INTRODUCTION

When bone scaffold material remains in the living body for a long period of time, it causes bone fracture or infection, so it is necessary for the prosthetic material to be absorbed and lost in the body in vivo. Coral is a material that has been applied to research and clinical applications as restorable bone scaffold material, absorbed by osteoclasts, and has been reported to be replaced with new bone tissue. Coral has a certain compressive strength and is expected as a scaffold for bone augmentation. Bone scaffold material must be porous, and it is important for the adhesion of...
angiogenesis and osteoblasts formed in the cavity. Montipora digitata exoskeleton is porous and has a communicative lumen with a diameter of approximately 200 μm.\textsuperscript{12-13} Collagen fiber is known as an extracellular matrix that becomes a scaffold for capillaries and bone formation\textsuperscript{14}, but the influence of oral exoskeleton to cell proliferation and collagen productivity of human fibroblasts has not been reported. In this study, human normal skin fibroblasts involved in bone regeneration and human periodontal ligament fibroblasts involved in regeneration of alveolar bone were cultured, Montipora digitata exoskeleton particles were added, and those without particles were treated as a control. Then cell proliferation and collagen production of these fibroblasts were observed.

MATERIALS AND METHODS

1. Montipora digitata exoskeleton-derived aragonite particles
Montipora digitata exoskeleton was pulverized and sieved to obtain particles of 850-1000 μm. The particles were immersed in 1 N sodium hydroxide under negative pressure for 7 days, and organic matter was removed. These particles were washed with ultrapure water, and neutralized in PBS. After sterilizing with autoclave for 20 minutes under an atmosphere of 2 atm and 121 °C, Montipora digitata exoskeleton-derived aragonite particles (hereinafter referred to aragonite particles) were prepared.

2. Cell culture
Normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany) and human periodontal ligament fibroblasts (HPLF, ScienCell\textsuperscript{TM}, CA, USA) were cultured at 37 °C, 5% CO\textsubscript{2}, and the cell density at seeding was 1.91-2.125 × 10\textsuperscript{5}. 10% Fetal bovine serum (FBS: GE Healthcare Life Sciences HyClone Laboratories, UT, USA) containing Dulbecco’s modified Eagle Medium (DMEM: Nacalai tesque, Kyoto, Japan) with 500 U/mL penicillin-500 μg/mL streptomycin (Nacalai tesque) was used as a medium. At a dose of 25 mg/well, aragonite particles were added after 6 hours incubation in the experimental group. Aragonite particles were not added to the control group.

3. Measurement of cell proliferation with WST method
NHDF and HPLF were cultured in 96 well microplate with flat bottom with flat bottom (IWAKI, Tokyo, Japan), and cell proliferation reagent (WST-1; Roche Applied Science, IN, USA) in an amount of 10 μL/well in the medium was reacted at 30 minutes 37 °C at 5% CO\textsubscript{2} conditions. After reacting the reagents for 30 minutes in the culture condition of 37 °C at 5% CO\textsubscript{2}, in both control and experimental groups, the culture supernatant 100 μL discolored by the reagent, was dispensed into each well (IWAKI). Absorbance of the supernatant formazan caused by WST-1 was measured with Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm. The statistical difference was determined by two-sided Student’s t-test. A difference of P < 0.05 was considered significant.

4. Morphological observation of fibroblasts by scanning electron microscope (SEM)
The morphology of fibroblasts and fibrous structure on the aragonite particles were observed with SEM (S-4000; Hitachi, Tokyo, Japan). After adding aragonite particles, NHDF and HPLF were cultured in 6 well microplate with flat bottom with Lid flat bottom (IWAKI). Tissues attached to the particles were pre-fixed with 2.5% Glutaraldehyde - 0.1M phosphate buffer (pH 7.4) and post-fixed with 4% OsO\textsubscript{4}. After dehydra-
tion with 10% ethanol, they were lyophi-
lized (VFD-21S; Shinku device, Ibaragi, 
Japan). Pt-Pd was vacuum deposited 
on the sample with an ion sputtering 
device. After the vacuum deposition, the 
samples were observed by SEM.

5. Determination of collagen fibers 
with hydroxyproline analysis
Twenty-eight days after adding arago-
nite particles, the cultured tissues were 
reacted to the reagent of total collagen 
assay kit (Hydroxyproline assay; Cosmo 
Bio, Tokyo, Japan) in the control group 
and the experimental group. After 
reacting the reagent, synthesized 
hydroxyproline was used to measure 
the absorbance with Softmax Pro soft-
ware (Molecular Devices, Sunnyvale, 
CA, USA) at a wavelength of 570 nm. 
These determinations were used as an 
index of collagen fiber production.

RESULT

1. Measurement of cell proliferation 
with WST method
Both HPLF and NHDF of the experi-
mental and control group showed cellu-
lar proliferation over the entire culture 
period. NHDF and HPLF showed sig-
nificantly higher formazan values in the 
experimental group than that in the 
control group on 1, 3, and 7 days after 
culture (Fig. 1).

2. Morphological observation of fi-
broblasts by SEM
At one day after culture, round-shaped 
HPLF and NHDF adhering to the ara-
gonite particles were observed. Seven 
days after culture, spindle-shaped HPLF 
and NHDF and the fibrous structure 
were observed on the outer layer of the 
aragonite particles and the surface of 
their lumen. Fourteen days after culture,

![Fig. 1](image_url)

Cell proliferation with WST method
On 1, 3, and 7 days after culture
Experimental group; fibroblasts cultured with aragonite particles.
Control group; fibroblasts cultured without aragonite particles.
* Student’s t-test (P<0.05)
Fig. 2A  Morphological observation of NHDS by SEM. (Arrows: NHDF)  Ar: Aragonite particles  

- a: 1 day after culture
- b: 7 days after culture
- c: 14 days after culture
- d: 28 days after culture

Fig. 2B  Morphological observation of HPLF by SEM.  (Arrows: NHDF)  Ar: Aragonite particles

- e: 1 day after culture
- f: 7 day after culture
- g: 14 days after culture
- h: 21 days after culture
spindle-shaped NHDF and HPLF with cell proliferation proliferated on aragonite particles. At twenty-one days after culture, proliferation of NHDF and HPLF and formation of fibrous structure were observed on the aragonite particles, which were almost entirely covered with fibroblasts and fibrous structures (Fig. 2A, 2B).

3. Determination of collagen fibers with hydroxyproline analysis
At twenty-eight days after culture, increases in the amount of hydroxyproline produced by HPLF and NHDF were measured in the experimental groups compared with the control groups (Fig. 3).

DISCUSSION
In the experimental groups in which HPLF and NHDF were cultured with the aragonite particles added, NHDF and HPLF increases in cell proliferation were observed as compared with the control groups cultured without aragonite particles. Moreover, proliferated spindle-shaped fibroblasts were observed on the outer layer of the aragonite particles and their lumen. Fibroblasts express integrin on the cell membrane surface as cell adhesion molecules. In the cells of integrin-expressed cells, Signal transduction occurs and the cell proliferation is enhanced as a result15-18. The α chain of integrin requires Ca++ in the domain structure and is activated by obtaining Ca++. In vivo, coral is a material to be dissolved by osteoclasts, and it is dissolved easily in acidic environment in vitro and then Ca++ is eluted20. The metabolism of cells grown on the aragonite particles may be due to local acidosis; Ca++ eluted from the aragonite particle was used for cell proliferation. In addition, the RGD binding site of the integrin β chain binds to fibronectin, vitronectin and fibrinogen18, 21. Also, the surface of the aragonite particles has a nano-level concavo-convex structure22.

![Fig. 3](image)

**Fig. 3** Determination of collagen fibers with hydroxyproline analysis. Amount of hydroxyproline on 28 days after culture. Experimental group; fibroblasts cultured with aragonite particles. Control group; fibroblasts cultured without aragonite particles. * Student’s t-test (P<0.05)
A rough surface structure promoted fibroblast adhesion and increased cell proliferation\textsuperscript{23}. NHDF and HPLF proliferated were observed in the outer layer of the porous aragonite particles and the surface layer of their lumen. From these facts, three-dimensional culture of fibroblasts using aragonite particles is possible\textsuperscript{13,24-27}.

At twenty-eight days after culture, the amount of hydroxyproline produced was significantly higher in the experimental group as compared with the control group. Hydroxyproline is the main component of collagen fibers and is responsible for the stability of collagen\textsuperscript{28}. The fibrous structure on the aragonite particle surface was also observed morphologically by SEM twenty-eight days after the particles addition. In the experimental group, the fibrous structure showed high values in both fibroblasts throughout the culture period. From this, the fibrous structure may be collagen fiber and fibroblasts on the aragonite particles surface enhanced cell proliferation and collagen fiber production\textsuperscript{29,30}.

In the SEM image (Figs. 2A and 2B), it was also observed that NHDF and HPLF wrapped the aragonite particles over time. When foreign matter in the living body and can not be absorbed by it, the matter is surrounded and encapsulated by granulation tissue and fibrous connective tissue\textsuperscript{31}. The phenomena observed in vitro in this experiment may be related to encapsulation of NHDF and HPLF wrapping aragonite particles.

CONCLUSION

In this experiment, it was presumed that cultured HPLF and NHDF evidenced enhanced cell proliferation and collagen formation by adding aragonite particles.

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REFERENCES

9) Uswatta SP, Okeke IU, Jayasuriya AC. Injectable porous nano-hydroxyapatite/chitosan/tripolyphosphate scaffolds with improved compressive strength for bone
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