Introduction

Bone formation by BMP-containing implanted materials has been studied to restore bone lost due to injury or disease [1]. However, clinical use of these materials is not widespread [2]. BMP has been employed in medical research together with carrier materials such as micro-sized hydroxyapatite [3], insoluble bone matrix [4], and demineralized dentine matrix [5]. The carrier retains and slowly releases BMP [4]. The bone-forming ability of implanted BMP-containing materials has been evaluated by implantation in muscles [6]. The granular nature of these materials necessitates anesthesia, inci-
sion, and suturing. BMP application might also be facilitated by injection of jelly BMP-containing biomaterials [7]. Very fine nHAP powder such as nHAP, together with an injectable jelly scaffold material, is effective as a carrier of BMP to induce new bone formation [8].

Jelly hydrogels such as polyethylene glycol [9], chitosan [10], alginate [11], and gelatin [12] have been studied as base materials for injection-type bone-forming materials. Hyaluronic acid (HLA) is a candidate base hydrogel material for injection [13] and is used to eliminate nasolabial folds [14]. A cross-linkable hyaluronic acid product, cHLA, was recently developed [15], but has not been investigated as a base material for injection-type bone-forming materials [16].

In this study, we prepared an injection-type bone-forming material consisting of cHLA, BMP, and nHAP (cHLA/BMP/nHAP) and examined its effect on bone formation in muscles of mice.

Materials and Methods
1. Materials
We used a cHLA kit (Hystem Kit 12.5 mL, Sigma-Aldrich, St. Louis, MO, USA), which consisted of HLAs with –SH functional groups (Glycosil) (ESI Bio, Alameda, CA, USA), a cross-linker (Extralink Lite, ESI Bio), and degassed (DG) water (ESI Bio). We also used recombinant human/mouse/rat (CHO cell-derived) BMP (R&D Systems, Minneapolis, MN, USA) and nHAP of mean diameter 40 nm (nano-SHAp [40 nm], SofSera, Tokyo, Japan). The nHAP particles were autoclaved prior to use.

2. Preparation of sols
The following procedure was performed on a clean bench. BMP (50 μg) was reconstituted with 4 mM HCl (0.25 mL total) containing 0.5 wt% bovine fetal albumin standard (fraction V) (Thermo Scientific Pierce, Waltham, MA, USA) as an adjuvant, and diluted in DG water (5 mL total volume). Next, freeze-dried Glycosil (50 mg) was reconstituted as a sol with DG water containing BMP (5 mL) on a vibrating mixer (Mild Mixer PR-12, Tokyo, Japan) for 30 min at room temperature (Liquid A). Extralink Lite was also diluted in DG water (2.5 mL) (Liquid B). Liquid B was mixed with Liquid A, yielding a viscous sol, and sterilized by membrane filtration (0.22 μm). The test sol was manually mixed with nHAP (20 μg) (Fig. 1 (a)) using a plastic spatula in a 35 mm plastic culture dish (cHLA/BMP/nHAP). The sol was chemically cross-linked and transformed to a gel for 20 min at room temperature. Prior to gelation, the sol could be injected using a syringe and needle (Fig. 1 (b)). The control sol was also prepared without addition of nHAP (cHLA/BMP).

Figure 1 (a) nHAP powder and (b) a drop of the cHLA/BMP/nHAP sol
3. Animal experiments
The Institutional Animal Care and Use Committee of Iwate Medical University approved the protocol used in this study (approval no. 25-015). Twenty 10-week-old male BALB/cAJcl mice (CLEA Japan, Tokyo, Japan) were used in this study. Groups of two to three mice were housed in pathogen-free cages and provided with a standard diet and water ad libitum. Under anesthesia with a mixture of isoflurane (3 vol%) and oxygen (0.5 L/min) gas generated by a carburetor (IV-ANE; Olympus, Tokyo, Japan), the test and control sols were injected into back subcutaneous intra-muscular tissue (0.2 mL = 1.33 μg of BMP) and thigh intra-muscular tissue (0.4 mL = 2.67 μg of BMP) (Figure 2 (a) and (b)) of the mice (n=10 each), respectively, using a 24-gauge needle. After feeding for 8 weeks, the animals were euthanized by CO2 inhalation.

4. Calcification analysis by CT
To evaluate calcification, three-dimensional (3D) images of the mice which received test cHLA/BMP/nHAP and control cHLA/BMP sol-gels were obtained at 8 weeks postoperatively using a 3D micro-CT system (eXplore Locus; GE Healthcare, Wilmington, MA, USA). The samples were scanned at 90 μm intervals at 80 kVp and 450 μA, resulting in generation of Vextus Factor compiled storage files (VFF data). After scanning, 3D image analysis software (MicroView v 2.2; GE Healthcare) was used to reconstruct 3D CT images. The instrumental opacity threshold value was set to 8,000 to minimize the interference caused by other bony tissues.

5. Visual inspection
Calcification in the intramuscular muscles was inspected visually and photographed.

6. Histological assessment
The subcutaneous and thigh intra-muscular tissues containing test cHLA/BMP/nHAP sol-gels were harvested and fixed in 10% neutral-buffered formaldehyde equivalent (Mildform, Wako Chemical, Osaka, Japan) for 8 weeks at 4°C. The tissues were dehydrated in a graded alcohol series, followed by xylene, and embedded in wax. The specimens in wax were next cut into 5 μm sections using a microtome (IVS-410, Sakura Finetek, Tokyo, Japan). The sections were transferred to slides and stained with hematoxylin and eosin (HE).

7. Statistical analysis
Non-parametric Chi-squared test was performed to assess the bone forming capability of the test and control sol-gel materials in two kinds of muscles of mice with SPSS v.16 software (SPSS, Inc., Chicago, IL, USA). P<0.01 was considered to induce a statistically significant difference.

Figure 2 Injection of the cHLA/BMP/nHAP sol into the (a) subcutaneous intra-muscular and (b) thigh intra-muscular tissues of a mouse.
Results

1. Calcification analysis by CT

CT images of the back subcutaneous intra-muscular tissue at 8 weeks after injection of the test cHLA/BMP/nHAP sol-gel showed formation of X-ray–opaque calcified areas of various sizes and shapes (Figure 3 (a), (b) and (c)). No calcification was observed in ten mice at 8 weeks after injection of the control cHLA/BMP sol-gel (Figure 3 (d)).

Representative CT images of the thigh intra-muscular tissue at 8 weeks after injection of the test cHLA/BMP/nHAP sol-gel indicated formation of X-ray–opaque calcified areas of various sizes and shapes (Figure 4 (a), (b) and (c)). No calcification was observed in ten mice at 8 weeks after injection of the control cHLA/BMP sol-gel (Figure 4 (d)).

Table 1 shows the calcification case counts by micro-CT at two intra-muscular locations of mice at 8 weeks after injection of test cHLA/BMP/nHAP and control cHLA/BMP sol-gels. cHLA/BMP/nHAP test sol-gel caused bone formation in both muscles of mice at 90% probability rate in back subcutaneous intra-muscular tissue and at 80% probability rate in thigh intra-muscular tissue, whilst cHLA/BMP control could not bring about any ossification in muscles. There was statistically significant difference in the bone forming capability between test cHLA/BMP/nHAP and control cHLA/BMP sol-gels in two kinds of muscles, respectively (p<0.01, n=10 each).

![Figure 3](image-url)  
Typical CT images of the back subcutaneous intra-muscular tissues of mice containing (a), (b), (c) the test cHLA/BMP/nHAP sol-gel and (d) control cHLA/BMP sol-gel at 8 weeks after injection. Ellipse, main calcified objects.
**Table 1** Calcification case counts by micro-CT at the back subcutaneous intra-muscular and thigh intra-muscular tissues of mice at 8 weeks after injection of test cHLA/BMP/nHAP and control cHLA/BMP sol-gels.

<table>
<thead>
<tr>
<th>Material</th>
<th>Injection site</th>
<th>Back subcutaneous intra-muscular tissue (n=10)</th>
<th>Thigh intra-muscular tissue (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calcified</td>
<td>Not-calculated</td>
</tr>
<tr>
<td>Test cHLA/BMP/nHAP sol-gel</td>
<td></td>
<td>9 (^{1})</td>
<td>1</td>
</tr>
<tr>
<td>Control cHLA/BMP sol-gel</td>
<td></td>
<td>0 (^{1})</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^{1}\); \(p<0.01\) \(^{2}\); \(p<0.01\)

**Figure 4** Representative CT images of the thigh intra-muscular tissues of mice containing (a), (b), (c) the test cHLA/BMP/nHAP sol-gel and (d) control cHLA/BMP sol-gel at 8 weeks after injection. Ellipse, main calcified objects.
2. Visual inspection
At 8 weeks after injection of the cHLA/BMP/nHAP sol-gel, calcification was evident in both the back subcutaneous and thigh intra-muscular tissues (Figure 5 (a) and (b)).

3. Histological analysis
Figure 6 (a) shows the back subcutaneous intra-muscular tissue of a mouse at 8 weeks after injection of the cHLA/BMP/nHAP sol-gel. Remnant material was distributed in the central one-third and lower one-third of the center-right muscle tissues. Round cartilaginous tissue was also detected (Figure 6 (b)). Figure 7 shows the different back subcutaneous intra-muscular tissue of a mouse at 8 weeks after injection of the cHLA/BMP/nHAP sol-gel. Blood vessels infiltrated into the area, which contained abundant bone marrow. Newly formed bone replaced cartilaginous tissue, implying endochondral ossification.

Figure 8 shows the thigh intra-muscular tissue of a mouse at 8 weeks after injection of the cHLA/BMP/nHAP sol-gel at the high magnification. Within remnant material, new bone matrix was infiltrating into and partially replacing cartilaginous tissue, implying endochondral ossification.

Discussion
In a preliminary study, we found that both cHLA and cHLA/nHAP sol-gels did not induce bone formation in muscles. Moreover, the cHLA/BMP/nHAP sol-gel did not induce bone formation in the back subcutaneous connective tissue, possibly due to use of low 1.33–2.67 μg BMP. Bone formation in connective tissue reportedly requires high-dose (i.e., 5–20 μg) BMP [17]. Because of its side effects [18], smaller quantities of BMP are preferred. Therefore, we evaluated and confirmed the ability of the cHLA/BMP/nHAP sol-gel, which contained a small quantity of BMP, to induce bone formation in muscle tissue.

Satellite muscle cells (stem cells) [19] restore muscles damaged by heavy exercise or injury. Multipotent muscle-derived mesenchymal stem cells migrate to damaged muscles, where they proliferate and differentiate into myoblasts [20]. Injection of BMP results in differentiation of mesenchymal stem cells to chondrocytes and osteoblasts. The rate and end result of this differentiation is determined by the environment at the injection site and the physiological activity of the mouse [4]. BMP also causes endochondral ossification of muscles [21].
**Figure 6** The back subcutaneous intra-muscular tissue of a mouse at 8 weeks after injection of the HLA/BMP/nHAP sol-gel. Upper image (a), low magnification. Gray, remnant material; yellow rectangle, area enlarged in the right image (b). RM, remnant material; C, cartilaginous tissue.

**Figure 7** The different back subcutaneous intra-muscular tissue of a mouse at 8 weeks after injection of the cHLA/BMP/nHAP sol-gel. NB, new bone; BM, bone marrow; V, blood vessels; C, cartilaginous tissue. C was being replaced by NB, implying endochondral ossification.

**Figure 8** The thigh intra-muscular tissue of a mouse at 8 weeks after injection of the cHLA/BMP/nHAP sol-gel. NB, new bone; BM, bone marrow; C, cartilaginous tissue; RM, remnant material; MU, muscle cells (myofiber). *C being replaced by NB, indicative of endochondral ossification.
The chLA/BMP/nHAP sol-gel induced endochondral ossification in the back subcutaneous intra-muscular tissue (Figures 3, 6, 7) and thigh intra-muscular tissue (Figures 4 and 8). This was characterized by replacement of cartilaginous tissue with new bone [22] (Figures 7 and 8). The sequence of the endochondral ossification is considered to be as follows [23]. First, in parallel with bio-absorption of the injected material and BMP release, cartilage anlage and chondrocytes formed in the remnant material (Figure 6 (b)) and proliferated due to the low oxygen and nutrient levels. Second, blood vessels infiltrated into the cartilaginous tissue (Figure 7). Third, new bone replaced the cartilaginous tissue (Figures 7 and 8). The new bone was irregular in shape (Figures 7 and 8) [4].

The bio-absorption of the chLA/BMP/nHAP sol-gel was slower in the back subcutaneous intra-muscular tissue than in the thigh intra-muscular tissue, possibly because of restricted blood supply and the consequent low level of oxygen in muscle [24].

We report here that > 80% of the mice showed calcification in muscle tissue (Table 1). The mice frequently touched or pushed the injected area and actively moved, leading to migration and fragmentation of the injected material. The less than 100% success rate may be due to bio-absorption of the injected material without inducing calcification or production of un-calcified chondrocytes in the remnant material.

An injectable bone-forming material comprises the sol-gel base material with fluidity, a growth factor, and a drug-delivery system. The chLA sol-gel base material used in this study remained in vivo over 8 weeks. During this time, chLA promoted BMP release from nHAP and provided sites for the formation of cartilaginous tissue. Any reduction in the amount of linker would likely accelerate the clearance of chLA. Polyethylene glycol [9], chitosan [10], and gelatin [12] can also be used as base materials. We used as a growth factor a small quantity [17] of BMP, which induced endochondral ossification [21-23]. The delivery of BMP [25] was facilitated by nHAP, consistent with a previous report [8]. The large surface area and electrical charge of nHAP facilitates retention of initially applied BMP and the subsequent slow release of BMP. Moreover, nHAP is angiogenic [26], which promotes bone formation by inducing dissolution of calcium and phosphate ions, and osteo-conductive [27].

The control chLA/BMP sol-gel could not produce bone in muscles, possibly because BMP was quickly lost from chLA base material in muscles. A BMP-carrier material such as nHAP is necessary to form bone in muscles.

Induction of bone formation by injected sols is easy and minimally invasive [7], and does not require sutures. The cartilage or bone formed can be applied to bone defects [28], in dentistry to extraction sockets [29], or to injured or damaged bone to accelerate new bone formation [30, 31]. This chLA/BMP/nHAP sol-gel osteo-inductive biomaterial has potential applications in dentistry and medicine.

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Competing interests
The authors declare that they have no competing interests to disclose.

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