

Research Article

Fish Collagen Promotes the Expression of Genes Related to Osteoblastic Activity

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Tilapia type I atelocollagen (TAC) is a strong candidate for clinical application as its biological scaffold due to a high degeneration temperature and biologically safe properties. The aim of this study was to confirm the biological effects of TAC *in vitro* on osteoblastic cells, simulating its clinical application. The proliferation and differentiation of typical preosteoblasts, MC3T3-E1 cells, were investigated using a microarray analysis, staining assay for mineralization, and real-time PCR analysis of the expression of mineralization-related genes. The mRNA expression of 10 genes involved in proliferation and differentiation increased after 3-day culture on an TAC gel, with an average balanced score ratio exceeding 1.5 compared to the control. After two weeks of culture, all three experimental groups showed stronger alkaline phosphatase staining than after one week. The genes expression of alkaline phosphatase, osteocalcin, and bone sialoprotein increased under the experimental conditions. The gene expression of osteopontin did not increase, and no statistical differences were noted among the three experimental groups. The present and previous findings suggest that TAC is not only a suitable alternative to collagen products originating from mammals but also a novel biomaterial with cell differentiation ability for regenerative medicine.

1. Introduction

The research and development of nonmammalian natural biomaterials will improve the safety of scaffold and carrier in regenerative medicine, which are meaningful and beneficial, due to the global prevalence of dangerous infectious diseases (zoonosis) such as bovine spongiform encephalopathy, avian and swine influenza, tooth-and-mouth disease in cows, pigs, and buffalos, Ebola hemorrhagic fever, and Zika fever [1]. Furthermore, collagen generated from porcine and bovine sources cannot be used due to religious objections in some countries. Fish, which are the most genetically distant relatives from mammals, have recently attracted attention as an alternative source of collagen. Fish collagen (FC) derived from the scales, skin, and bone has interested many laboratories worldwide for its bioactive properties, such as excellent biocompatibility, low antigenicity, high biodegradability, and cell proliferation potential [2, 3].

The weakest point of fish collagen is its relatively low denaturation temperature (T_d), which indicates a poor stability in clinical applications. However, our recent experiment using calorimetry revealed that T_d of collagen solution derived from the tropical fish, tilapia (tilapia type I atelocollagen [TAC]), was 35–36°C [4], indicating that the abovementioned weak point has been completely overcome. In addition, the biological safety of TAC has been confirmed using various test methods recommended with ISO standards [5]. The long-term storage of TCA under frozen conditions for over 1 year has already been demonstrated in our laboratory (unpublished data). These data and findings strongly support TAC as a candidate for clinical application as a biological scaffold.

In the present experiment, we confirmed the biological effects of TAC *in vitro* on osteoblastic cells simulating its clinical application as a suitable alternative to collagen products

TABLE 1: Primers designed for RT-PCR analyses of genes related to cell proliferation and differentiation.

Gene name	Oligonucleotides
Matrix metalloproteinase 13 (Mmp13)	Forward: AGGCCTTCAGAAAAGCCTTC Reverse: GGTCCTTGGAGTGATCCAGA
Wnt inhibitory factor 1 (Wif1)	Forward: GAGTGTCCGGATGGGTCTA Reverse: TGGTTGAGCAGTTTGCTTTG
Receptor activity modifying protein 1 (Ramp1)	Forward: GCGGTATCCTCCTGAAAACA Reverse: CAGTCCTCCAGTTGGACCAT
SMAD family member 6 (Smad6)	Forward: ACGGTGACCTGCTGTCTCTT Reverse: AGCGAGTACGTGACCGTCTT
Platelet-derived growth factor, D polypeptide (Pdgfd)	Forward: TCAGCTGTGTGCTCAACAAA Reverse: ATTGGGCCTGGCTTACTTCT
Chordin-like 1 (Chrd1)	Forward: TGGTCTTTGCTTTCCCATGT Reverse: CCCAGGTGTTCTCTGAAAGC
Septin 4 (Sept4)	Forward: TTCAGGTCCAAAAGCCAGTT Reverse: TGACTTCCCTATCCCTGCTG
Fibroblast growth factor receptor 3 (Fgfr3)	Forward: ACAAGGACCGTACTGCCAAG Reverse: ACCTGGTAGGCACAGGACAC
Lumican (Lum)	Forward: TTCTCTCTTGCCTTGGCATT Reverse: CACTGCAGGTCTGTGACGTT
Vitamin D receptor (Vdr)	Forward: CGGAAATGGGTACCAAAATG Reverse: CACGTAGCAAGCGCTATGAA
Ribosomal protein S17 (rpS17)	Forward: GCATATCATGCAACGCTTTC Reverse: GGAGCTTCAGCATCTCCTTG

originating from mammals. The proliferation and differentiation of MC3T3-E1 cells, which are typical preosteoblasts, were investigated using a microarray analysis, staining assay for mineralization, and real-time PCR analysis (RT-PCR) to determine the expression of calcification-related genes.

2. Materials and Methods

2.1. Cell Culture for Passage. The used MC3T3-E1 cells derived from mouse calvaria were the typical preosteoblasts. They were cultured in a 100 mm dish at a density of 1.5×10^6 cells in α -MEM (Gibco, Palo Alto, CA, USA) with 10% fetal bovine serum (FBS) (MP Biomedicals, Santa Ana, CA, USA) and penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) and then incubated [6].

2.2. The Dissolution of TAC. TAC (0.6%) produced by solubilized tilapia skin was supplied by Nippi Inc., Biomatrix Institute (Ibaraki, Japan). A diluted 0.1% TAC solution in 1.5-fold concentration PBS (-) (pH 7.4) was used for the biological experiments.

2.3. Total RNA Isolation for Microarray. For FC gel group, 1 mL of 0.1% TAC was added to a 60 mm culture dish. TCA solution was gelled at 37°C for 30 min in a condition of 5% CO₂ and air. MC3T3-E1 cells were seeded in a 60 mm culture dish covered with or without TAC gel at a density of 1.5×10^6 cells. After 3-day culture, total RNA from cells was isolated

using TRIzol reagent (1 mL/10 cm²) (Invitrogen, Carlsbad, CA, USA) and purified in accordance with the manufacturer's instructions [6].

2.4. Microarray of Gene Expression for Checking Proliferation and Differentiation [6]. The cRNA was amplified and hybridized to an Affinity Mouse Genome 430 2.0 array (for 39,000 transcriptional products), scanned by an Affymetrix scanner, and calculated using the Affymetrix Expression Console™.

2.5. Filter Criteria for Data Analysis [6]. We then established the criteria for upregulated genes (Z score > 3.0, ratio > 1.5-fold).

2.6. RT-PCR following Microarray. The MC3T3-E1 cells were seeded and cultured ($n = 3$ for FC group, $n = 3$ for control group) under the same conditions as in the experiment for total RNA isolation for microarray. The primers are indicated in Table 1. The analyses of RT-PCR were carried out according to the similar methods to our recent study [6], except the following two steps. The first was that the protocol of amplification and quantification was 40 cycles (58-59°C, 1 min). The second was that, in the final calculations, the data were normalized by ribosomal protein S17.

2.7. Alkaline Phosphatase (ALP) Staining. For the FC gel group, 90 μ L of 0.1% TAC was poured into each well. TCA solution was gelled at 37°C for 30 min in an atmosphere of

TABLE 2: Primers designed for RT-PCR analyses of gene related to calcification.

Gene name	Oligonucleotides
Alkaline phosphatase (ALP)	Forward: GTTGCCAAGCTGGGAAGAACAC Reverse: CCCACCCCGCTATTCCAAAC
Osteopontin (OPN)	Forward: CATTGCCTCCTCCCTCCCGGTG Reverse: GCTATCACCTCGGCCGTTGGGG
Osteocalcin (OCN)	Forward: AGGGAGGATCAAGTCCCG Reverse: GAACAGACTCCGGCGCTA
Bone sialoprotein (BSP)	Forward: TGTCTGCTGAAACCCTTC Reverse: GGGGTCTTTAAGTACCGGC
Ribosomal protein S17 (rpS17)	Forward: GCATATCATGCAACGCTTTC Reverse: GGAGCTTCAGCATCTCCTTG

5% CO₂ and air. The MC3T3-E1 cells were cultured in a 24-well plate at a density of 5×10^4 cells/well and incubated for 1 or 2 weeks. The following four groups ($n = 3$ wells for each group and each week) were designed for comparison: (1) the control group (culture medium: α -MEM with FBS), (2) FC-positive group (culture medium: α -MEM with FBS), (3) FC-negative and BGP-positive group {culture medium: α -MEM supplemented with FBS, 50 μ g/mL ascorbic acid (Wako, LTD., Osaka, Japan), and 10 mM β -glycerophosphate ([BGP] nakarai tesque, Kyoto, Japan)}, (4) FC-positive and BGP-positive group (culture medium: α -MEM with FBS, 50 μ g/mL ascorbic acid, and 10 mM BGP). The medium was renewed every three days. The ALP Staining Kit (Stemgent, Inc., Cambridge, MA, USA) was used to stain for ALP expression in accordance with the maker's instructions.

2.8. RT-PCR for Calcification-Related Genes. For the FC gel group, 1 mL of 0.1% TAC was added to a 60 mm culture dish. TCA solution was gelled at 37°C for 30 min in a condition of 5% CO₂ and air. MC3T3-E1 cells were seeded in a 60 mm culture dish covered with or without TAC gel at a density of 1.5×10^6 cells. The abovementioned four groups were also prepared for this experiment ($n = 3$ for each group). After three days of culture, RT-PCR was applied to check the expression of four calcification-related genes: ALP, osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP). All the methods were similar to the abovementioned methods. The primer designs for these genes are represented in Table 2.

2.9. Statistical Analyses. All data were represented as the mean \pm standard deviation (SD). The statistical significance ($P < 0.05$) of the differences between the two different experimental groups was demonstrated using a paired Student's *t*-test.

3. Results

3.1. Genes Expression to Proliferation and Differentiation. Microanalysis revealed 72 of the upregulated genes (Z score > 3.0). The following 10 genes related to proliferation and differentiation of osteoblastic cells were compared with the control using the RT-PCR: vitamin D receptor (Vdr), lumican (Lum), matrix metalloproteinase 13 (Mmp13), SMAD family

TABLE 3: Genes expression represented in cell proliferation and differentiation.

Gene name	Z score	Ratio ^a	Confirmation ^b
Vdr	3.9	1.6	1.5
Lum	4.9	1.7	1.5
Mmp13	12.2	4.4	3.5
Smad6	3.7	1.5	2.0
Pdgfd	3.4	1.5	1.6
Wif1	6.3	2.0	5.1
Fgfr3	4.8	1.7	2.0
Sept4	6.5	2.1	2.3
Ramp1	4.3	1.6	3.2
Chrd11	6.0	1.9	3.9

^aThe change raw signal ratio. ^bConfirmed by the RT-PCR data (triplicate samples).

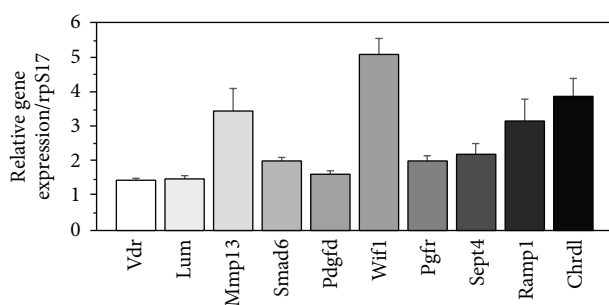


FIGURE 1: RT-PCR for 10 genes in relation to cell proliferation and differentiation. Each bar indicates the SD of triplicate samples.

member 6 (Smad6), platelet-derived growth factor D (Pdgfd), Wnt inhibitory factor 1 (Wif1), fibroblast growth factor receptor 3 (Fgfr3), septin 4 (Sept4), receptor (calcitonin) activity modifying protein 1 (Ramp1), and chordin-like 1 (Chrd11) (Table 3). The RT-PCR analyses revealed that the mRNA expression of these 10 genes also increased after 3-day culture on FC gel, with an average balanced score ratio exceeding 1.5 compared to the control (Figure 1).

3.2. Alkaline Phosphatase Staining (Figure 2). In group (1) after 2 weeks of culture, very-weak-intensity ALP staining

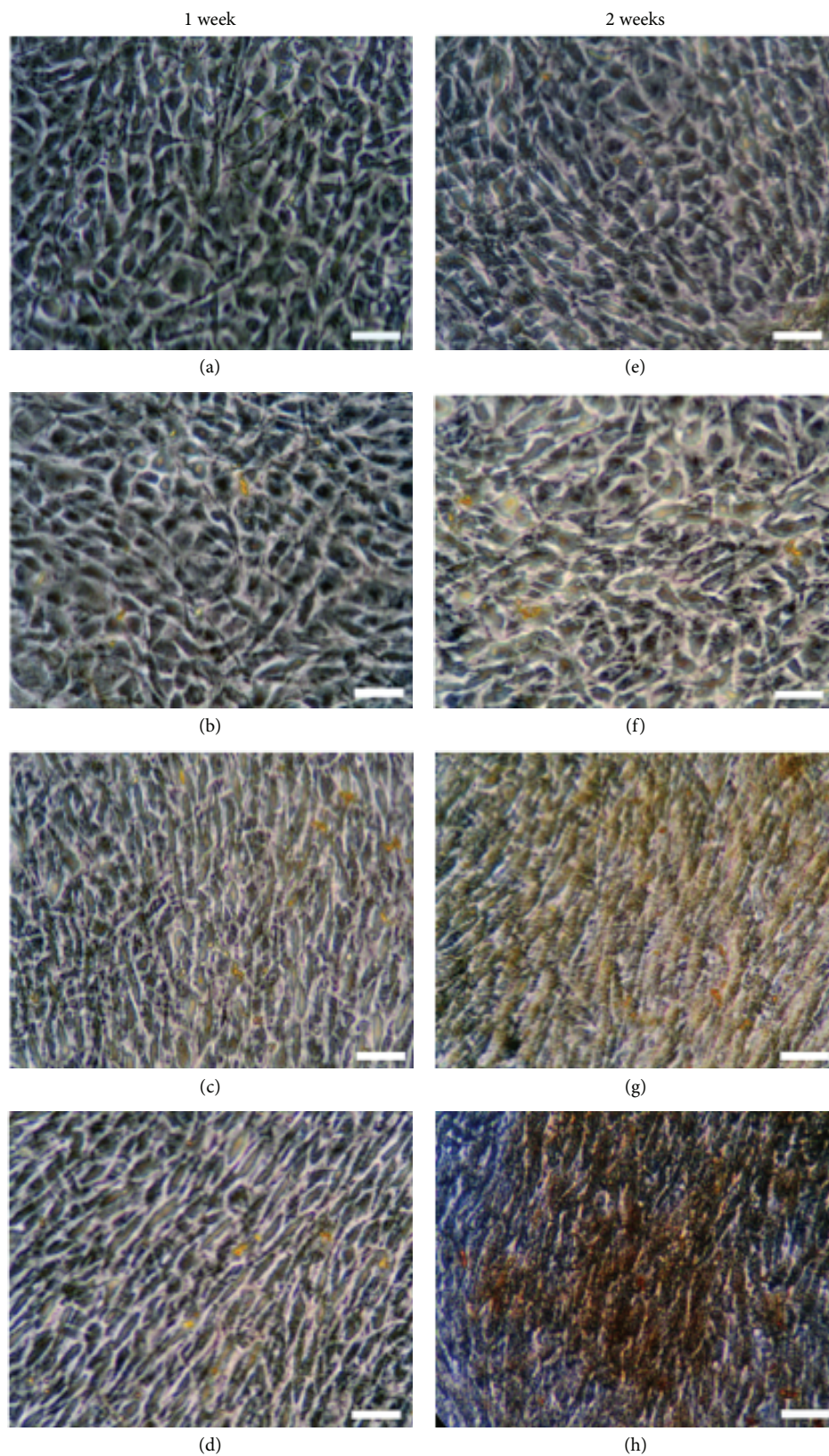


FIGURE 2: Representative ALP staining photographs after one or two weeks of culture: (a, e) FC-negative group, (b, f) FC-positive group, (c, g) FC-negative and BGP-positive group, and (d, h) FC-positive and BGP-positive group. ALP staining shows either a yellow or brown color. Although the typical pavement-like shape is observed at (a), (e), and (b), (f), on the whole, the cell morphology changes to a spindle-like cell shape (c, g, and d, h). Scale bar = 15 μm .

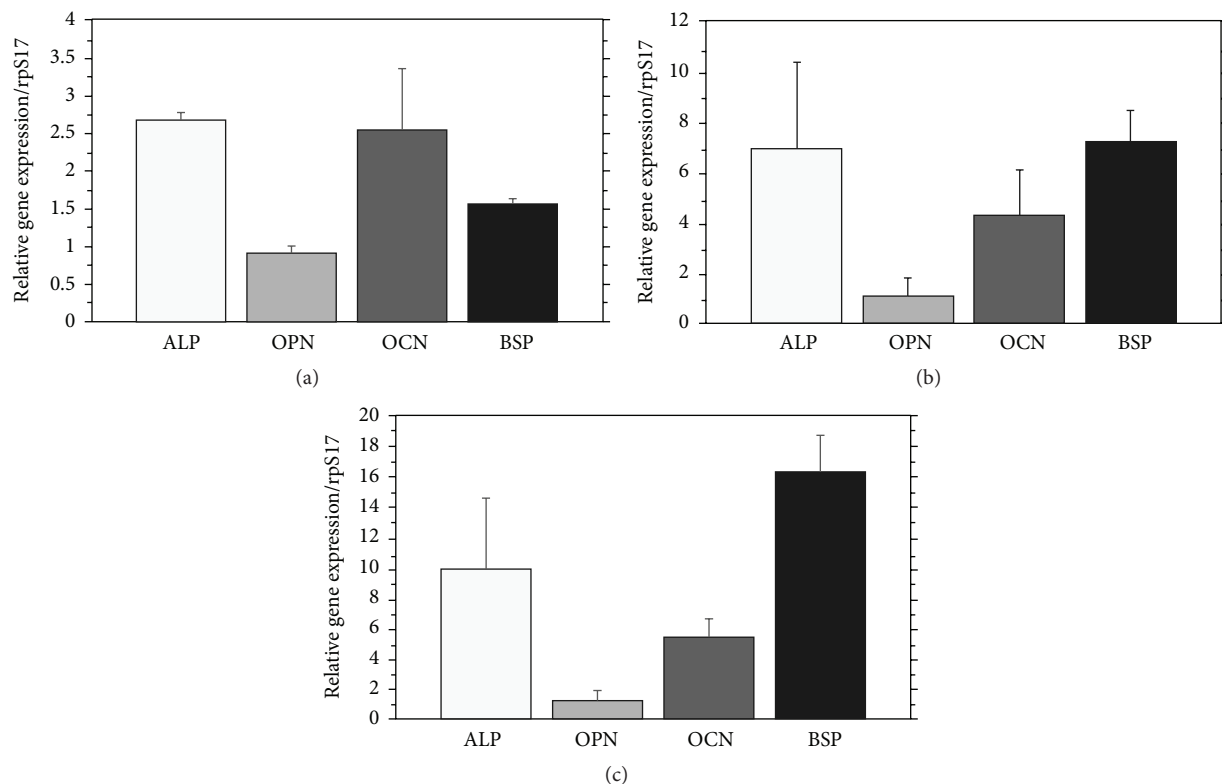


FIGURE 3: RT-PCR for four calcification-related genes: (a) FC-positive group (group 2), (b) FC-negative and BGP-positive group (group 3), and (c) FC-positive and BGP-positive group (group 4). Each bar represents the SD of triplicate samples.

was observed, similar to that in group (2) after 1 week. After 2 weeks of culture, each group showed stronger staining than after 1 week. On the whole, the spindle-like shape was observed in groups (3) and (4) (Figures 2(c), 2(g), and 2(d), 2(h)). The intensity of staining increased with increasing group number from group (2) to group (4).

3.3. Gene Expression for Calcification. The RT-PCR analyses demonstrated that the mRNA expression of four calcification-related genes in three experimental groups was analyzed after 3-day culture, compared to the control group (1) (Figure 3). Although the gene expression of ALP, OCN, and BSP increased with increasing group number from group (2) to group (4), a significant increase was recognized in BSP gene expression ($P < 0.01$). The raw data of OPN expression of OPN was around 1.0 in three groups, although there was a significant increase between group (2) and group (3) ($P < 0.05$).

4. Discussion

Cell culture method using the type I collagen gel was first reported by Elsdale and Bard [7]. The cells have well grown within a three-dimensional (3D) structure, as the conditions more closely resemble those of the *in vivo* condition of the osteoblast than the traditional two-dimensional (2D) cultures. Cells growth in 3D gels showed positive mineral staining and induction of osteoblastic marker genes earlier

than cell growth in 2D cultures [8]. Although the present culture conditions were not the exact 3D gel culture system, the present environment was remarkably similar to a 3D culture environment, as a low-concentration TCA solution was gelled to mimic wet and elastic conditions [9].

Microarray and RT-PCR analyses for checking the genes involved in proliferation and differentiation in MC3T3-E1 cells demonstrated that the Z score data using the standard deviations presented the effect of FC on MC3T3-E1 cells more clearly than the original ratio results. Ten genes, including Wif1, a secreted modulator of Wnt proteins [10], increased expressions compared with a control condition. This datum is the first to reveal that FC directly accelerates the activity of MC3T3-E1 cells in order to maintain the preosteoblast nature.

In the present experiment, the function of MC3T3-E1 cells was accelerated after three-day culture in FC-positive or BGP-positive group, which was confirmed by the increase of the expression of calcification-related genes, except OCN. ALP is an early expression marker essential for osteoblast proliferation [11], which was well demonstrated by the present ALP staining findings and the typical change in the cell shape. Furthermore, the time lapse between mRNA expression (3 days) and protein synthesis (7 days) seemed reasonable. Although OPN, OCN, and BSP are generally used as differentiation markers for osteoblasts [11–13], OPN was also reported to act as a mineralization inhibitor to regulate crystal growth [14, 15]. The present PCR data of OPN clearly demonstrated this osteoclastic function of OPN.

Although the intracellular signal transduction pathway and the related gene expression in osteoblast differentiation have been widely investigated [16–22], the direct trigger mechanisms for osteoblast proliferation and differentiation by collagen have not yet been clarified. Recently, an interesting study revealed that adhesion of human mesenchymal stem cells on tilapia collagen *in vitro* was faster and higher than that on porcine collagen or a noncoated surface, probably due to the texture flexibility and surface softness of the tilapia scale collagen, making the binding between collagen and integrin easier to trigger and stimulate both proliferation and differentiation of preosteoblasts [23]. A 0.1% TAC solution was used to make a gel in the present FC group. The nature of this gel is very soft, even after 2 weeks in the CO₂ incubator, and is thought to be possessed of sufficient flexibility and surface softness. This means that cultured cells may also behave as they would in a 3D culture system.

Proper biodegradation and suitable biocompatibility are indispensable for biomaterials. The biodegradation of collagen is closely linked to and dependent on the constitution of amino acids in a given species. Compared to bovine collagen, the contents of methionine, isoleucine, leucine, hydroxylysine, and lysine are lower in TAC [24]. As these amino acids are related to the collagen stiffness, the tilapia collagen can be easily degraded *in vivo*. The characteristics of these amino acids give TAC its abovementioned nature.

From our previous data [4, 5] and the present results, TAC may represent not only a promising alternative to mammalian and avian collagen products but also a novel biomaterial with cell differentiation ability. The final goal with TAC is its clinical application as a scaffold in regenerative medicine. The enzymatic digestion time for atelotreatment and the elimination of enzyme in TAC are controlled to a clinical level (personal communication). The preliminary experimental data concerning pulp regeneration [24] using TAC as a scaffold revealed no rejection reaction on the newly regenerated pulp tissue in dogs (unpublished data). These findings suggest that TAC may be useful for clinical application after further animal experiments.

5. Conclusion

The present *in vitro* experiments using osteoblasts clearly revealed that TAC is not only a suitable alternative to collagen products originating from mammals for cell proliferation and differentiation as the scaffold for regenerative medicine.

Competing Interests

The authors declare no conflicts of interests in association with this study.

Authors' Contributions

Mark Luigi Fabian Capati and Ayako Nakazono contributed equally to this work.

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