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Highlights

- Type A, B, and C gelatins were produced from a caviar by-product, sturgeon skin
- Type A and C gelatins had high gelatin content (>97%) and thick gelatin bands
- Type A and C gelatin gels (1%) crosslinked with 5 mM EDC were heat-stable
- These gels showed higher mechanical properties than porcine gelatin gel
- Type A gelatin showed the best mineral deposition in pre-osteoblast culture

Valorization of sturgeon skin to produce biomedical-grade gelatin

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Abbreviations:
AHG, type A head gelatin; ALP, alkaline phosphatase; ASG, acid-pretreated type A gelatin; BSG, alkali-pretreated type B gelatin; CSG, alkali-acid-pretreated type C gelatin; EDC, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; EU, endotoxin units; FBS, fetal bovine serum; LMM, low molecular mass; MEM, Minimum Essential Medium; MES, 2-(N-morpholino)ethanesulphonic acid; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; PSG, porcine skin gelatin; RGD, arginine-glycine-aspartic acid sequence

Abstract:

Gelatin, a collagen derivative used in tissue engineering, is often sourced from mammals; however, this raises concerns regarding diseases found in both humans and mammals. Therefore, this study aimed to valorize sturgeon skin, a processing by-product of caviar, to produce marine gelatin as a safe alternative to mammalian gelatin. To that end, acid-pretreated type A (ASG), alkali-pretreated type B (BSG), and alkali-acid-pretreated type C (CSG) gelatins were extracted from sturgeon skin. The ASG and CSG had high yield (dry weight basis) at 66.6% and 57.7%, respectively, with >97% gelatin content in samples, thicker α- and β-bands with minimal low molecular mass fragments in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and <0.2 endotoxin units per mg. A heat-stable (37 °C) gelatin gel was developed using 1% (w/v) gelatin with 5.0 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) crosslinking. The ASG and CSG gels showed higher elastic modulus and stress at break than porcine biomedical-grade type A skin gelatin (PSG). Proliferation of L929 fibroblasts, ATDC5 pre-chondrocytes, and MC3T3-E1 pre-osteoblasts on ASG, CSG, and PSG gels and control (cell culture well) were similar. The alkaline phosphatase activities of MC3T3-E1 cells cultured on the ASG, CSG, and PSG gels showed earlier activation than on the control, whereas mineral deposition was highest on the ASG gel. Considering the simple extraction, yield, and mineralization-inducing ability of MC3T3-E1 cells, ASG was the most suitable biomedical-grade gelatin alternative to mammalian gelatin. The present study proposes a novel valorization method for sturgeon skin to produce gelatin, which has biomedical applications.

Keywords: Valorization, sturgeon skin, gelatin, biomedical application

1. Introduction

Overusing natural resources promotes the search for alternate ways to meet human needs. A circular economy aligns with the Sustainable Development Goals (SDGs) by focusing on creating, preserving, and recovering natural resources in industries [1]. To achieve the economic, social, and environmental goals endorsed by SDGs in 2015, waste materials will be decreased through prevention, reduction, recycling, and reuse by Vision 2030 [2]. Thus, one of the nine blue changes of Asian aquaculture is defined by the Food and Agriculture Organization (FAO) as "repurposing, reuse and recycling, and the valorization of discarded waste" [3]. Waste or by-products from fisheries are on the rise owing to increased fish production and subsequent utilization [3]. For example, sturgeon aquaculture has increased globally to produce the primary commercial item caviar [4], but the remaining parts of sturgeon, sometimes even muscles, are discarded. Consequently, a large quantity of sturgeon processing by-products is produced. New fish by-product-based ingredients offer value-added, eco-friendly, and nutritious food options, reducing economic loss and environmental impact [3]. Thus, novel approaches of valorizing sturgeon by-products for zero industrial waste, blue transformation, and sustainability are required [5]. Leading valuable products obtained from aquatic by-products are collagen and its derivatives, such as gelatin and hydrolysates, and many studies have been carried out to produce them from fish heads, bone, scale, and skin [6–8]. In the case of sturgeon processing by-products, head, skin, swim bladder, cartilage, fin, and notochord have been previously used to prepare collagen [9–13], gelatin [14–16], and active peptides [5, 13, 17] for the food, cosmetics, pharmaceutical, and biomedical industries. However, few studies have explored the production of biomedical-grade gelatin for potential industrial applications, especially from aquatic sources.

Gelatin is a collagen derivative and single-stranded biopolymer widely used in tissue engineering, such as a biomaterial for cellular scaffolds [18, 19, 20] as a hydrogel [21] and cryogel [22]. It is less expensive than collagen and has identical amino acid residues [23]. Presently, gelatins used in tissue engineering are commonly produced from mammals [21, 24]. However, considering concerns about common diseases among humans and other mammals, marine-based gelatin is globally accepted as a safe and potential alternative [21].

Some studies have demonstrated the potential application of fish gelatin in tissue engineering. For example, gelatin from fish-scale collagen (fish species was not identified), modified using phenolic acid, followed by EDC crosslinking, showed high swelling ability, gel strength, biodegradability, and ability to support fibroblast adhesion and proliferation [25]. The study concluded that the gelatin was suitable for use in wound healing [25]. In addition, commercial cold-water fish gelatin has been used as a hydrogel for cell culture [21]. After crosslinking with horseradish peroxidase, the hydrogel exhibited high mechanical and biodegradable properties, supported fibroblast proliferation, and upregulated collagen and fibronectin gene expression [21]. Zeng et al. [22] used 0.3% (v/v) glutaraldehyde to crosslink 6% (w/v) commercial cold-water fish gelatin solution for injectable gelatin microcryogels. The microcryogels positively upregulated stemness gene expression and improved the secretion of growth factors from human adipose-derived stem cells [22]. Moreover, the microcryogels accelerated wound healing significantly in a mouse model [22]. The data highlight the potential utilization of fish gelatin as a biomaterial in tissue engineering.

Therefore, the aim of the present study was to develop a novel method for producing highly purified biomedical-grade gelatin with negligible contents of non-gelatinous components and low molecular mass (LMM) fragments from sturgeon by-products. First, the extraction methods of acid-pretreated type A (ASG), alkali-pretreated type B (BSG), and alkali-acid-pretreated type C (CSG) gelatins from sturgeon skin were optimized. Second,

crosslinking with EDC was performed to facilitate the use of gelatin in cell culture. Lastly, cell proliferation activities of fibroblasts, pre-chondrocytes, and pre-osteoblasts were evaluated. Moreover, cell differentiation activities were assessed using the pre-osteoblasts.

2. Materials and methods

2.1. Materials and reagents

Sturgeon heads and skin were obtained from an aquaculture farm (Bifuka Shinko Kosha, Bifuka, Japan) and stored at –20 °C. Type A sturgeon head gelatin (AHG) and purified collagen were prepared according to the methods in our previous studies [12, 14]. Biomedical-grade type A porcine skin gelatin (PSG) (cell culture grade, bloom value ~ 2.9 N) was obtained from Sigma-Aldrich (St. Louis, MO, USA), Endospecy® ES-24S Kit (Lot # 758115) was obtained from Seikagaku Corporation (Tokyo, Japan), and Control Standard Endotoxin (CSE, Lot No. 249025, 10 ng/vial) was obtained from Associates of Cape Cod Inc. (East Falmouth, MA, USA). Mouse fibroblast L929 (RCB1451) and pre-chondrocyte ATDC5 (RCB0565) cells were obtained from RIKEN (Tsukuba, Japan), and pre-osteoblast MC3T3-E1 cells (Subclone-4) were obtained from ATCC (Manassas, VA, USA). Cell culture media and serum were purchased from Gibco (Grand Island, NY, USA), Sigma Aldrich, and Life Technologies Corporation (Grand Island, NY, USA). Other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise notified. All reagents used were of analytical grade.

2.2. Production of gelatins

A mixture of kalamtra (*Huso dauricus* × *Acipenser schrenckii* × *Acipenser transmontanus*) and bester (*Huso huso* × *Acipenser ruthenus*) sturgeon skin [5] was used to extract ASG, BSG, and CSG, as outlined in Supplementary Fig. 1. Skin samples were heated 50 °C for 30 min and blended for 30–50 s (Supplementary Fig.1). Such procedures soften the pretreated sturgeon skin and facilitate a short extraction time with minimum pretreatment with acid and alkali chemicals. Type A head gelatin (AHG) was extracted from kalamtra sturgeon heads, as described in our previous study [14], with minor modifications. In the present study, bones and scales were removed from the mixed head tissues for gelatin extraction. The yield was calculated on a dry weight basis.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

After dissolving the gelatin (w/v 0.2%) in distilled water, heating at 50 °C for 20–30 min, and mixing by pipetting, SDS-PAGE (7.5% gel) and Tricine-SDS-PAGE (15.5% gel) were performed as described in our previous study [14]. Using captured images of the 7.5% SDS-PAGE gels and Image-J version 1.52 (National Institutes of Health, Bethesda, MD, USA), the intensities of the gelatin bands (α, β, and γ) and LMM bands (<100 kDa) were quantified. For comparison, PSG (cell culture grade, bloom value ~ 2.9 N; Sigma-Aldrich, St. Louis, MO, USA) was used as the standard.

2.4. Amino acid composition and gelatin content in the total protein fraction

The amino acid composition and gelatin content in the total protein fraction of gelatins were quantified as described in our previous study [14]. Briefly, triplicates of each gelatin sample were mixed and hydrolysed in 6 M HCl at 110 °C for 24 h. Furthermore, the

hydrolysates were dried via evaporation and dissolved in a citric acid buffer solution, after which the amino acid concentration was quantified using an automated amino acid analyser (L-8900; Hitachi High-Technologies Corporation, Tokyo, Japan). Tryptophan was not quantified because it cannot be detected using the method.

Gelatin content (%) in the total protein fraction (GCp) was calculated as Hyp in the sample per Hyp in the purified collagen, where Hyp is the number of hydroxyproline residues per 1000 amino acid residues. Hyp is completely collagen (gelatin)-specific amino acid and used to calculate the gelatin content in both protein fraction and total sample fraction of gelatin samples [14]. Purified collagen obtained from a mixture of bester and kalamtra sturgeon skin [12, 14] was used as a standard and defined to contain 100% collagenous proteins.

2.5. Proximate composition and gelatin content in the total sample fraction

The proximate composition of samples was measured according to the method of Islam et al. [14]. Briefly, the gelatin content (GCs) and non-gelatinous protein content (NGCs) in samples were calculated using the following formulae:

$$\text{GCs (\%)} = [\frac{\text{Hyp of sample}}{\text{Hyp of purified collagen}}] \times 100$$

$$\text{NGCs (\%)} = 100\left(\frac{\text{GCs}}{\text{GCp}}\right) - \text{GCs}$$

where Hyp is the hydroxyproline content (nmol/µg dry weight of the sample). GCp is the gelatin content in total proteins, calculated as above.

The other components, such as lipids and ash, were not considered in the analyses because they were only present in trace amounts, except for head gelatin, which contained a significant amount of ash [14].

161

162 *2.6. Endotoxin level*

163

164 The endotoxin levels in gelatin samples were measured using a Limulus Amebocyte
165 Lysate assay (Endospecy® ES-24S Kit), according to the manufacturer's instructions. The
166 control standard endotoxin (CSE) was obtained from *Escherichia coli* O113:H10.

167

168 *2.7. Rheological properties of gelatins*

169

170 The gelling and melting temperatures of the gelatin samples were measured following
171 the method used by Islam et al. [14]. Briefly, 2.5 mL of the gelatin solution (w/v 6.67%) was
172 transferred to each test tube (7.5 cm height × 1.2 cm diameter), and each test was repeated
173 three times. The temperatures were decreased or increased at a rate of 0.5 °C/min in an
174 incubator (FMU-054I; Fukushima Industries Corp., Osaka, Japan). PSG was used as the
175 standard.

176

177 *2.8. Preparation, crosslinking, and thermal stability analysis of gelatin gels*

178

179 To create a gel, gelatin was dissolved in endotoxin-free water at 1% (w/v), heated at
180 50 °C for 20–30 min, and pipetted to mix well. Subsequently, the gelatin solution (225
181 µL/well) was pipetted to a 24-well culture plate and kept overnight at 4 °C to form a gel. The
182 gel thickness was 1.2 mm.

183 For crosslinking, 1 mL of 0.2 mM, 1 mM, 5 mM, or 10 mM EDC in 0.1 M 2-(N-
184 morpholino) ethanesulfonic acid (MES) buffer (pH 5.5) was added to each well and kept 1 h
185 at 4 °C, followed by 6 h at room temperature (21 °C). The efficacy of EDC and gel stability

were determined by observing the melting of gels after incubating at 37 °C for 48 h, according to the method of Luo et al. [26], with minor modifications.

2.9. Mechanical properties of gelatin gels

The mechanical properties of gelatin gels (1%) were tested in compression and tensile modes using a texture analyser (TA.XT Plus; Stable Micro Systems, USA). Gelatin gels were prepared in 35-mm diameter dishes (2.25 mL/dish) and crosslinked with 5.0 mM EDC as described above. A flat-head probe (10 mm diameter, sensitivity load of 0.01 N, and crosshead speed of 0.2 mm/s) was used to draw a stress-strain curve of each sample. The elastic modulus (kPa) was calculated using the stress-strain curve, and the stress at break (kPa) was recorded to show gel strength according to the method of Poursamar et al. [27].

2.10. Cell culture experiments

Fibroblast L929, pre-chondrocyte ATDC5, and pre-osteoblast MC3T3-E1 cells were used in the present study. Cell culture experiments were conducted, as reported in Islam et al. [5] and Moroi et al. [10]. All cells were grown in 24-well culture plates with a density of 1.2 $\times 10^4$ cells/well. The growth media of each cell line were as follows: Minimum Essential Medium (MEM, Gibco, Grand Island, NY, USA) supplemented with 5% foetal bovine serum (FBS, Gibco) for L929; Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12, Sigma Aldrich) supplemented with 1.25% of 2 mM L-Glutamine (Gibco) and 5% FBS for ATDC5; and MEM-alpha (MEM-α, Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% FBS for MC3T3-E1. MC3T3-E1 cells were seeded and maintained in the growth medium for one day. Afterward, the growth medium

was replaced with the osteogenic medium, which was supplemented with 10 mM β-glycerophosphate (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and 0.285 mM L-ascorbic acid (Fujifilm Wako Pure Chemical Corporation). All media were supplemented with a mixture of 1% (v/v) penicillin and streptomycin (Thermo Fisher Scientific, Grand Island, NY, USA) and renewed at 3-day intervals. The cell morphology was observed under a phase-contrast microscope (DMI600B; Leica, Wetzlar, Germany) during the cell incubation periods.

2.11. Cell proliferation assay

The viable cell number was determined by measuring the DNA content. The culture media were discarded, and cells were rinsed twice with phosphate-buffered saline (PBS, pH 7.4). Following this, 300 µL/well of PBS with 0.4% (w/v) papain was added to gelatin gel wells, and the gels were hydrolysed at 37 °C for 2.5 h in a cell culture incubator. The cell suspension of each well was collected in plastic tubes and centrifuged at 12 000× *g* for 10 min at 4 °C to obtain cell pellets. The pellets were washed thrice with PBS and stored at –20 °C until use. The cells in control wells without the gelatin gels were rinsed with PBS and incubated in PBS at 37 °C for 2.5 h in a cell culture incubator. After discarding the PBS, cell culture plates were stored at –20 °C.

To measure DNA content, samples in plastic tubes or in the cell culture wells were thawed at 21 °C for 30 min. Cell lysate buffer [0.5% (v/v) proteinase K solution with 0.01% (w/v) SDS in PBS] was added (225 µL/tube or 225 µL/well), pipetted 10–12 times, and incubated for 1 h at 21 °C to digest the cells. The samples were diluted with PBS when necessary. The Hoechst 33258 solution (2 µg/mL) was added to each sample, and DNA content was measured using a fluorescent plate reader (ARVO X4; PerkinElmer, Waltham,

MA, USA) with an excitation/emission wavelength pair of 355 nm/460 nm. Salmon sperm DNA was used as the standard.

2.12. Alkaline phosphatase (ALP) assay

ALP activity, a general marker of osteoblast differentiation, was measured according to the method used by Nagai et al. [28] with minor modifications. Briefly, the culture medium was discarded, and the cells cultured on gelatin gels were washed twice with PBS. Subsequently, the gels were mixed with 225 µL/well of lysis buffer (0.5% Triton X-100, 150 mM NaCl, and 10 mM HEPES) for 1 h at 4 °C. The lysis buffer solution with a gel in each well was then transferred to plastic tubes and homogenized on ice at 10 000 rpm (Physcotron; Nition Irika Kikai Seisakusho Co., Ltd., Tokyo, Japan). The supernatant was obtained via centrifugation (21 000× *g* for 15 min at 4 °C) using a CF15RXII centrifuge (Hitachi, Koki, Tokyo, Japan) and used for the measurement of ALP activity and DNA content. For ALP measurement, the supernatant was incubated with ALP substrate buffer (4 mg/mL *p*-nitrophenyl phosphate, 0.02 M $MaCl_2$, 0.1 M Tris-HCl at pH 8.8) at 37 °C for 30 min. After stopping the reaction with the addition of 0.2 M NaOH, the absorbance was measured at 405 nm using a microplate reader (Infinite F50; Tecan Japan, Kawasaki, Japan). ALP activity was quantified using a standard curve with *p*-nitrophenol (*p*-NP) as the standard and normalized with the DNA content. The DNA content (mg) was measured as described in Section 2.10.

2.13. Mineralization assay

To assess the differentiation of pre-osteoblastic MC3T3-E1 cells to osteoblasts, cells were cultured in the osteogenic medium, and calcium deposition was assessed using the

method of Tevlek et al. [29] with minor modifications. In brief, after the osteogenic medium was discarded, the wells were washed twice with PBS. Cells were fixed with 70% ethanol for 1 h on ice, washed with distilled water, and stained with 40 mM Alizarin Red S solution (pH 4.2; Wako Pure Chemical Industries Ltd., Osaka, Japan) for 15 min at 21 °C. The samples were then washed thrice with distilled water at 1-h intervals and dried at 45 °C for 30 min. The calcium deposition that reacted with Alizarin Red S was directly imaged under a stereomicroscope (Nikon SM 2800; Nikon Corp., Tokyo, Japan).

Calcium deposition was quantified after solubilizing the stain with 1 mL of 10% (w/v) cetylpyridinium chloride at 37 °C for 30 min with gentle shaking. Subsequently, 100 µL of each sample was transferred to a 96-well plate, and the absorbance was measured at 570 nm using a microplate reader (Infinite F50). A mixture of Alizarin Red S stock solution and 10% (w/v) cetylpyridinium chloride solution was used as the standard.

2.14. Data analysis

Samples of the three replicates were mixed for SDS-PAGE, Tricine SDS-PAGE, and amino acid analysis. Data are presented as the mean ± SE, if applicable. One-way ANOVA and the Tukey-Kramer test were performed using Microsoft Excel add-in statistical software version 2.12 (Social Survey Research Information Co., Ltd., Tokyo, Japan) for statistical comparisons. The significance was set at $p < 0.05$.

3. Results and discussion

In the present study, yield, proximate composition, and endotoxin levels of the gelatins were compared with type A head gelatin, which was the highest-quality gelatin from sturgeon

heads reported in our previous study [14]. Biomedical-grade type A porcine skin gelatin (PSG) was used as the standard for comparison.

3.1. Yields of gelatins

The yields (%, dry weight gelatin/dry weight sample) of gelatins extracted from the sturgeon skin and head are given in Table 1. The yield was highest in ASG, followed by CSG, BSG, and AHG. Acid has a higher ability to swell the tissues than alkali [14, 15]; thus, the recoveries of ASG and CSG were significantly higher than that of BSG (Table 1). The lower yield of AHG than skin gelatins might be due to the dominance of collagen-poor muscle and gill tissues in the mixed tissue of the sturgeon head. Fish skins are reportedly the best source of gelatin due to the presence of an abundant amount of dermis. When the yields were re-calculated into dry-weight gelatin/wet-weight tissue, the yields of ASG (27.7 ± 0.5%), CSG (24.0 ± 0.8%), and BSG (8.1 ± 0.3%) were higher than those of Amur sturgeon skin gelatins (5.0–24.1%) [30–32], suggesting high extraction efficiency of the present method. In addition, the yields of ASG and CSG were significantly higher than that of camel skin gelatin (14.1%, dry weight gelatin/dry weight tissue [33], showing that sturgeon skin would be more suitable for industrial applications.

3.2. SDS-PAGE analysis

Although the same amount of gelatin samples (5 μg/lane) was loaded onto the 7.5% SDS-PAGE gel, the intensities of β-bands of gelatin were significantly higher in ASG than in the others (Fig. 1A, B). In contrast, the intensity of α-bands of gelatin was higher in BSG than in the others. These data showed that more covalent crosslinks between the α-chains

were destroyed after alkali pretreatment than acid pretreatment. However, γ-bands of gelatin were still visible on the SDS-PAGE gel in ASG and BSG, similar to the Amur sturgeon skin gelatins [31, 32]. The samples on the SDS-PAGE gel did not show a positive reaction to Alcian blue staining (data not shown), indicating that the contamination of the samples with acidic sugars, such as chondroitin sulfate and hyaluronic acid, was trace.

Compared with the commercial biomedical-grade PSG, all sturgeon gelatins showed thicker α-, β-, and γ-bands and much weaker bands of LMM fragments (<100 kDa) (Fig. 1A, B). There were no distinct LMM fragments (<30 kDa) in the sturgeon gelatins according to Tricine SDS-PAGE assay (Fig. 1C). The hydrolyzed collagen bands with a molecular mass of <30 kDa are not considered gelatin owing to a lack of gel-forming ability [34]. Thus, SDS-PAGE and Tricine SDS-PAGE data evidenced that the sturgeon skin gelatins had higher gelatin content with less amount of LMM fragments than commercial biomedical-grade PSG, implying much potential in biomedical applications.

3.3. Amino acid composition and proximate composition

The amino acid composition of the samples is shown in Table 2. The gelatin content in the protein fraction of samples (GCp) was somewhat similar, although a slightly higher value was observed in ASG, followed by CSG, AHG, and BSG (Table 2). The hydroxyproline residues of present samples (62–64 residues/1000 residues) were higher than the Amur sturgeon skin gelatin (39–51 residues/1000 residues) [31]. These data indicated that the gelatin content in the present samples was higher than that in the gelatins of Nikoo et al. [31] because hydroxyproline residues are specific for collagen and gelatin and serve as markers of their contents [14].

The gelatin contents in the total sample weight (GCs) is given in Table 3. ASG and CSG showed higher gelatin contents (>97.8%) than others. Alkali pretreatment destroys non-collagenous proteins without affecting collagen structure [35, 36]. Therefore, BSG may contain more non-collagenous peptides, resulting in lower gelatin contents (Table 3). Non-collagenous peptides may also be produced but were not included in CSG, possibly because the acid treatment after the alkali treatment washed out the peptides. The effect of acid pretreatment after the alkali pretreatment also restored CSG yield (Table 1) and gelatin content (Table 3). The lower gelatin content in AHG might be attributed to collagen-poor tissues in the sturgeon head [14]. Our study is the first to report fish gelatin samples with >97% gelatin content.

3.4. Endotoxin levels

The endotoxin levels [endotoxin units (EU)/mg] of samples are presented in Table 4. The lowest levels of endotoxin were observed in CSG and ASG, followed by in BSG, suggesting that the acid pretreatment was more effective in removing endotoxins in the skin sample than the alkali pretreatment. The higher endotoxin levels in AHG may originate from higher endotoxin contents in the head tissue. Although the usable limit of endotoxins may vary with sample application, the endotoxin levels should ideally be less than 0.5 EU/mL or 20 EU/device for a medical device directly or indirectly in contact with the cardiovascular and lymphatic systems [37, 38]. If the gelatin gel is used at 1%, the lowest endotoxin-containing CSG gel becomes 1 EU/mL; thus, further endotoxin removal must be considered in future research. As the endotoxins are derived from gram-negative bacteria contaminated with the raw material, immediate freezing of the skin for storage or processing of the skin for gelatin extraction is crucial. Additionally, using endotoxin-free water and devices in the

cleanroom for gelatin extraction may lower the endotoxin levels of gelatin. Moreover, the gelatin samples can be sterilized by supercritical CO_2 inactivation or by gamma radiation. Both techniques are employed for the sterilization of biomaterials [39, 40].

As AHG and BSG showed lower yields (Table 1), lower gelatin content (Tables 2 and 3), and higher endotoxin levels (Table 4) than ASG and CSG, further experiments were performed on ASG and CSG only.

3.5. Crosslinking of the gelatin gel

The melting temperature of fish gelatin is usually lower than a human's body temperature (37 °C). Thus, increasing the melting temperature to more than 37 °C is essential for the application of fish gelatin in tissue engineering. For this purpose, non-toxic chemical crosslinkers, such as genipin and EDC, are widely used to increase the stability of collagen- or gelatin-based biomaterials [9, 10, 24, 41]. Genipin is an excellent natural crosslinker [41] found in *Genipa americana* fruit extract; however, it produced an intense blue color after crosslinking with gelatins in the present study (data not shown). Thus, sturgeon skin gelatins were crosslinked with EDC.

ASG was used to determine the optimal EDC-crosslinking method as a representative sample (Supplementary Table 1). Before crosslinking, the gel-forming ability was tested using 0.5%, 1%, and 2% ASG solution (w/v). Samples were kept at 4 °C overnight. The 0.5% ASG did not make a gel, while 1% and 2% ASG made gels. The gel hardness increased when the ASG concentration was increased. Thus, the 1% ASG gel was used to determine EDC-crosslinking effects under a 0.1 M MES buffer at pH 5.5 (Supplementary Table 1). The gels crosslinked with 0.2 mM EDC melted after incubating at 37 °C for 48 h, while those crosslinked with 1.0 mM, 5.0 mM, and 10 mM EDC were stable at 37 °C (Supplementary

385 Fig. 2). The gels crosslinked with 1.0 mM EDC were softer than those crosslinked with >5.0
386 mM EDC. Scheffel et al. [42] found that collagen crosslinked with 0.1−0.5 M EDC was not
387 toxic to odontoblast-like cells. EDC is widely used to crosslink gelatins from other sources at
388 varying concentrations in acidic conditions [24, 43], but only a few studies report the
389 crosslinking of fish gelatin hydrogels with EDC or EDC coupled with N-hydroxysuccinimide
390 (NHS) [21, 25]. EDC-NHS has been used to increase the crosslinking efficiency of gels but
391 has a toxic effect [44]; thus, NHS was not used in the present study.
392
393 *3.6. Rheological and mechanical properties*
394
395 The gelling and melting temperatures are shown in Table 5. Fish gelatin is a widely
396 used biopolymer that commonly has lower rheological properties than mammalian gelatin
397 [45]. The fish samples also showed a lower gelling temperature than PSG (Table 5). ASG
398 showed a higher gelling temperature than CSG (Table 5) and Amur sturgeon skin gelatin
399 (13–14 °C) [30, 32]. The higher intensities of α- and β-bands (Fig. 1) as well as the higher
400 gelatin content in ASG (Table 3), all of which indicate a higher amount of undamaged gelatin
401 molecules in the sample, might be the reason for the higher gelling temperature in ASG than
402 in CSG because a similar correlation was observed in the head gelatins of sturgeon [14] and
403 skin and bone gelatins of Nile perch [46]. Hence, the amount of undamaged gelatin molecules
404 in the sample determines the gelling temperature of the gelatin.
405 The melting temperatures of ASG and CSG (Table 5) were higher than those of Amur
406 sturgeon skin gelatins (20.3–22.6 °C) [31, 32]. Moreover, in contrast to the gelling
407 temperature, the melting temperature of CSG was higher than that of ASG and PSG,
408 indicating that it was unaffected by the amount of undamaged gelatin molecules in the
409 sample. Possible stabilizing effects of the crosslinking among α-chains, which may be

partially destroyed by acid- or alkali-acid-pretreatments as indicated by the amount of β- and γ-bands in SDS-PAGE (Fig. 1), may not relate to the melting temperature as well. Therefore, there was an unknown factor affecting the melting temperature of CSG.

Without EDC-crosslinking, 1% gels fabricated from ASG and CSG showed lower elastic modulus and stress at break than PSG (Supplementary Table 2), suggesting that ASG and CSG formed softer and weaker gels than PSG. Without crosslinking, fish gelatins commonly showed weaker gels than mammalian gelatins, which is attributed to the lower amino acid content of fish gelatin [47]. With EDC-crosslinking, ASG and CSG gels showed significantly greater elastic modulus and stress at break than PSG gel (Table 6). This may be owed to the higher amount of undamaged gelatin molecules in ASG and CSG than PSG (Fig. 1) because the crosslinking among larger molecules may have contributed to the greater elastic modulus and stress at break. Recently, Michelini et al. [48] reported that the elastic modulus and stress of 15% (w/v) gelatin gels after crosslinking with 0.31 mM of N,N'-methylenebis (acrylamide) or glutaraldehyde were 29.7−42.4 kPa and <15−20 kPa in porcine skin, 14.2−14.3 kPa and <10 kPa in bovine skin, and 22.3−26.6 kPa and <10 kPa in cold-water fish skin, respectively. The elastic modulus of 1% ASG and CSG was close to that of bovine skin but lower than that of porcine and cold-water fish skin. In contrast, the values of stress at break of 1% ASG and CSG were much higher than those in the previous report. Therefore, ASG and CSG can be novel alternatives to mammalian and fish gelatins, which have higher stress at break. However, the desired mechanical properties of sturgeon gelatins can be manipulated easily after increasing or decreasing their concentrations and crosslinkers, providing more options in biomedical applications.

3.7. Proliferation and morphology of cells

435 The cellular activities on the ASG and CSG gels were compared with those on the cell
436 culture well without any gelatin (control well) and PSG gel.
437 Cell proliferation was assessed by measuring the DNA content after incubating each
438 cell line in its specific culture media. The L929 proliferation on the gelatin gels was slightly
439 slower than that on the control well (Fig. 2A). In contrast, MC3T3-E1 and ATDC5 cells did
440 not show significant differences in proliferative activity among the groups (Fig. 2B, C).
441 Gelatin gel from cold-water fish-skin or fish-scale collagen had no cytotoxic effects on L929
442 or NIH3T3 cells [25, 48]. Phenolic acid-modified gelatin from fish-scale collagen (after
443 crosslinking with 0.1 M caffeic acid, 0.2 M EDC, and 0.2 M NHS in 0.1 M HEPES buffer)
444 also induced the attachment and proliferation of NIH3T3 cells [25]. These data and the
445 present results suggest that the fish gelatin gel with EDC-crosslinking had no cytotoxic
446 activity.
447 Cell morphology is essential for understanding cell features and functions [10] in
448 tissue engineering. In the present study, L929 fibroblasts, ATDC5 pre-chondrocytes, and
449 MC3T3-E1 pre-osteoblasts on the PSG, ASG, and CSG gels showed distinct shapes,
450 especially before reaching confluence, compared with those on the control well. However,
451 morphological differences among the gelatin groups were not obvious. This study used the
452 morphologies of the MC3T3-E1 cells as the standard (Fig. 3). The morphologies of L929 and
453 ATDC5 cells are supplied in Supplementary Materials (Figs. 3 and 4).
454 One hour after the seeding, MC3T3-E1 cells on the control well showed a polygonal
455 shape with short pseudopods (data not shown). The cells maintained this polygonal shape on
456 Day 1 (Fig. 3A). Soon after reaching confluence (Day 3), cells were polygonal but became
457 smaller (Fig. 3B). However, they changed their shape to a more elongated one on Day 7 (Fig.
458 3C) as they increased their number. In contrast, a spindle-like, elongated shape with long,
459 thin pseudopods were observed when MC3T3-E1 cells were cultured on PSG, ASG, and

460 CSG gels over the incubation period (Fig. 3D–L). However, no distinct differences in cell
461 morphologies were observed among the gelatin gel groups (Fig. 3D–L). On Day 7, the cell
462 shape on gelatin gels was more elongated than that of the cells on the control well (Fig. 3C,
463 F, I, L), demonstrating more cell-spreading features of gelatin gels. Morphological changes of
464 MC3T3-E1 cells, such as cell spreading and flattening, correlate with osteogenic
465 differentiation [49]. Moreover, the MC3T3-E1 cells on the collagen fibrils, which had an
466 elongated cell body, showed higher ALP activity, an early differentiation marker of
467 osteoblasts [10]. These morphological data suggest a higher osteoblast differentiation-
468 inducing ability of gelatin gels than the control well. Thus, we examined the ALP activity and
469 mineralization activity, markers of osteoblast differentiation, of MC3T3-E1 cells in
470 subsequent experiments.
471
472 *3.8. ALP activity*
473
474 The ALP activity was measured as an early biomarker of osteoblast differentiation
475 [50]. The MC3T3-E1 cells were seeded and incubated for one day in the growth medium;
476 further, the medium was changed to the osteogenic medium (Day 0) to induce cell
477 differentiation.
478 The ALP activities of MC3T3-E1 cells in all gelatin groups were activated
479 significantly at Day 1 (Fig. 4), before cells got any differentiation stimuli from the culture
480 medium. The data suggest that early osteoblastic differentiation of MC3T3-E1 cells was
481 promoted by the signals from gelatins through the cell-surface ECM receptors such as
482 integrins. Moroi et al. [10] demonstrated similar activation of ALP activities, without any
483 stimuli from the culture medium, in MC3T3-E1 cells cultured on the thin layer of gels made
484 of collagen fibrils. Possibly, the gelatin gels offered more arginine-glycine-aspartic acid

485 (RGD) sequences to the cells and induced active cellular differentiation. Similarly, previous
486 studies have showed that RGD groups in gelatin hydrogels induced ALP activity in
487 osteoblasts [51, 52].
488 Differences in ALP activities among gelatin groups were not evident at any time point
489 (Fig. 4). The data indicated that all gelatin gels accelerated the osteoblastic differentiation
490 with similar potency. Rapid osteoblast differentiation is essential for early tissue formation in
491 subchondral bone development [50]. Therefore, the gelatin gels used in the present study are
492 suitable for bone tissue engineering.
493
494 *3.9. Mineralization activity*
495
496 Mineral deposition reflects osteogenic activity and is regarded as a marker for late
497 osteoblast differentiation [53]. Therefore, the mineralization of MC3T3-E1 cells was
498 examined using Alizarin Red S, a calcium-sensitive dye. On Day 14, the first sign of calcium
499 deposition was observed in all groups. A significantly higher calcium deposition was
500 recorded on gelatin gels compared with the control (Fig. 5A, B). Moreover, the ASG gel
501 showed the highest staining among the gelatin gels, and the difference between ASG and
502 PSG was significant (Fig. 5B), suggesting better performance of ASG in the osteogenic
503 differentiation of MC3T3-E1 cells. Shin et al. [54] revealed that higher mineralization in
504 MC3T3-E1 cells was observed on the harder material. Similarly, more calcium deposition in
505 MC3T3-E1 cells cultured on a high-mechanical-stress collagen-fibrin hybrid gel was reported
506 [55]. Hence, the slightly higher calcium deposition in the ASG group (Fig. 5B) may be
507 attributed to its higher mechanical strength compared to the CSG and PSG groups (Table 6).
508 As previously suggested in Section 3.8, the reason why gelatin gels induced distinct
509 functionality in MC3T3-E1 cells may be related to the motifs or domains of the gelatins that

bind integrins, which are cell surface receptors responsible for cell adherence to the extracellular matrix and regulation of cellular proliferation and differentiation [56, 57]. The distinct cell morphology on gelatin gels may also relate to the integrin-extracellular matrix interactions. Further studies on integrin expression and intracellular signaling on gelatin gels are warranted.

4. Conclusions

The present study proposes a novel and industrially suitable valorizing method for sturgeon skin, a processing by-product of caviar production, for production of biomedical-grade gelatin. The findings revealed that the gelatin produced after a mild acid pretreatment of sturgeon skin exhibited excellent characteristics and functionalities as biomedical-grade gelatin, which can be utilized as scaffolds for tissue engineering. Compared with the commercially available PSG gels, the ASG and CSG gels exhibit higher elastic modulus and stress at break. Moreover, ASG and CSG gels increased ALP activity and mineralization in MC3T3-E1 cells, suggesting that they accelerated osteoblast differentiation. Considering the novel extraction method, yield, and mineralization-inducing ability in MC3T3-E1 cells, ASG is an excellent biomedical-grade gelatin alternative to mammalian gelatin. This is the first study to report novel production of marine gelatin with >97% gelatin content, which would be useful for fabricating biomaterials in future biomedical applications. For this purpose, studies fabricating appropriate scaffolds, such as three-dimensional porous scaffolds for bone and cartilage engineering and sheet scaffolds for skin engineering, are required. Consequently, the findings of the present study could accelerate the valorization of sturgeon skin for application in various industries in the near future.

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Author Contributions

Md Rashidul Islam produced the biomedical-grade gelatins and conducted all experiments excluding analysis of the mechanical characteristics of gelatin gels. He also wrote the original draft and revised the manuscript. **Shunji Yunoki** conducted analyses of the mechanical properties of gelatin gels. **Kazuhiro Ura** and **Yasuaki Takagi** supervised the planning and experiments and revised the manuscript. All co-authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data supporting the findings of this study are available in the Supplementary material.

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Figure Captions

Fig. 1.

Banding patterns of gelatins. **(A)** 7.5% SDS-PAGE banding patterns, **(B)** intensity of α-, β-, and γ-bands and low molecular mass bands (<100 kDa) in SDS-PAGE, and **(C)** 15.5% Tricine SDS-PAGE banding patterns of porcine skin, sturgeon head, and sturgeon skin gelatins. M, molecular marker; PSG, type A porcine skin gelatin; AHG, type A sturgeon head gelatin; ASG, type A sturgeon skin gelatin; BSG, type B sturgeon skin gelatin; CSG, type C sturgeon skin gelatin. All gelatins were loaded as 5 μg/lane. Positions of α-, β-, and γ-bands are shown with arrows. Different letters in each band show significant differences ($p < 0.05$) in **B**.

Fig. 2.

Growth of cells on gelatin gels. **(A)** Growth of L929 fibroblasts, **(B)** ATDC5 pre-chondrocytes, and **(C)** MC3T3-E1 subclone-4 pre-osteoblasts on the cell culture well (control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin gelatin (ASG) gel, and type C sturgeon skin gelatin (CSG) gel. All gelatin gels were 1% and crosslinked with 5 mM EDC. Columns and bars indicate the mean value ± standard error ($n = 6$). Different letters in the same culture period indicate a significant difference ($p < 0.05$).

Fig. 3.

Phase-contrast microscopic images of MC3T3-E1 subclone-4 pre-osteoblasts cultured on the cell culture well (control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin gelatin

781 (ASG) gel, and type C sturgeon skin gelatin (CSG) gel. All gelatin gels were 1% (w/v) and
782 crosslinked with 5 mM EDC. Scale bars =100 μm.

783

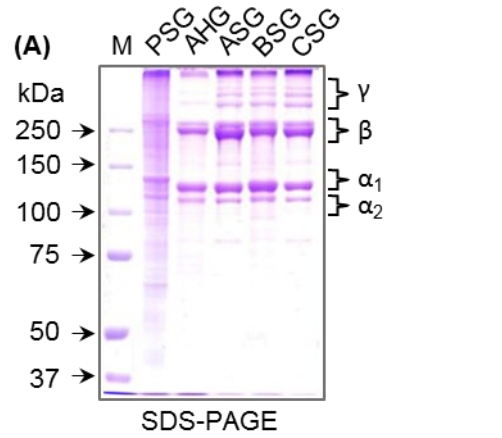
784 **Fig. 4.**

785 Alkaline phosphatase (ALP) activity of MC3T3-E1 subclone-4 pre-osteoblasts cultured on
786 the cell culture well (control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin
787 gelatin (ASG) gel, and type C sturgeon skin gelatin (CSG) gel. All gelatin gels were 1% and
788 crosslinked with 5 mM EDC. Columns and bars indicate the mean value ± standard error (n =
789 6). Different letters in the same culture period indicate a significant difference ($p < 0.05$).

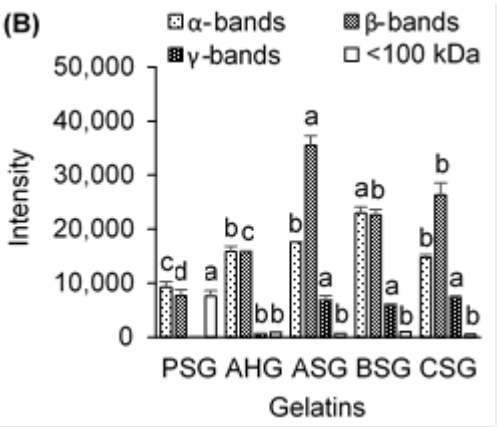
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791 **Fig. 5.**

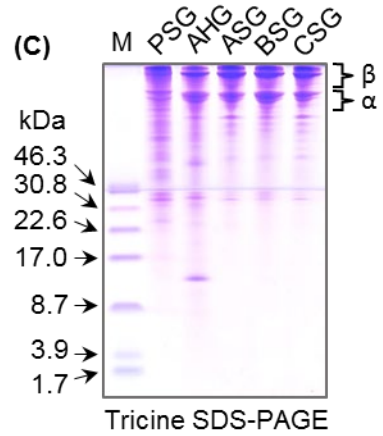
792 Mineralization of MC3T3-E1 subclone-4 pre-osteoblasts cultured on the cell culture well
793 (control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin gelatin (ASG) gel, and
794 type C sturgeon skin gelatin (CSG) gel. **(A)** Alizarin Red S staining and **(B)** its quantification.
795 All gelatin gels were 1% and crosslinked with 5 mM EDC. Columns and bars indicate the
796 mean value ± standard error (n = 6). Different letters in the same culture period indicate a
797 significant difference ($p < 0.05$).



798



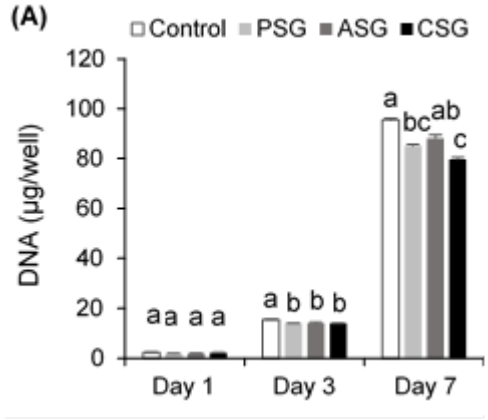
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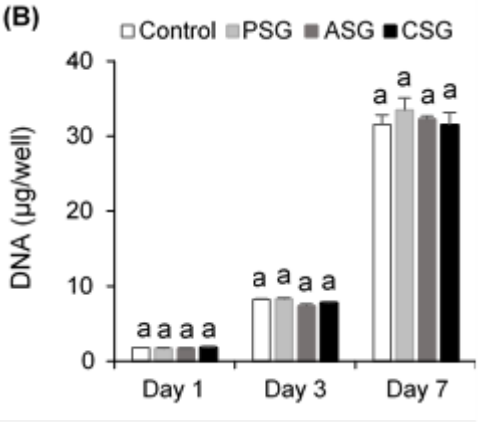
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801 **Fig. 1.**

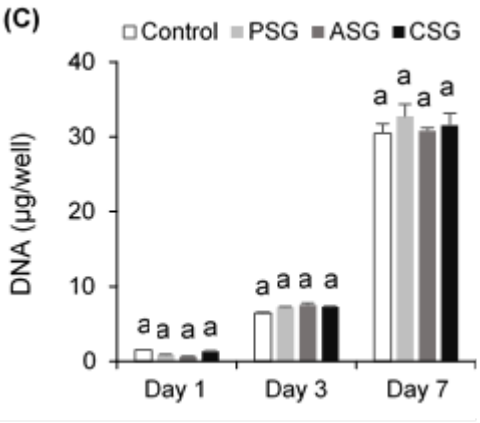
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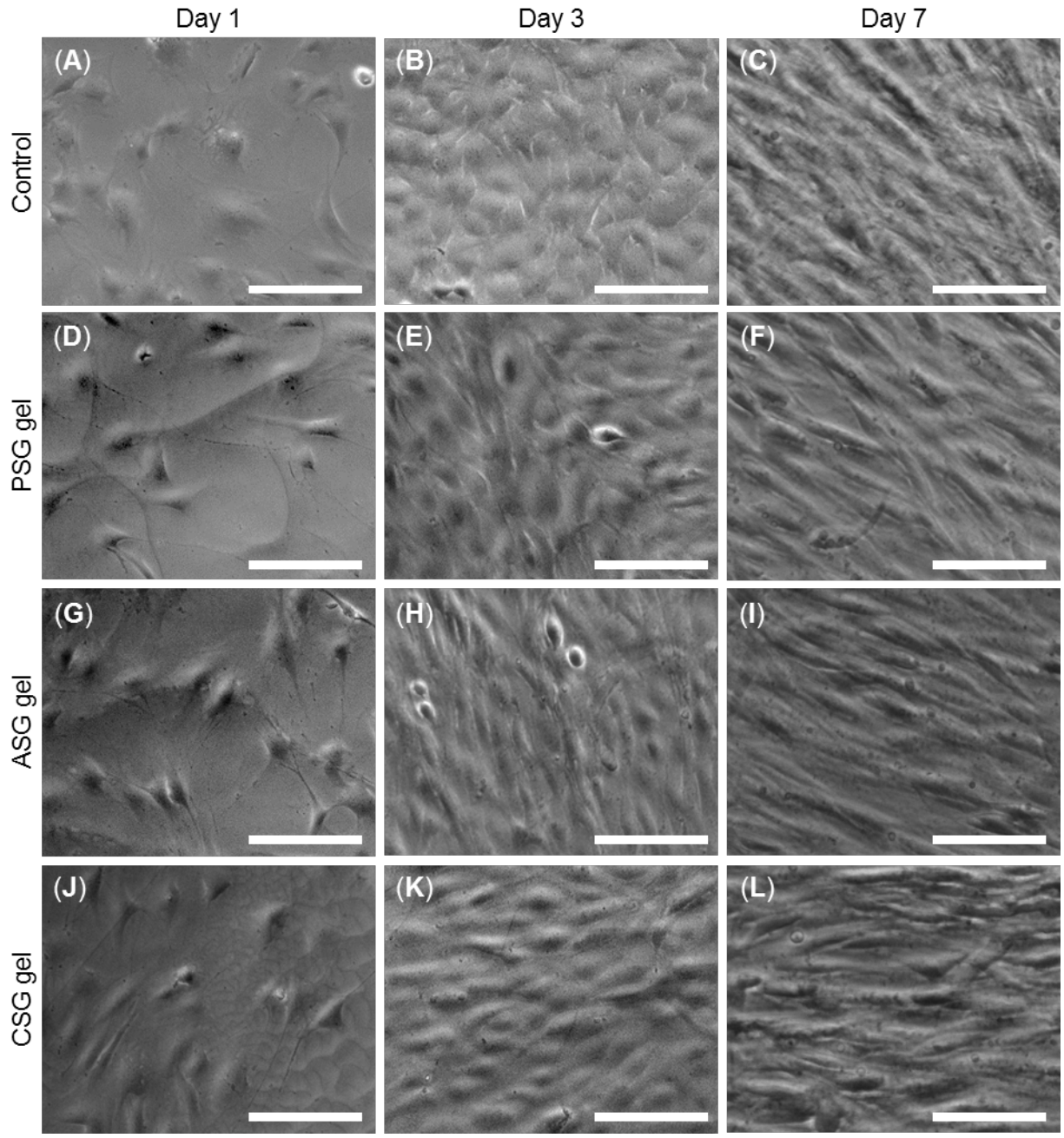
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804



805 **Fig. 2.**



806

807 **Fig. 3.**

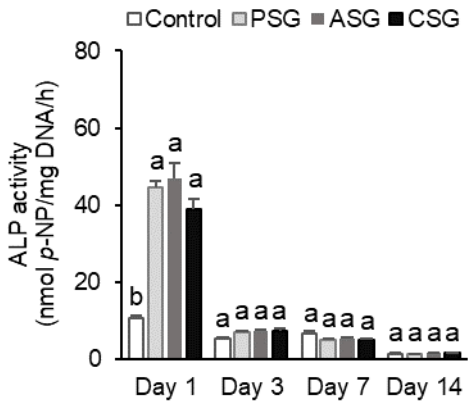

Control
PSG
ASG
CSG
ALP activity
(nmol p-NP/mg DNA/h)
80
60
40
20
0
b
a
a
a
a a a a
a a a a
a a a a
Day 1
Day 3
Day 7
Day 14

Fig. 4.

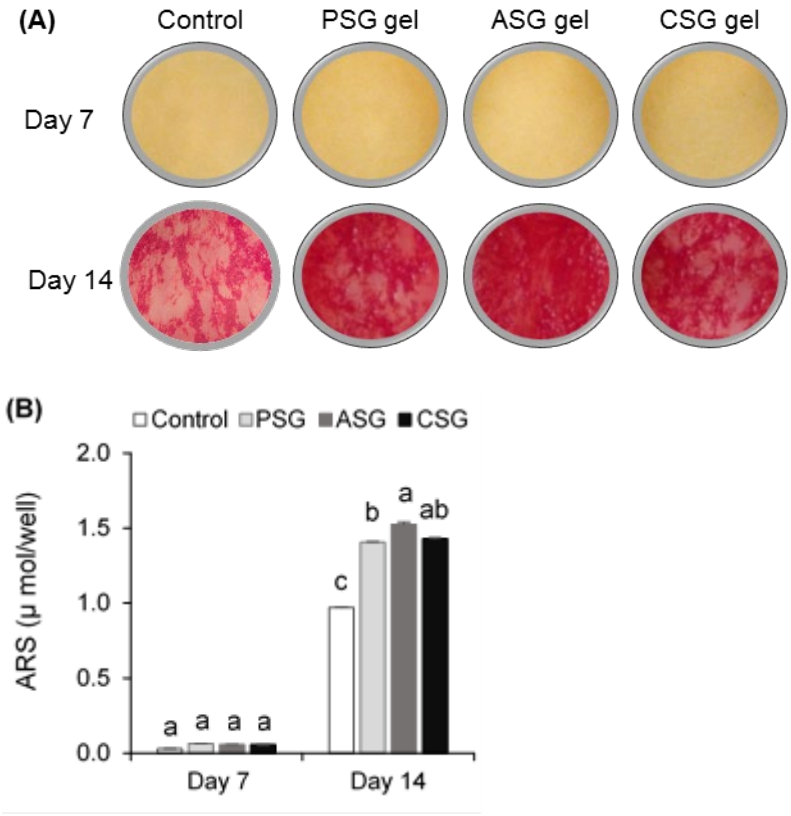

(A)
Control
PSG gel
ASG gel
CSG gel
Day 7
Day 14
(B)
Control
PSG
ASG
CSG
ARS (µ mol/well)
2.0
1.5
1.0
0.5
0.0
a
a
a
a
c
b
a
ab
Day 7
Day 14

Fig. 5.

Table 1

Yields (%, dry-weight gelatin/dry-weight sample; mean ± SE) of gelatins produced from sturgeon head and skin.

Sources	Types of gelatin	Yields (%)
Head	AHG	17.4 ± 1.7^{d}
Skin	ASG	66.6 ± 1.2^{a}
	BSG	19.4 ± 0.8^{c}
	CSG	57.7 ± 1.8^{b}

AHG, type A head gelatin; ASG, type A skin gelatin; BSG, type B skin gelatin; CSG, type C skin gelatin. All values are the mean ± standard error of three replicates. Different letters in the superscript show a significant difference ($p < 0.05$).

Table 2

Amino acid composition (residues/1000 residues) and gelatin content (% in the protein fraction) of gelatins extracted from sturgeon head and skin.

Amino acids	Amino acid composition (residues/1000 residues)			
	AHG	ASG	BSG	CSG
Ala	113	115	113	115
Arg	52	53	52	53
Asp	53	49	53	50
Cys	1	0	1	0
Glu	75	73	75	73
Gly	329	341	328	340
His	9	5	6	5
Hylys	8	8	8	8
Hyp	63	64	62	63
Ile	14	12	14	12
Leu	22	18	22	19
Lys	31	25	26	25
Met	11	11	11	11
Phe	16	14	15	14
Pro	108	119	114	118
Ser	50	49	50	49
Thr	24	24	25	24
Tyr	5	4	6	4
Val	18	16	19	17
Gelatin content (%)*	96.2	98.5	94.9	97.1

*Gelatin content (%) in the protein fraction of the sample. Values of each sample were obtained from the mixed samples of three replicates. AHG, type A head gelatin; ASG, type A skin gelatin; BSG, type B skin gelatin; CSG, type C skin gelatin.

825 **Table 3**

826 Proximate composition (%) of gelatins extracted from sturgeon head and skin.

Content (%)	Proximate composition (%)			
	AHG	ASG	BSG	CSG
Gelatin*†	74.7	99.1	93.7	97.8
Non-gelatinous proteins**†	3.0	0.9	5.0	2.2
Others	22.3	0.0	1.3	0.0
Total	100.0	100.0	100.0	100.0

827 All values are expressed as a percent in the sample weight. *Gelatin and **non-gelatinous protein
828 contents (%) in the total sample fraction. †Values were obtained from the mixed samples of three
829 replicates. AHG, type A head gelatin; ASG, type A skin gelatin; BSG, type B skin gelatin; CSG, type
830 C skin gelatin. Others would be considered as ash and carbohydrates.

Table 4

Endotoxin level of gelatins extracted from sturgeon head and skin.

Factors	Gelatins			
	AHG	ASG	BSG	CSG
Endotoxin (EU/mg)	1.2±0.4[a]	0.2±0.1[b]	1.1±0.4[a]	0.1±0.0[b]

AHG, type A head gelatin; ASG, type A skin gelatin; BSG, type B skin gelatin; CSG, type C skin gelatin. All values are the mean ± standard error of three replicates. Different letters in the superscript show a significant difference ($p < 0.05$).

836 **Table 5**

837 Rheological properties of gelatins.

Properties	Gelatins		
	PSG	ASG	CSG
Gelling temperature (°C)	23.2±0.2[a]	19.8±0.4[b]	17.2±0.5[b]
Melting temperature (°C)	31.4±0.1[b]	26.1±0.4[c]	34.7±0.5[a]

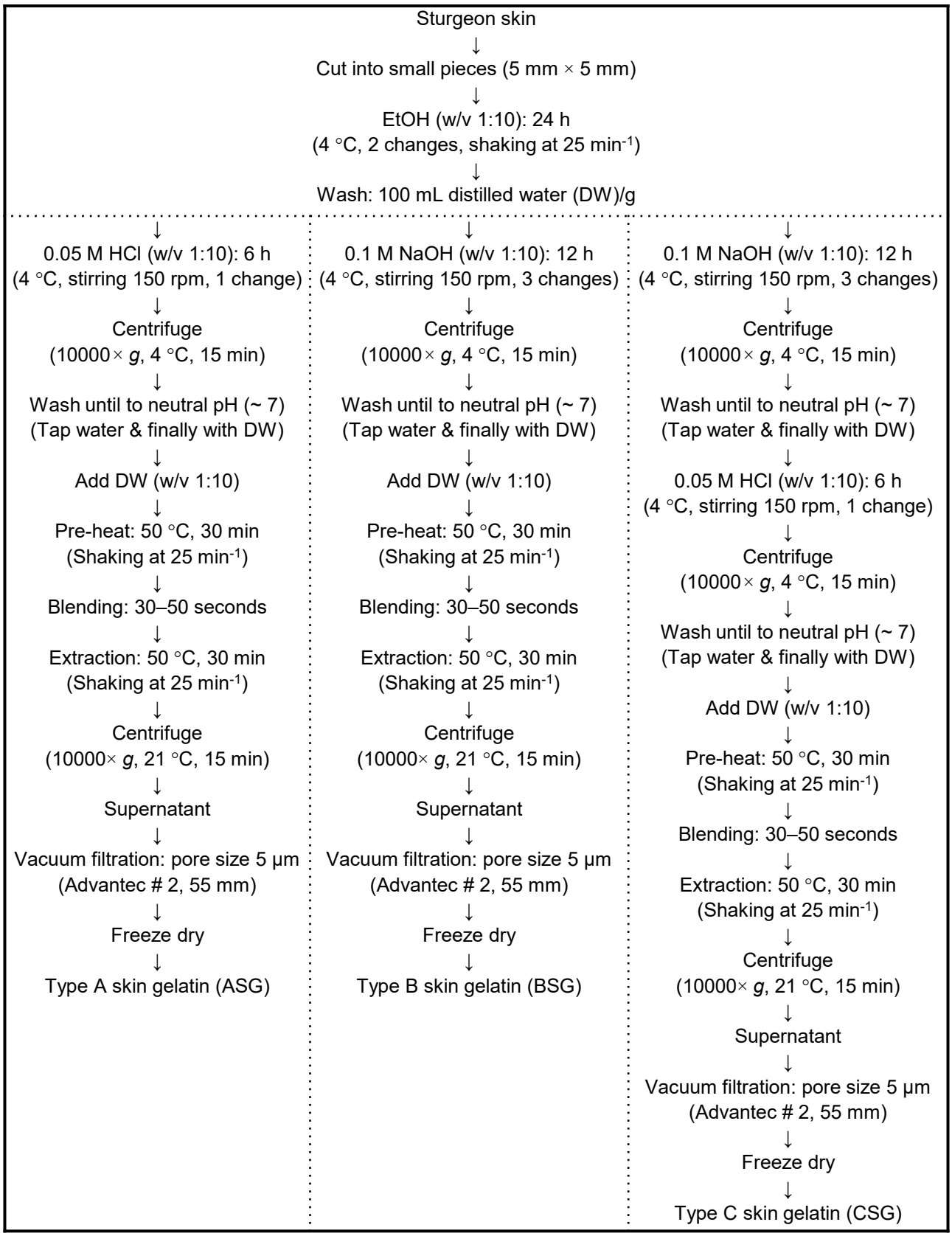
838 PSG, type A porcine skin gelatin; ASG, type A skin gelatin; CSG, type C skin gelatin. All values are
839 the mean ± standard error of 3–5 replicates. Different letters in the superscript of the same row show a
840 significant difference ($p < 0.05$).

Table 6

Mechanical properties of gelatin gels after crosslinking with 5 mM EDC.

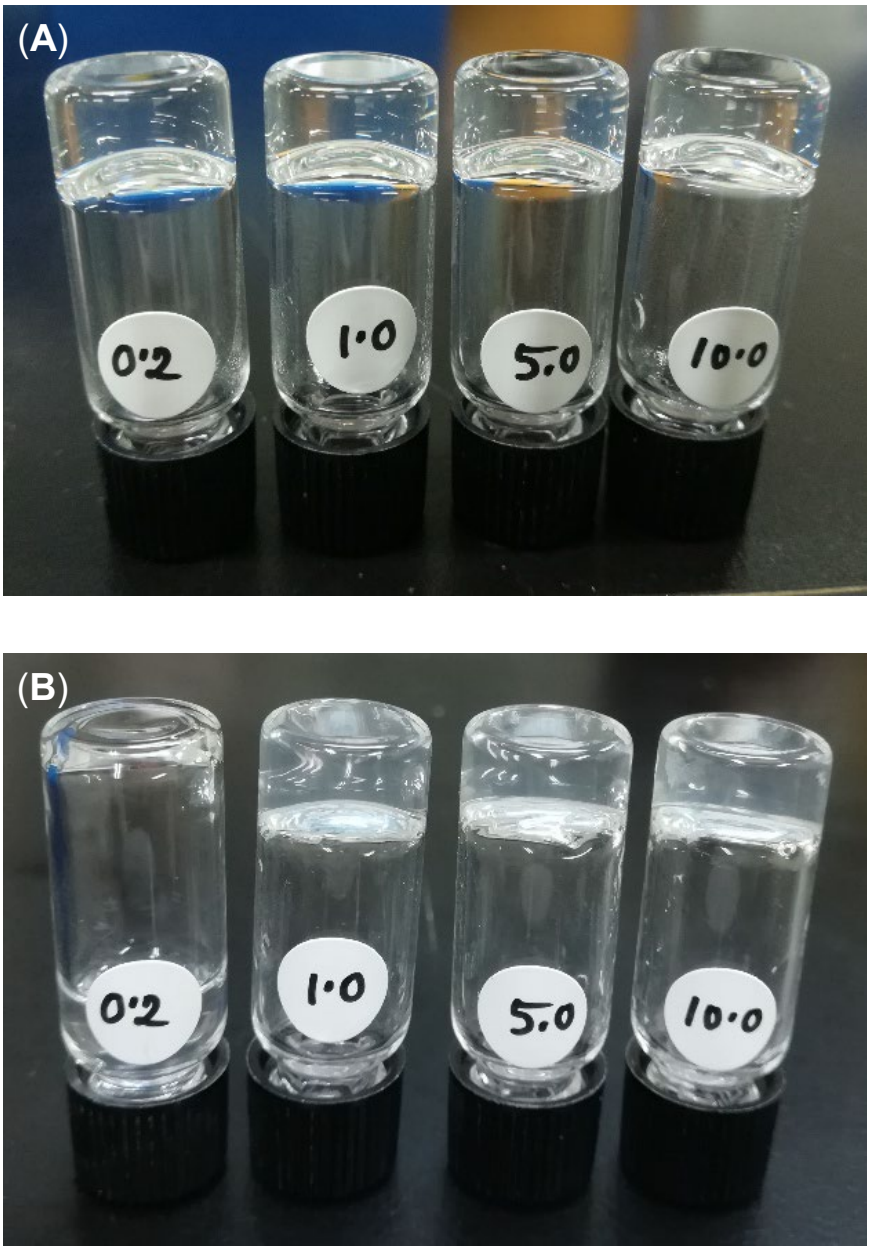
Properties	Gelatins		
	PSG	ASG	CSG
Elastic modulus (kPa)	4.4±0.2[b]	10.3±0.5[a]	12.2±0.29[a]
Stress at break (kPa)	78.5±10.5[b]	184.2±14.4[a]	126.1±9.8[a]

PSG, type A porcine skin gelatin; ASG, type A skin gelatin; CSG, type C skin gelatin. All values are the mean ± standard error of 3–5 replicates. Different letters in the superscript of the same row show a significant difference ($p < 0.05$).



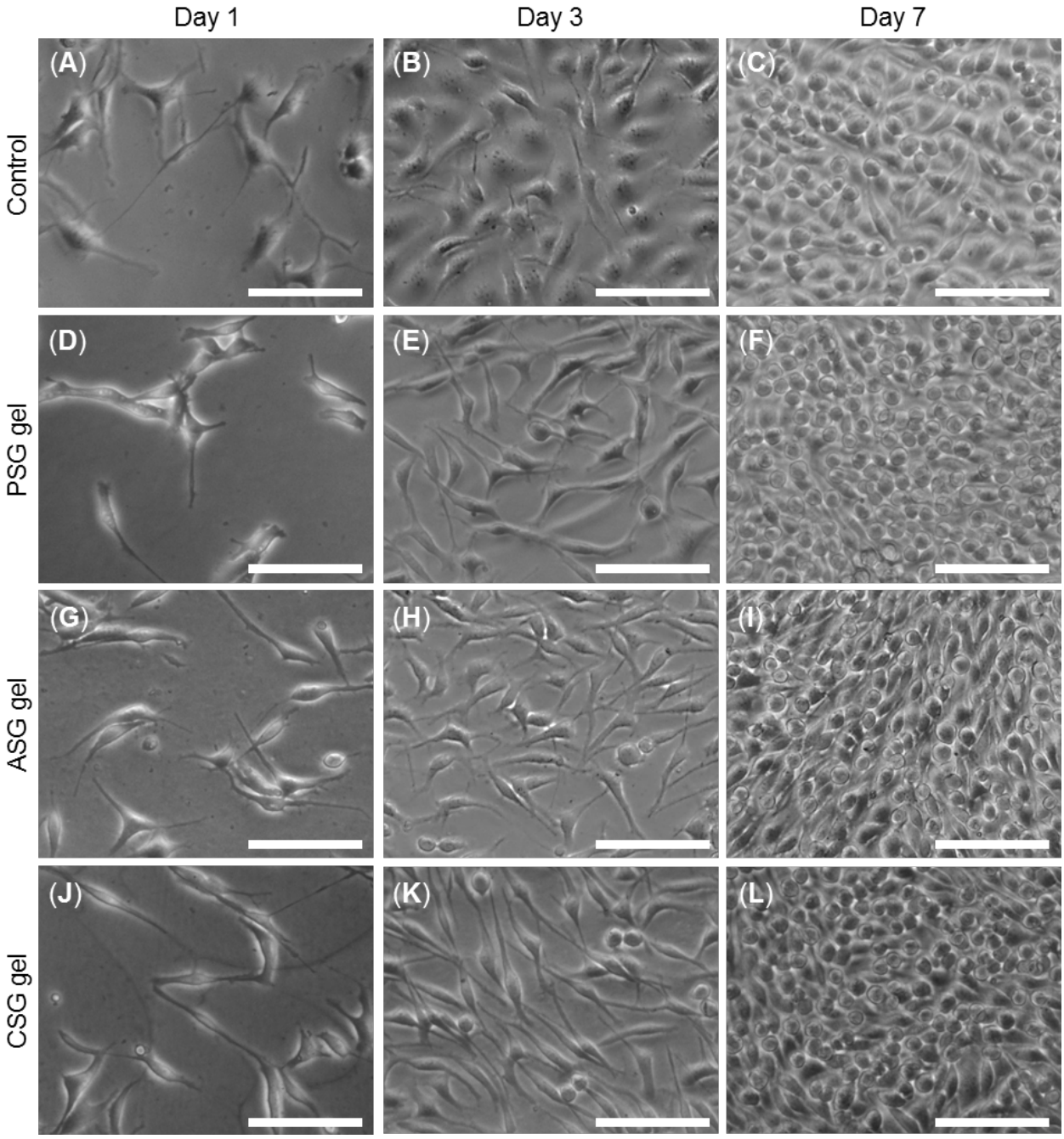
Supplementary Fig. 1.

Outline of the extraction methods of the different types of gelatins from sturgeon head tissue and skin.



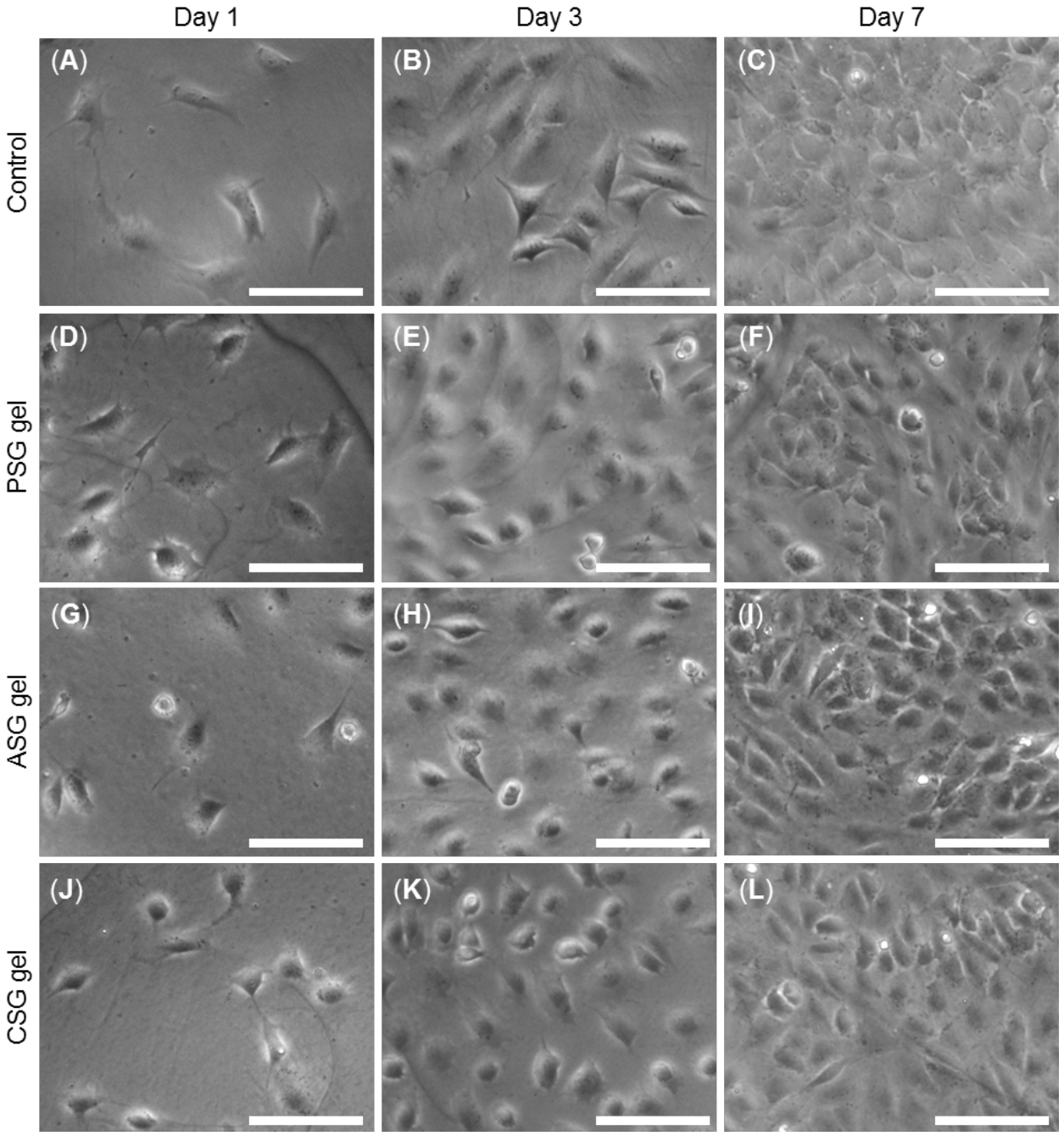
Supplementary Fig. 2.

Crosslinking of gelatin gels (type A skin gelatin, 1% w/v) with 0.2–10.0 mM EDC and its stability before (**A**) and after (**B**) their incubation at 37 °C for 48 h.



Supplementary Fig. 3.

Phase-contrast microscopic images of L929 fibroblast cultured on the cell-culture well (Control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin gelatin (ASG) gel, and type C sturgeon skin gelatin (CSG) gel. All gelatin gels were 1 % (w/v) and crosslinked with 5 mM EDC. Scale bars =100 μm. One hour after seeding, L929 cells attached to CWB surface showed polygonal shape, whereas those on the PSG, ASG, and CSG gel surfaces showed elongated/spindle shape (data not shown). On Day 1, L929 cells on all gelatin gels showed more elongated spindle shape extending their pseudopod to form a cell network (D–J) and Day 3 (E–K) than on CWB (A, B). On Day 7, both on gelatin gels and CWB, cell bodies were rounded and become small after reducing the size to increase their number (C–L). There was no distinct variation in the morphologies of L929 cells among ASG, CSG and PSG (D–L).



Supplementary Fig. 4.

Phase-contrast microscopic images of ATDC5 pre-adipocyte cultured on the cell-culture well (Control), type A porcine skin gelatin (PSG) gel, type A sturgeon skin gelatin (ASG) gel, and type C sturgeon skin gelatin (CSG) gel. All gelatin gels were 1 % (w/v) and crosslinked with 5 mM EDC. Scale bars =100 μm. After one hour of ATDC5 cell seeding, a polygonal shape was observed on CWB and a round or spindle shape was found on gelatin-gels (data not shown). When the cells cultured on CWB, a polygonal shape with short pseudopod was observed on Day 1 and continued to maintain this shape on Day 3 and Day 7 (A–C). In contrast, a slightly spindle or spherical cell bodies with short pseudopod was found when cells cultured on gelatin-gels at Day 1, 3 or 7 (D–L). No distinct cell morphologies were found on each incubation period among the gelatin gels fabricated from ASG, CSG or PSG (D–L).

Supplementary Table 1

Crosslinking of gelatin gels (type A skin gelatin, 1% w/v) and its stability at 37 °C for 48 h.

EDC (mM)	Gel stability
0.2	-
1.0	++
5.0	+++
10.0	+++

EDC, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; -, gel melted; ++, gel not melted and comparatively soft; +++, gel not melted and comparatively hard.

Supplementary Table 2

Mechanical properties of gelatin gels without crosslinking.

Properties	Gelatins		
	PSG	ASG	CSG
Elastic modulus (kPa)	4.3±0.7[a]	-	1.4±0.3[b]
Stress at break (kPa)	4.1±0.4[a]	-	1.6±0.1[b]

PSG, type A porcine skin gelatin; ASG, type A skin gelatin; CSG, type C skin gelatin. All values are the mean ± standard error of 3–5 replicates. Different letters in the superscript of the same row show a significant difference ($p < 0.05$). -, Data were not available due to the softness of ASG.