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Original Article

Evaluation of a hydroxyapatite-crosslinked fish gelatin membranes

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KEYWORDS
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Abstract *Background/purpose:* Porcine collagen is widely used in regenerative therapies to generate membranes for bone augmentation. However, porcine or bovine gelatin or collagen is often not appropriate for patients with creed and religious beliefs or for allergic reasons. In this study, we evaluated the potential of fish gelatin to generate membranes.
Materials and methods: Fish gelatin and hydroxyapatite (HAp) were used at three different ratios (2:0, 2:1, 2:1.5, and 2:2) to prepare gelatin-hydroxyapatite (G-HAp) membranes via freeze-drying and heat-crosslinking. The surface morphology and cell attachment of G-HAp membranes were observed using scanning electron microscopy and confocal laser microscopy. G-HAp membrane was placed at the bottom of a well plate, and MC3T3-E1 cells were seeded on it. Cell viability and cytotoxicity were tested after 1 and 3 days of culture. Alkaline phosphatase (ALP) and alizarin red staining was performed at 10 and 21 days, respectively.
Results: Viability of cells on G-HAp membrane with the gelatin:HAp ratio of 2:1.5 was significantly higher than that on membranes with other gelatin:HAp ratios. ALP and alizarin red

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staining showed that ALP-positive areas and calcium deposition were the highest on G-HAp membrane with the gelatin:HAp ratio of 2:1. These membranes showed negligible cytotoxicity. *Conclusion:* Fish-derived G-HAp membranes have the potential to promote osteogenic differentiation of MC3T3-E1 cells with negligible cytotoxicity.

Introduction

With the increasing variety and effectiveness of materials for clinical guided bone regeneration (GBR) in dental implant treatment, it is important to allow for patients' requests based on cultural diversity.[1] Porcine and bovine collagens are widely used for generating membranes and bone substitutes in regenerative therapy.[2] However, the use of pig or cattle gelatin/collagen is often not preferred by patients for creed and religious beliefs or for allergic reasons.[3,4] Therefore, identifying alternative sources of gelatin/collagen to improve GBR surgery results is necessary.[5—7]

Peptides of fish collagen have excellent bioavailability attributable to their relatively small particle sizes and antioxidant potential.[8] Owing to its water solubility, biodegradability, easy extraction, and low immunogenicity, fish collagen has potential applications as a biomaterial source.[9,10] Fish gelatin is widely accepted by people of all religions and cultures and not associated with the risk of bovine spongiform encephalopathy (BSE) outbreaks.[11] Additionally, fish skin is a major waste generated by commercial fish-processing industries and causes environmental pollution.[12,13] Its use represents an emerging concept that has attracted widespread attention for effectively and sustainably utilizing resources, energy, and infrastructure while ensuring that the quality of human life is not effected.[14]

Fish collagen peptides have specific amino acid compositions with high concentrations of proline, glycine, and hydroxyproline.[15] Peptides containing hydroxyproline are not completely digested into free amino acids after ingestion and can be detected in the blood, leading to collagen synthesis through cellular activation and growth in the skin, joints, and bones.[16] Oreochromis niloticus (Nile tilapia) has characteristics of high-temperature resistance, adaptability to captivity, fast growth, and cost-effectiveness, and the use of decellularized tilapia skin for medical purposes, such as healing skin wounds[17] and generating acellular dermis matrix,[18] skin graft,[19] and tendon regeneration material,[20] has been reported.[21]

Hydroxyapatite (HAp) promotes stromal cell function and osteogenic activity because of its similarity to bone minerals, biocompatibility, and mechanical properties that enable membranes to withstand static pressure from soft tissues and provide adequate space for bone regeneration.[22] Therefore, here, we used gelatin derived from Nile tilapia skin and investigated the potential of gelatin-hydroxyapatite (G-HAp) membranes with different contents of HAp powder.

Materials and methods

Membrane preparation

Gelatin from fish skin was a slightly acid-treated, water-soluble, light-yellow powder (Lot No: 181128, CAS RN®: 9000-70-8; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Porcine gelatin was an alkali-treated yellow powder (MORINAGA Milk Industry Co., Ltd., Tokyo, Japan).

To prepare 10 mL of gelatin solution, 200 mg of water-soluble warm-water-fish gelatin and porcine gelatin was added into four different tubes and heated at approximately 40 °C after adding 10 mL of deionized water until gelatin was completely dissolved. Thereafter, 0, 100, 150, or 200 mg of HAp (Bio Medical Science, Tokyo, Japan) was added to the tubes. The mixture was then poured from each of the tubes into two round pans (40-mm diameter) while stirring and allowed to settle for 1 h in a refrigerator. During this time, gelatin and HAp were layered and solidified, and then placed in a freezer at −70 °C for 24 h and 3days for freeze-drying. Thereafter, crosslinking was initiated by heating the mixture at 150 °C for 6 h under pressure to obtain G-HAp membranes. Four different amounts of HAp were used with the same gelatin concentration, that is, the gelatin:HAp ratio in the G-HAp membranes was 2:0, 2:1, 2:1.5, and 2:2 (referred to as 2:0 G-HAp, 2:1 G-HAp, 2:1.5 G-HAp, and 2:2 G-HAp, respectively).

Both fish- and pig-derived G-HAp membranes were placed in cell culture medium to compare their structural stability at 37 °C. As G-HAp membrane from porcine gelatin collapsed and ripped in the culture medium within 24 h, further experiments including cell culture were carried out using G-HAp membrane derived from fish gelatin.

Characteristics of G-HAp membrane derived from fish skin

To observe the state of membrane after crosslinking under heat and pressure, scanning electron microscopy (SEM) (S-4500; Hitachi Ltd., Hitachinaka, Japan) was performed, and the shape characteristics of the surface, cross-section, and bottom surface of the samples were observed. Three sets of G-HAp membranes (2:1, 2:1.5, and 2:2) were sputter-coated with Pt, and their surface and cross-sectional morphologies were observed (acceleration voltage, 15 kV; magnifications, ×100, × 130, and × 300).

Cell culture preparation

MC3T3-E1 cells (RIKEN Cell Bank, Ibaraki, Japan) were seeded at a density of 1×10^5 cells/well in a growth medium (10% fetal bovine serum with minimum essential medium) and were grown for 2 days until they reached more than 90% confluence. The cells were then incubated in an osteogenic medium (10% fetal bovine serum, minimum essential medium, 10 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, and 10 nM/mL dexamethasone) at 37 °C under 5% CO_2. The medium was changed every 2 days. G-HAp membranes were punched at a diameter of 6.35 mm; their surfaces were disinfected under ultraviolet light for 15 min. Thereafter, the membranes were placed in the wells of a 96-well culture plate. The cells were seeded on the membranes at a density of 1×10^5 cells/well in 250 μL of osteogenic medium and incubated at 37 °C under 5% CO_2.

Assessment of cell viability and proliferation

Cell proliferation was observed on days 1 and 3 using Cell Counting Kit-8 (CCK-8; Wako, Osaka, Japan) according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 was added to each sample and incubated at 37 °C for 60 min. The absorbance of each sample was measured using a microplate reader at 450 and 620 nm.

Adhesion of MC3T3-E1 cells on gelatin membranes

Cells cultured on G-HAp membranes were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0) for more than 4 h, washed thrice with phosphate buffer, and postfixed with 1% OsO_4 in phosphate buffer (pH 7.0) for 2 h. The fixed cells were washed thrice with phosphate buffer and dehydrated with a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100%), with each 15-min step performed thrice. Thereafter, the cells were treated with a mixture of ethanol and isopropanol for 5 min, followed by treatment with pure isoamyl acetate for approximately 1 h. Subsequently, critical point drying was performed using liquid CO_2. The gold-palladium-coated dehydrated samples were then subjected to ion coating (E102; Hitachi) and observed using SEM (JSM-7900F/JED-2300; JEOL, Tokyo, Japan).

Fluorescent staining of MC3T3-E1 cells cultured on G-HAp membranes

After 3 days of culture, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Thermo Fisher Scientific, Waltham, MA, USA) fluorescent-stained images of MC3T3-E1 cells were captured three dimensionally using a confocal laser microscope (BZ-X700; Keyence, Osaka, Japan) to observe the condition of the cells cultured on the four materials.

Analytical alkaline phosphatase staining

After 10 days of cell culture, the membranes were removed together with the medium, and the cells were washed four times with PBS, fixed with 3.7% formaldehyde, washed with PBS, dried for 3 min, stained with ALP solution at 37 °C for 15 min, washed with distilled water (DW), and observed for color changes. The stained areas were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Analytical alizarin red staining

After 21 days of cell culture, the membranes were removed together with the medium, washed three times with PBS, fixed for 15 min with 10% formalin, washed three times with DW, stained with 1% alizarin red solution for 1–5 min, washed again with DW, and mounted with glycerin. The stained areas were quantified using ImageJ software and compared.

Cytotoxicity assay

A cytotoxicity assay kit (LDH-Cytotoxic Test; FUJIFILM Wako Pure Chemical Corporation) was used to measure cytotoxicity after 5 days of culture of cells in 96-well cell culture plates, according to the manufacturer's instructions. On day 5 of culture, the culture supernatant was transferred to a new culture plate, mixed with the cytotoxicity assay reagent, and incubated for 1 h at approximately 25 °C. Thereafter, 100 μL of stop solution was added into each well, and the absorbance of the samples was measured at 570 nm using the iMark Microplate Reader (Bio-Rad, Hercules, CA, USA).

Statistical analyses

Prism 9.3.1 software (GraphPad Software, LA Jolla, CA, USA) was used for statistical analysis of data. One-way (multiple comparisons with Bonferroni test) tests were used to evaluate the significance of differences in the comparisons. The results were considered statistically significant at $P < 0.05$.

Results

Dissolution and thickness of G-HAp membranes

G-HAp-cross-linked membranes fabricated using porcine or fish gelatin showed differences in dissolution in the cell culture medium for 24 h, 3 days, and 5 days (Fig. 1). The thickness of three different G-Hap membranes (2:1, 2:1.5, and 2:2) measured using a digital meter (Code No: 4-484-01, model BDC 100; AS ONE Corporation, Osaka, Japan) was 0.68 ± 0.10 mm, and the thickness of 2:0 G-HAp was 0.65 ± 0.16 mm, with no significant difference.

Surface morphology of cross-linked G-HAp membranes

The surface and cross-sectional morphologies of the membranes, observed using SEM, are shown in Fig. 2. In terms of morphological structure, the accumulation of HAp particles was mainly below the membrane, and gelatin was on the top of the membrane owing to the difference in mass; the obtained membrane had two layers: an upper HAp layer and

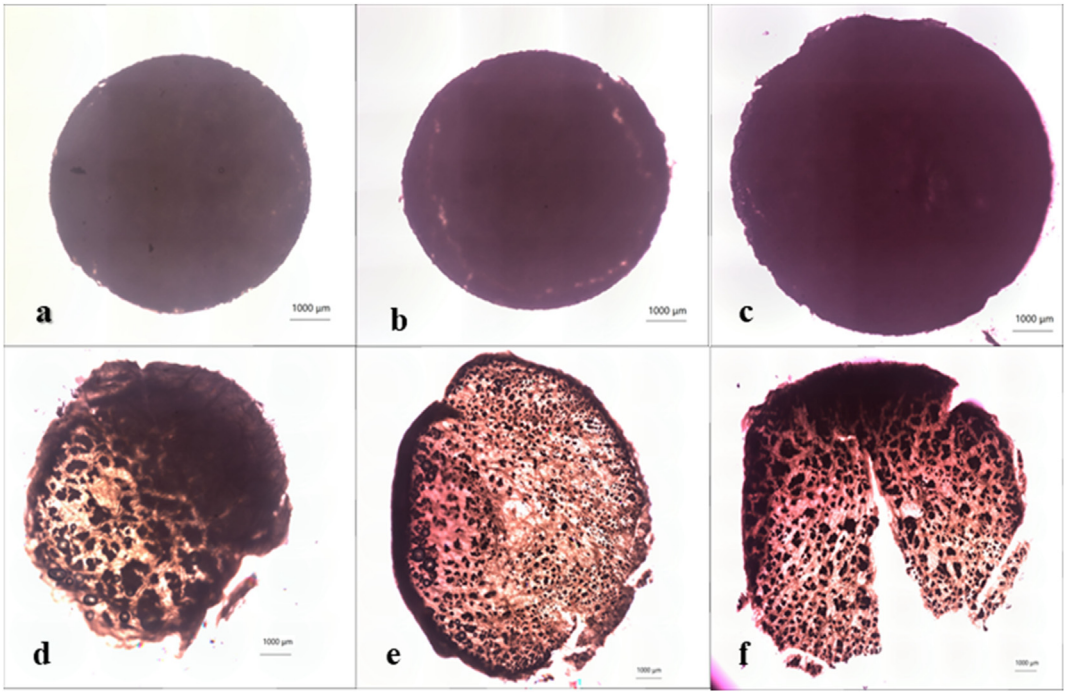

Figure 1 **Solubility of fish gelatin-/porcine gelatin-derived 2:2 G-HAp membranes. a-c:** fish gelatin-derived G-HAp membranes in culture medium at 24 h **(a)**, 3 days **(b)**, and 5 days **(c)**. **d-f:** porcine gelatin-derived G-HAp membranes in culture medium at 24 h **(d)**, 3 days **(e)**, and 5 days **(f)**.

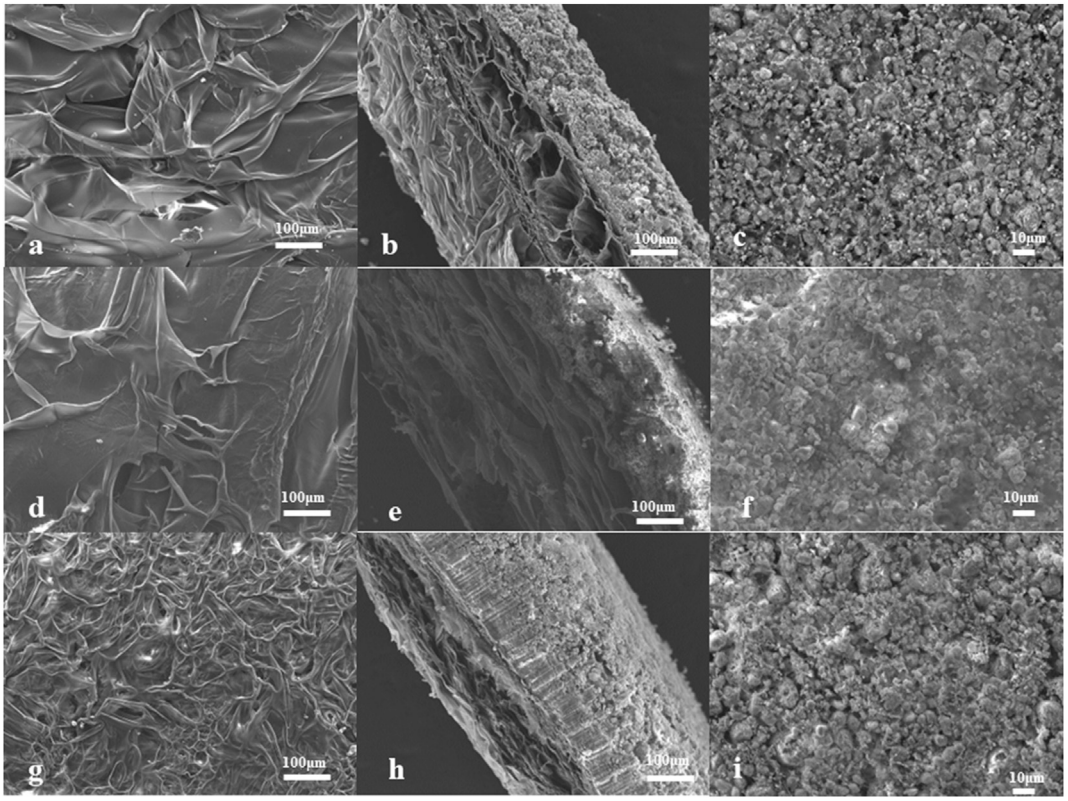

Figure 2 **SEM surface images of fish gelatin cross-linked with HAp. a-c:** 2:1 G-HAp membranes (**a:** top, **b:** cross section, and **c:** bottom). **d-f:** 2:1.5 G-HAp membranes (**d:** top, **e:** cross section, and **f:** bottom). **g-i:** 2:2 G-HAp membranes (**g**: top, **h:** cross section, and **i:** bottom).

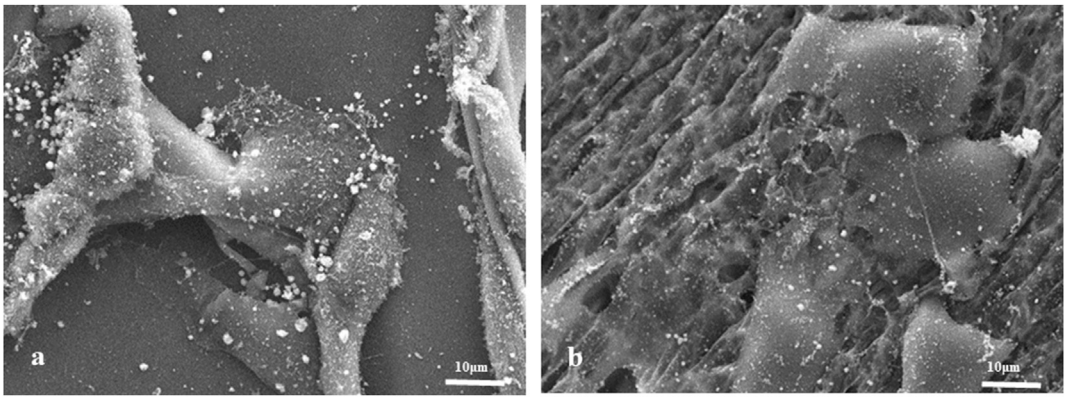

Figure 3 **SEM images of MC3T3-E1 cells cultured on 2:0 fish gelatin membranes at 1 day (a) and 3 days (b).**

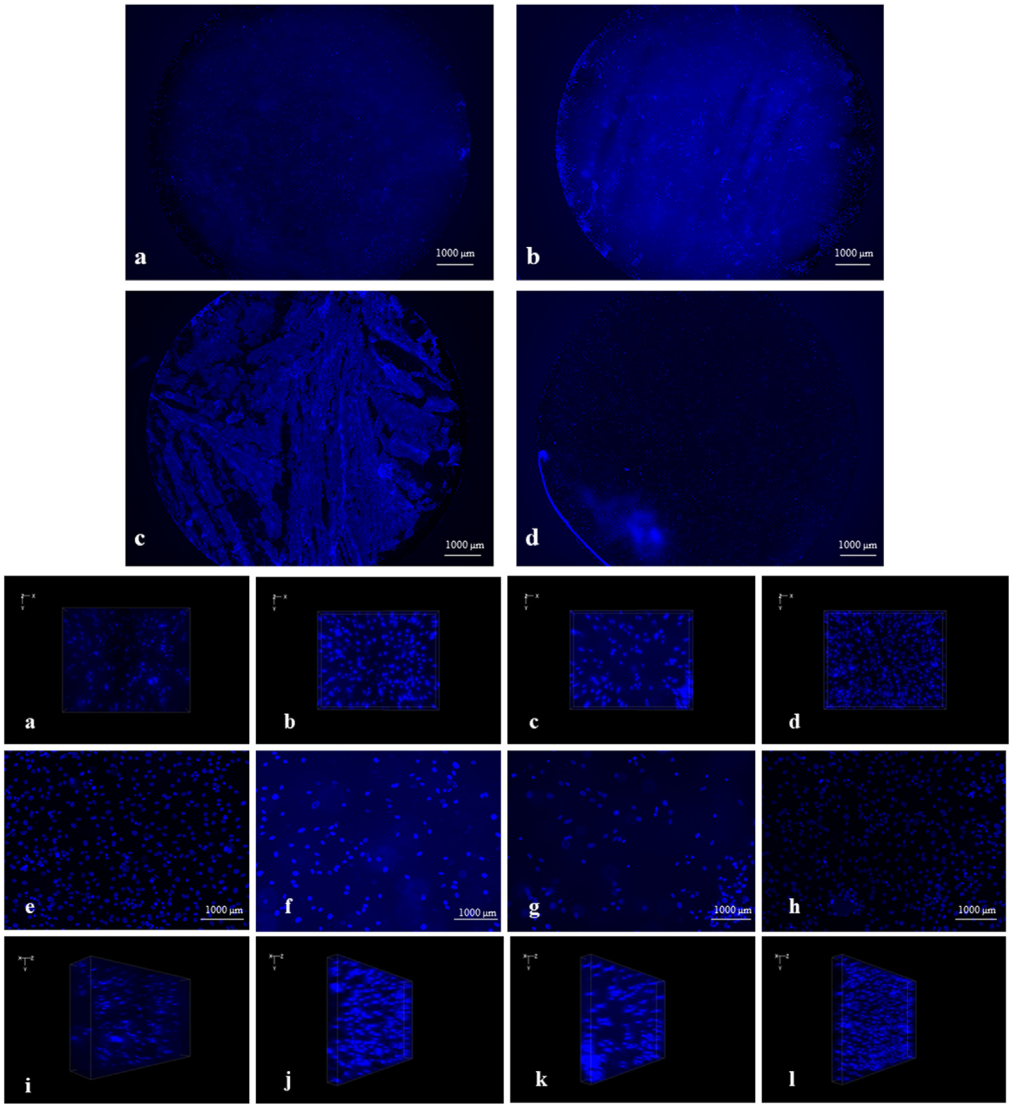

Figure 4 **A: DAPI fluorescent stained images of MC3T3-E1 cells cultured for 3 days on top of the membranes. a:** 2:1 G-HAp membrane, **b:** 2:1.5 G-HAp membrane, **c:** 2:2 G-HAp membrane, and **d:** 2:0 G-HAp membrane. **B: DAPI fluorescent 3D-images of MC3T3-E1 cells cultured for 3 days on top of the membranes. The vertically arranged pictures groups (a, e, i), (b, f, j), (c, g, k), and (d, h, l) represent a, e, i:** 2:1 G-HAp membranes, **b, f, j:** 2:1.5 G-HAp membranes, **c, g, k:** 2:2 G-HAp membranes, **d, h, l:** 2:0 G-HAp membranes, respectively. **The horizontally arranged pictures groups (a—d), (e—h), and (i—l) represent a-d: overview image, e-h: high-magnification image, and i-l: cross section image, respectively.**

a lower gelatin layer (Fig. 2). The surface of the membrane was wavy on the top (Fig. 2 a, d, g) and rough at the bottom (Fig. 2 c, f, i), and the cross-section area of the mesoregion between the upper and lower layers is presented in Fig. 2 b, e, h.

SEM observations of MC3T3-E1 cell adhesion on the gelatin membranes

After cell culture for 1 (Fig. 3a) and 3 days (Fig. 3b), the cells on the surface of 2:0 G-HAp membrane were observed using SEM. The surface of gelatin became rough, and cells proliferated on the gelatin membrane during the 3 days of culture (Fig. 3b).

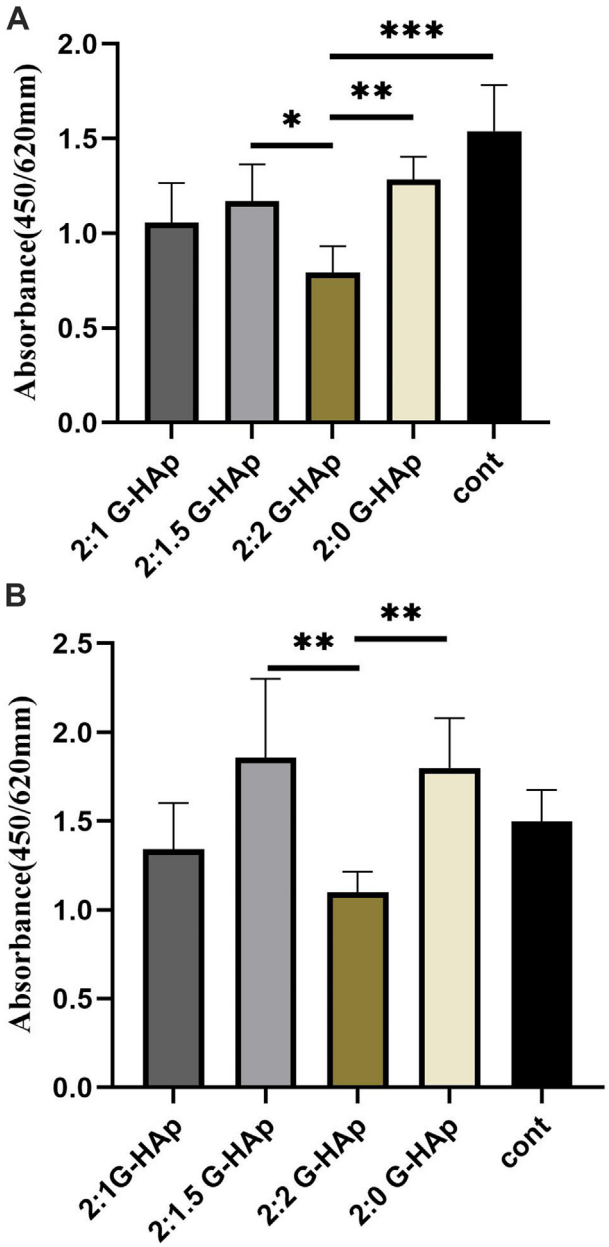

Figure 5 Cell proliferation on day 1 (A) and day 3 (B). Cell proliferation on 2:1.5 G-HAp membrane was the highest among the different membranes.

Nuclear staining and fluorescent confocal laser microscopy

The results of DAPI fluorescent staining of G-HAp membranes are shown in Fig. 4A, wherein the stained nuclei of cells grown on the top of the membranes can be seen (a: 2:1 G-HAp; b: 2:1.5 G-HAp; c: 2:2 G-HAp; and d: 2:0 G-HAp) on day 3 ($\times$ 4 magnification). The migration state of cells cultured on different G-HAp membranes, 2:1 G-HAp (a, e, i), 2:1.5 G-HAp (b, f, j), 2:2 G-HAp (c, g, k), and 2:0 G-HAp (d, h, l), are shown in Fig. 4B.

Cell viability and proliferation

Cell proliferation on day 3 was significantly higher than that on day 1 in all groups. Notably, cell proliferation on 2:1.5 G-HAp on day 3 was significantly higher than that on 2:2 G-HAp (Fig. 5).

Cytotoxicity

The lactate dehydrogenase assay showed no significant difference among the G-HAp membranes and negative control (Fig. 6).

Alkaline phosphatase-positive area

The results of ALP staining are shown in Fig. 7A. After culturing MC3T3-E1 cells for 10 days, the ALP activity of cells at the bottom of the culture dish was detected after removing the membranes. The ALP-stained areas of cells cultured on 2:0 G-HAp membrane and control were similar

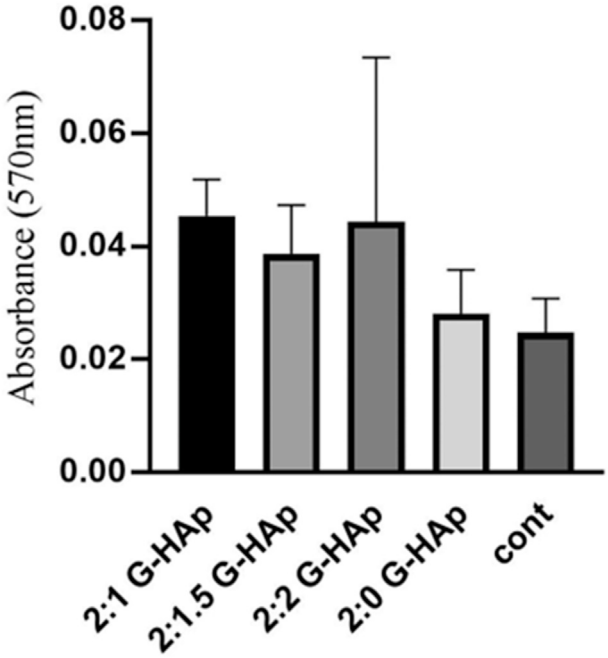

Figure 6 Cytotoxicity test of fish gelatin membrane and different G-HAp membranes. There was no significant difference in cytotoxicity between the G-HAp membranes and fish gelatin membranes compared to the control group. ($P < 0.05$).

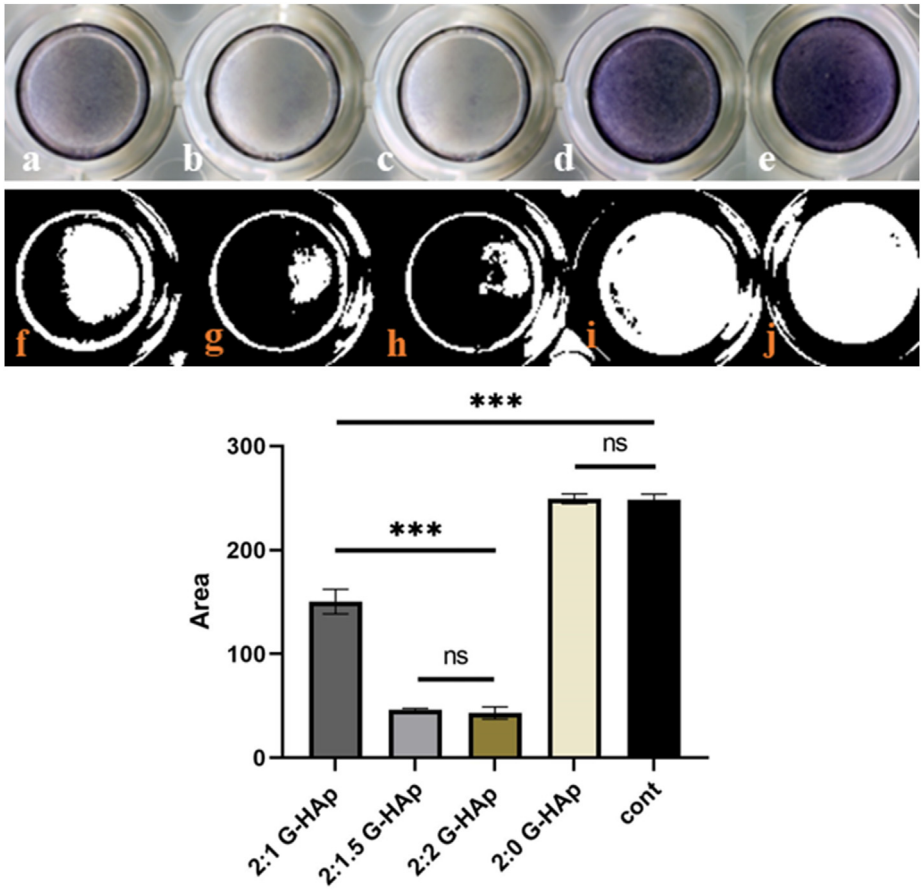

Figure 7 ALP staining after 10 days of culture. A: ALP-positive area (blue); **a, f:** 2:1 G-HAp membrane, **b, g:** 2:1.5 G-HAp membrane, **c, h:** 2:2 G-HAp membrane, **d, i:** 2:0 G-HAp membranes. **e, j:** control group. **(a—e:** staining area, **f—j:** measuring range). **B:** Calculation using ImageJ software.

(Fig. 7A: d, e), whereas the 2:1, 2:1.5, and 2:2 G-HAp groups showed a significant decrease or low response compared to the control group (Fig. 7A: a, b, c). The 2:1 G-HAp group showed significantly higher staining intensity than the 2:1.5, and 2:2 G-HAp groups (Fig. 7B).

Calcium deposition

Alizarin red staining at 21 days (Fig. 8A) showed that 2:1 G-HAp membrane was the best in terms of calcium deposition (Fig. 8B).

Discussion

We evaluated the preliminary properties of fish gelatin as a new source of gelatin membranes cross-linked with HAp. Both HAp and gelatin can be used as biomaterials for bone regeneration.[23,24] However, the existence of some deficiencies in the commonly used sources of gelatin inspired us to conduct this study.[23,24]

Fish-derived gelatin has a unique property of gelling at low temperatures and distinct gelation properties owing to its specific composition of amino acids.[25] Although some studies have reported a lower gel strength of fish-derived gelatin than porcine or bovine gelatin,[26] other studies have suggested that fish-derived gelatin may have superior strength, and higher flexibility, elasticity, and heat resistance.[27] Gelatin strength, the ability of gelatin to form a stable gel under specific conditions, is measured by the force required to deform the gel. Gelatin, with a high gel strength, can form a strong and rigid gel that is suitable for various biomedical applications owing to its antimicrobial properties, rigidity, and persistence. Here, the G-HAp membranes derived from porcine gelatin degraded and collapsed in the culture medium within 24 h, whereas the fish-derived G-HAp membranes maintained their shape even after 21 days of culture. However, the melting point of porcine gelatin is generally 20—30 °C, and the melting point of fish gelatin is even lower. This is due to the lower hydroxyproline content in fish gelatin than in porcine gelatin. Thus, future comparative experiments using porcine-derived gelatin from other manufacturers are also considered necessary. The fish gelatin evaluated was produced from the skin and bones of tilapia from warm-water regions, such as Brazil and Thailand. Gelatin from the skin of cold-water fish has higher strength than that from the skin of warm-water fish. The strength of gelatin can be influenced by other factors, including type of fish, specific part of fish used, process temperature, and extraction techniques. Therefore,

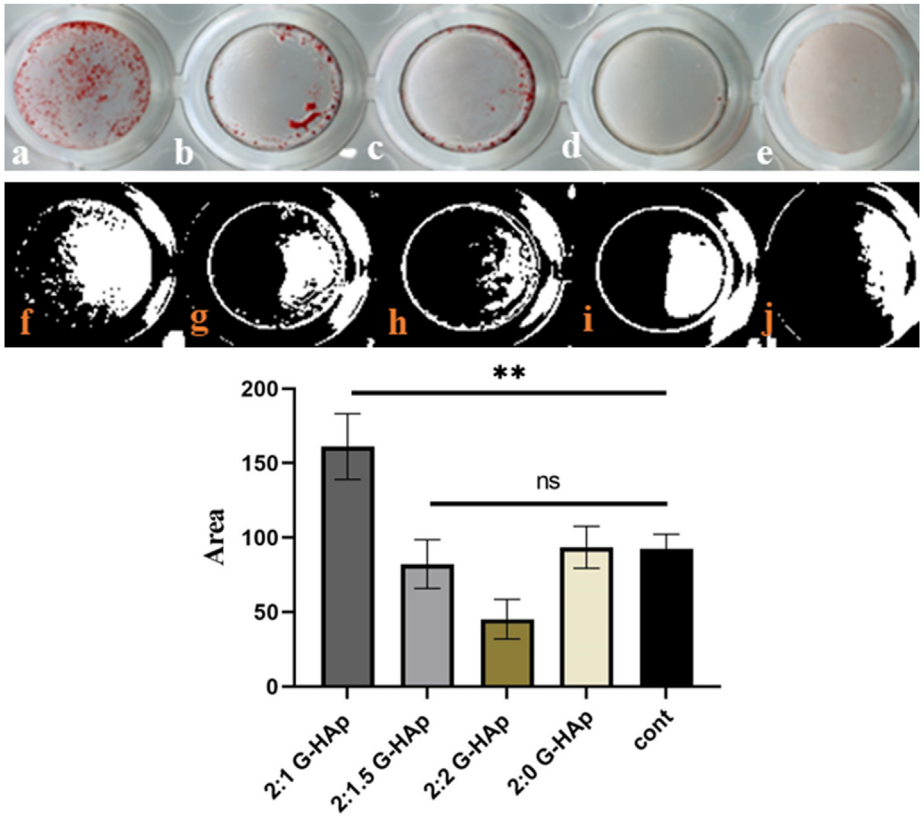

Figure 8 Mineralized nodule formation. A: Calcium nodules (red) were observed using alizarin red S staining on day 21. **a, f:** 2:1 G-HAp membrane, **b, g:** 2:1.5 G-HAp membrane, **c, h:** 2:2 G-HAp membrane, **d, i:** 2:0 G-HAp membrane. **e, j:** control group. (**a—e:** staining area, **f—j:** measuring range.). **B:** Calculation using ImageJ software.

considering the relevant factors, it is necessary to conduct further experiments to determine the optimal conditions for producing membranes for bone regeneration.

Additionally, fish-derived gelatin can be obtained as a byproduct of fish processing, thereby developing ethical products from a circular economic perspective. Therefore, fish-derived gelatin has lower environmental impact than conventional gelatin.[28,29]

A comparison of the properties of fish gelatin before and after crosslinking has shown that fish gelatin does not significantly change after heat crosslinking.[30] Therefore, fish gelatin can be considered to have relatively stable crosslinking characteristics.[31]

The thicknesses of the crosslinked fish-gelatin membranes formed using four different concentrations of HAp here was the same as those of commercial collagen membranes. The microscopic characteristics of the crosslinked membrane of fish gelatin and HAp showed a rough and wavy surface with a network space, allowing cells to proliferate and migrate into the membrane.[32,33] Cells cultured on the membrane were observed inside the membrane on day 3 and expressed ALP at the bottom of the well plate on day 10. This finding indicates that cells passing through fish G-HAp-crosslinked membranes may have osteogenic potential.[34] Although the positive result in the ALP test was the best for the 2:0 G-HAp and negative control group, 2:1 G-HAp membrane showed significantly higher staining intensity than the 2:1.5 and 2:2 G-HAp groups.

Notably, the calcium deposition results for the 2:1 HAp group was the most significant. However, the membrane was removed for both ALP and alizarin red staining, and cells in the higher-concentration G-HAp membranes might be trapped by HAp and could not pass through it. Cytotoxicity tests showed no significant difference among the groups, suggesting the HAp and fish gelatin membranes were nontoxic.[35]

Overall, fish gelatin is a potential alternative to porcine or bovine gelatin, and fish-derived G-Hap membrane containing less than half the total amount of HAp is favorable for osteogenic differentiation and mineralization with calcium deposition.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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