreous powder (300μm-1 mm) was packed and then sterilized with ethylene oxide gas.

Co-culture of nacre and mouse osteoblasts

The cell line of mouse osteoblast (MC3T3-E1) was obtained from the Riken Cell Bank. The culture medium was a combination of alpha-modified Eagle's medium (MEM; Cellgro®, Mediatech), 10 % fetal bovine serum (Iwaki), and 1 % L-glutamine (Bio Whittaker). The osteoblasts after six passages were then cultured in 35mm plastic dishes (Iwaki) with the decalcified (300μm-1mm) or the undecalcified (300-425μm) nacreous powder. Each 35mm dish contained 30000 mouse osteoblasts. We designed three different experimental groups (Group A, B, and C) and cultured for four weeks. The medium was changed every three days.

The group A consisted of ten dishes. Each dish contained only one grain of undecalcified nacreous powder (size: 300-425μm). The group B consisted

of ten dishes. Each dish contained only one grain of decalcified nacreous powder (size: 300μm-1mm). The group C consisted of twenty dishes. Each dish contained one grain of decalcified nacreous powder (size: 300μm-1mm) or/and one grain of undecalcified nacreous powder (size: 300-425μm). The undecalcified nacre was placed within 1mm from the decalcified one.

We stained the samples by the methods described below. Before staining, all samples were fixed at 4 ℃ for one hour with 2% glutaraldehyde solution in 0.1 M sodium phosphate buffer at pH 7.2. After fixation, the samples were gently washed two times with distilled water.

For the observation of samples, an inverted light microscope was used (Eclipse TE300, Nikon). The staining outcomes were also evaluated with computer software (Scion Image, Scion Corporation) and graded according to the criteria:

1. Score of the thickness of newly formed tissue (Table 1). The distance from the margin of a nacreous chip to the coincident outermost margin of stained area represented the thickness. The thickest part was chosen for scoring. Grading was made as the followings: without obviously positive stain scored 0 point, thickness less than 10 micron scored 1 point, thickness between 10 and 20 micron scored 2 points, and thickness more than 20 micron scored 3 points.
2. Score of the spreading area of newly formed tissue (Table 2). The stained area around the nacreous chip represented the diameter. Grading was made as the followings: without obvious spreading area scored 0 point, spreading area of less than 50% of the nacreous circumstance scored 1 point, spreading area of more than 50% but less than 100% of the nacreous circumstance scored 2 points, and spreading area of 100% of the nacreous circumstance scored 3 points.
3. Staining positivity was graded ranging from (−) to (+++). (Table 3).

Alizarin red S stain for calcium

One percent of alizarin red S (Wako) in 0.1% ammonia solution (Nacalai Tesque) was prepared by Dahl's method[5]. Two milliliter of alizarin red S solution was added to each selected sample. The samples were incubated at room temperature for five minutes and then washed well with distilled water.

Table 1. Points from Rule 1 on defining thickness of newly formed tissue*

	Not Obvious	<10μm	≧10μm, <20μm	≧20μm
Points [X]	0	1	2	3

*The definition of thickness was described in the section of "Materials and Methods".

Table 2. Points from Rule 2 on defining spreading area of newly formed tissue*

	Not Obvious	<50%	≧50%, <100%	100%
Points [Y]	0	1	2	3

*The definition of spreading area was described in the section of "Materials and Methods".

Table 3. Grading table

	Grade(−)	Grade(+)	Grade(++)	Grade(+++)
Points [(X+Y)/2]*	≧0and<1	≧1and<2	≧2and<3	3

*X=points from Rule 1. Y = points from Rule 2. Please refer to Table 1 and 2.

Von Kossa stain for phosphates

A modification of von Kossa method[19] was used to explore phosphates. Two milliliter of 2% silver nitrate (Nacalai Tesque) solution was added to each test sample and the samples were exposed under strong light for one and a half hours. After that, the samples were treated with 5% of sodium thiosulfate solution for three minutes.

Mallory's trichrome and van Gieson stains for collagen

Three different solutions were used during Mallory's trichrome staining[7,15]. Solution A contained 1g of acid fuchsine (Nacalai Tesque) dissolved in 100 ml of distilled water. Solution B contained 1g of phosphomolybdic acid (Nacalai Tesque) dissolved in 100ml of distilled water. Solution C was prepared by dissolving 2.0g of Orange G (Certistain®, Merck), 0.5g of methyl blue (Chroma-Gesellschaft), and 2.0g of oxalic acid (Nacalai Tesque) in 100ml of distilled water. The samples were stained by solution A for two minutes, solution B for two minutes, and solution C for 15 minutes respectively. The samples were rinsed thoroughly. Mallory's trichrome staining was performed at room temperature.

The van Gieson solution was prepared by mixing 100ml of saturated picric acid (Nacalai Tesque) with 10ml of 1% acid fuchsine (Nacalai Tesque) solution. The samples treated with this solution were incubated at room temperature for five minutes.

Alcian blue stain

Alcian blue of pH 2.5 (Nacalai Tesque) was added to each dish of samples. Then they were placed at room temperature for five minutes and the samples were washed well with distilled water.

RESULTS

All samples from three groups (the group A, B, and C) were stained after culturing mouse osteoblasts (MC3T3-E1) with nacre (*Hyriopsis schlegeli*) for four weeks. The meshed tissue around the undecalcified nacre was more than that around the decalcified nacre in all groups. In group C, the undecalcified nacre formed more prominent meshed tissue than the decalcified one and the distribution of meshed tissue around decalcified nacre did not show any polarity, that is, toward or away from the undecalcified nacre. Therefore, after culturing for four weeks, interaction between the undecalcified nacre and the co-cultured decalcified nacre was unlikely.

Table 4 shows the quantitative differences of the positively stained tissue around decalcified and undecalcified nacre.

Table 4. The quantitative analysis of different staining methods on nacre-induced meshed tissue

Groups / Staining Methods	Group A (undecalcified)		Group B (decalcified)	
	Nacreous chip 1	Nacreous chip 2	Nacreous chip 1	Nacreous chip 2
Alizarin Red S	26μm, 100%(+++)	21μm, 100%(+++)	0μm, 0%(−)	0μm, 0%(−)
Von Kossa	29μm, 100%(+++)	22μm, 100%(+++)	0μm, 0%(−)	0μm, 0%(−)
Van Gieson	55μm, 100%(+++)	46μm, 100%(+++)	16μm, 50−100%(++)	42μm, 50−100%(++)
Mallory Trichrome	65μm, 100%(+++)	32μm, 50−100%(++)	28μm, 100%(+++)	12μm, 0−50%(+)
Alcian Blue	0μm, 0%(−)	0μm, 0%(−)	0μm, 0%(−)	0μm, 0%(−)

* Group C had both decalcified and undecalcified nacreous chips in one dish at the same time and the 4-week's outcome had no obvious differences from Group A and B, so we did not put the data here.
# The grading method was described in the section of "Materials and Methods".
§ The data inside were arranged as "Rule 1-thickness, Rule 2-spreading area (Rule 3-grade)".

Fig. 1. Alizarin red S stain, 100x, 4 weeks. (A) Group A: an undecalcified nacreous chip. (B) Group B: a decalcified nacreous chip. (C) Group C: decalcified and undecalcified nacreous chips. [Arrow = positively stained area. DN = decalcified nacre. uDN = undecalcified nacre. Medium = alpha-Eagle's medium+10% fetal bovine serum+1% glutamine. Cells = mouse osteoblasts.]

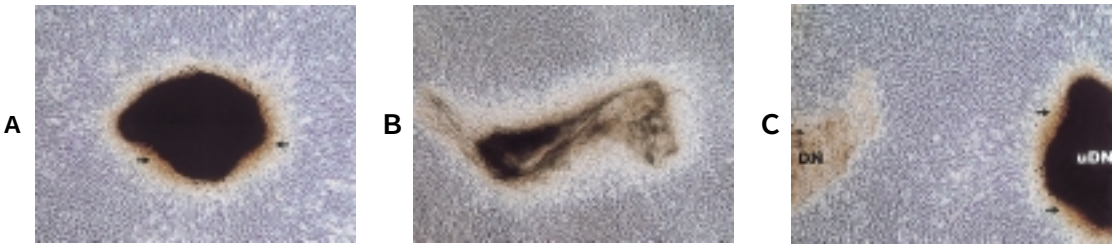

Fig. 2. Modified von Kossa stain, 100x, 4 weeks. (A) Group A: an undecalcified nacreous chip. (B) Group B: a decalcified nacreous chip. (C) Group C: decalcified and undecalcified nacreous chips. [Arrow = positively stained area. DN = decalcified nacre. uDN = undecalcified nacre. Medium & cells = the same as Fig.1.]

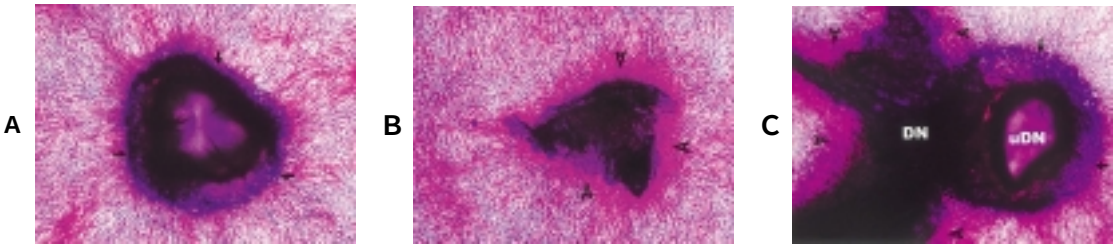

Fig. 3. Mallory stain, 100x, 4 weeks. (A) Group A: an undecalcified nacreous chip. (B) Group B: a decalcified nacreous chip. (C) Group C: decalcified and undecalcified nacreous chips. The blue-stained area is less prominent around the decalcified nacre. [Arrow = positively stained area of undecalcified nacre. Arrowhead = positively stained area of decalcified nacre. DN = decalcified nacre. uDN = undecalcified nacre. Medium & cells = the same as Fig.1.]

Alizarin red S solution stains calcium-containing material to a red color. In group A, the meshed tissue that surrounded the undecalcified nacre was stained red (Fig.1). Contrarily, alizarin red S did not positively stain the meshed tissue around the decalcified nacre in group B. Alizarin red S staining in group C demonstrated that positive staining of the undecalcified nacre and negative staining on the decalcified nacre, the same as groups A and B, respectively. Therefore, interaction between the decalcified and undecalcified nacre was not evident.

Phosphates are stained yellow after treating samples by the von Kossa method[19]. Meshed tissue in group A was stained yellow, but, in group B, the meshed tissue was not positively stained (Fig.2). In group C, the meshed tissue around undecalcified nacre was stained yellow as the outcome in group A. The tissue around decalcified nacre was stained negatively as shown in group B. It was considered that the undecalcified nacre did not facilitate the formation of phosphates around decalcified nacre.

Collagens could be separately stained as dark blue with Mallory's trichome or orange with van Gieson staining methods. The meshed tissue was

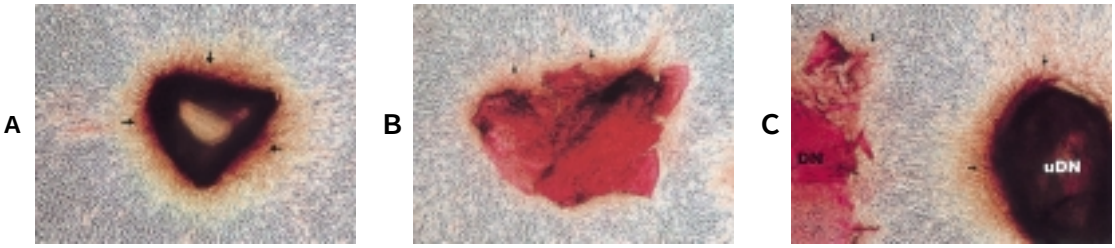

Fig. 4. Van Gieson stain, 100x, 4 weeks. (A) Group A: an undecalcified nacreous chip. (B) Group B: a decalcified nacreous chip. (C) Group C: decalcified and undecalcified nacreous chips. The orange-stained area is less prominent around the decalcified nacre. [Arrow = positively stained area. DN = decalcified nacre. uDN = undecalcified nacre. Medium & cells = the same as Fig.1.]

Fig. 5. Alcian blue stain, 100x, 4 weeks. (A) Group A: an undecalcified nacreous chip. (B) Group B: a decalcified nacreous chip. (C) Group C: decalcified and undecalcified nacreous chips. None of them is positively stained. [DN = decalcified nacre. uDN = undecalcified nacre. Medium & cells = the same as Fig.1.]

positively stained by both methods in all groups (Fig.3, Fig.4). We also found that the meshed tissue around the undecalcified nacre was stained more prominently than the tissue around the decalcified nacre. The stained area corresponded with the area of white meshed tissue. Thus, the meshed material was supposed to be mainly composed of collagens.

Alcian blue stain is used to demonstrate the cartilaginous chondroitin sulfate by staining it by blue color[9,24]. In our study, we could not stain the meshed tissue in all groups (Fig.5). This demonstrated that the meshed tissue did not contain cartilaginous tissue.

DISCUSSION

Biomineralization of mollusks involves crystal nucleation and accumulation, and is controlled by various proteins, which are mainly secreted by the outer mantle epithelium of the shell. The proposed functions of these proteins are similar to those of osseous proteins.[3,4,17,18] Due to this similarity, we considered that nacre may have the ability to induce osteogenesis.

The osteogenetic potential of seawater mollusk has previously been reported[1-3,11-14,21], but freshwater mollusk has not yet been tested. Therefore, in this paper, we showed that the nacre of freshwater mollusk *Hyriopsis schlegeli* can induce mineralization when co-cultured with mouse osteoblasts *in vitro*. Following decalcification with ethylenediaminetetraacetic acid (EDTA), the nacre loses its potential to induce mineralization.

According to the outcomes obtained, two questions may arise: firstly, whether it is appropriate to conclude that the meshed tissue is an osteoid tissue, and, secondly, whether calcium in the medium and/or in the nacre itself makes false-positive outcomes of various stainings.

As shown in this paper, the mineralized meshed tissue formed around the undecalcified nacre contains calcium, collagens, and phosphates, which are the main constituents of osteoid and cartilage. Mouse osteoblasts were the only cells present in the culture medium. The meshed tissue is supposedly produced by these osteoblasts. In addition,

the outcome from alcian blue stain suggests that the meshed tissue is unlikely to be cartilage. These results suggest that the meshed tissue is more osteoid-like.

The staining methods that we chose have been used as specific stains for more than 20 years. Therefore, these methods serve well to primarily identify biomineralization. The samples were fixed in a glutaraldehyde solution and washed well with distilled water after fixation to eliminate influential factors from the medium. If other influence did exist in alizarin red S staining, there would have been numerous red areas in every sample, not only the area surrounding the nacre. In our studies, there was no such problem.

Alizarin red S solution had been neutralized by ammonia to pH 6.4 before use[5], so that the alizarin red S solution may not decalcify the nacre. If the calcium is immediately released by the undecalcified nacre itself during staining, the released calcium should also be washed out. Thus, interference of the culture media with the present staining procedures is unlikely.

The von Kossa staining has long been erroneously thought to stain calcium-containing materials to black color by reduced silver. In fact, the von Kossa staining is not a specific detecting method for calcium ions. It is the organic compounds but not the calcium phosphates to react with von Kossa solution to form black deposits[19]. However, it was noted that the von Kossa solution is specific for identifying phosphates by staining them yellow or yellowish brown.[19]

In conclusion, we considered that undecalcified nacre of the freshwater mollusk *Hyriopsis schlegeli* can induce mouse osteoblasts to form osteoid *in vitro*, but EDTA-decalcified nacre cannot induce osteoid formation.

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マウスの骨芽細胞(MC3T3-E1)を用いた淡水真珠貝 *Hyriopsis schlegeli* の骨誘導に対する影響

― *in vitro* における脱灰と未脱灰粉の比較 ―

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抄　　録

淡水真珠貝 *Hyriopsis　schlegeli* の真珠層が骨誘導能を有することを調べるために以下の実験を行った．EDTA で脱灰した粉状の真珠層（大きさ0.3－1㎜）及び未脱灰の真珠層（大きさ300－425μm）をマウスの骨芽細胞（MC3T3-E1）と四週間培養した．本実験において三つのグループ（A－C）を設定した．全てのグループの培養液は alpha 変法の Eagle 培養液,10%のウシ胎仔血清（FBS）及び１%のL－グルタミンの混合液を使用した．グループA（n＝10）は未脱灰の真珠粉のみ，グループB（n＝10）は脱灰した真珠粉のみで，個々のシャーレに一粒各々の真珠粉をシャーレの中心に置いた．グループC（n＝20）は個々のシャーレの中心に一粒の脱灰した真珠粉と一粒の未脱灰の真珠粉を距離を約１㎜あけて置いた．第四週の終わりに全ての培養サンプルを alizarin red S 液，von Kossa 液，van Gieson 液，Mallory 液及び alcian　blue 液で染色した．その結果，脱灰した真珠粉の周囲よりも，未脱灰の真珠粉の周囲に網状の新生組織の形成が顕著であった．脱灰した真珠粉の周囲の新生組織はコラーゲンを特異的に染色する van　Gieson 液と Mallory 液に染色された．未脱灰の真珠粉の周囲の新生組織は alcian　blue 液を除くその他の全ての染色液で染色され，これによりこの新生組織は類骨質であることが示された．以上のことから，未脱灰の淡水真珠貝 *Hyriopsis　schlegeli* の真珠層は骨誘導能を持ち，脱灰した真珠層は骨誘導能を持たないことが示された．