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Percutaneous Nonviral Delivery of Hepatocyte Growth Factor in a Fracture Gap Promotes Bone Repair in Rabbits: A Preliminary Study

Running title: HGF on Fracture Healing

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Abstract

Hepatocyte growth factor (HGF) was initially identified in cultured hepatocytes and subsequently reported to induce angiogenic mitogen, morphogen, and antiapoptotic activity in various tissues. These properties suggest a potential influence of HGF on bone healing. We asked if gene transfer of human HGF (hHGF) into a fracture gap with a hemagglutinating virus of Japan-envelope (HVJ-E) vector promotes bone healing in rabbits.

HVJ-E that contained either hHGF or control plasmid was percutaneously injected into the fracture gap of rabbit tibias on Day 14. The fracture gap was evaluated by radiography, pQCT, mechanical tests, and histology at Week 8. The expression of hHGF was evaluated by RT-PCR and immunohistochemistry at Week 3. Radiography, pQCT, and histology suggested the hHGF group had faster fracture healing. Mechanical tests demonstrated the hHGF group had greater mechanical strength. The injected tissues at 3 weeks expressed hHGF mRNA by RT-PCR. hHGF-positive immunohistochemical staining was observed in various cells at the fracture gap at Week 3. The data suggests delivery of hHGF plasmid into fracture gap promotes fracture repair, and HGF could become a novel agent for the treatment of bone fractures.

18 **Introduction**

19 The treatment of bone fractures has advanced rapidly in recent years. Various implants
20 for treating fractures have been developed.[3,20] However, complex fractures remain a
21 challenge and often require prolonged fixation. External fixation is preferred by many
22 surgeons method to stabilize such fractures. However, external fixators are still
23 associated with nonunions, pin track infections, and contracture of adjacent joints, some
24 of which relate to the length of the required immobilization.

25 More rapid bone repair within the fracture gap would avoid the complications that result
26 from prolonged immobilization. Moreover, Einhorn[16] concluded enhancement of the
27 fracture repair process would ensure rapid restoration of function. The ability of injured
28 patients to return earlier to daily life would not only have a substantial economic impact
29 on society, but would also improve the overall physical and mental wellbeing of the
30 patients. Therefore, a compelling need still exists for a safe and effective way to enhance
31 bone repair.

32 Many studies report enhancement of fracture healing with hormones or growth factors,
33 such as IGF-I,[30] BMP,[17, 29, 51] FGF-2,[4, 10, 42] VEGF,[13] and PTH.[2] Recently, another growth
34 factor called hepatocyte growth factor (HGF) functions as a powerful and versatile factor
35 with angiogenesis, mitogen, morphogen, and antiapoptotic activity. HGF was originally
36 identified from plasma and serum as a molecule that simulated DNA synthesis in rat and
37 human hepatocytes in 1989.[36, 37] Multiple studies subsequently confirmed the role of
38 HGF in enhancing hepatocyte function.[21, 25, 27, 28, 39, 45, 47, 49, 50, 52] However, little is known
39 about the influence of HGF on bone healing, especially *in vivo*. [24] Given the fact that

40 many growth factors influence various tissues, the question arises as to whether HGF
41 influences bone healing.

42 We therefore hypothesized gene transfer of hHGF directly into fracture gaps using the
43 HVJ-E vector would promote tibia fracture healing in radiograph, computer tomograph,
44 mechanical test ,and histology in rabbits. Furthermore, we tested the mRNA and protein
45 expression of hHGF to prove the effect of the injected gene.

46 **MATERIALS AND METHODS**

47 In a preliminary study we delivered high human HGF (hHGF) concentrations
48 percutaneously and therefore less invasively than an open method to rabbit tibia fracture
49 gaps. We utilized a novel, nonviral vector, the hemagglutinating virus of Japan envelope
50 (HVJ-E), to deliver hHGF genes to fracture gaps. The HVJ-E vector is effective for gene
51 transfer both in vitro and in vivo.[26] Fracture models were prepared in 50 mature female
52 Japanese white rabbits weighing 2.5 to 3 kg. The rabbits were equally divided into two
53 groups of 25 rabbits each, the hHGF group and the control vector group. Human HGF
54 cDNA (2.2 kb) was inserted between the EcoRI and NotI gaps of the pUC-Sr expression
55 vector plasmid to produce an hHGF expression vector. A pcDNA 3.1(-) plasmid DNA
56 vector (Invitrogen, San Diego, CA) with the same structure, but lacking the hHGF cDNA,
57 was used as a control vector. On Day 7 after injection of HVJ-E, three rabbits in each
58 group were assessed for hHGF mRNA. At 3 and 8 weeks postoperatively, two animals
59 from each group were sacrificed for immunohistochemistry. At 8 weeks, the remaining
60 40 animals were euthanized and equal numbers used for histological and mechanical

61 testing. The experimental protocol was approved by the Committee on the Ethics of
62 Animal Experiments of Kanazawa University.

63 HVJ (also known as Sendai virus) envelope vector was prepared as described
64 previously.[26] Briefly, the virus was purified by centrifugation and inactivated by UV
65 irradiation, which disabled the replication capacity of the virus completely without
66 affecting the cell membrane fusing capability of the envelope. HVJ envelope (5 AU) was
67 mixed with 50 μg of either HGF or pcDNA3.1(-) plasmid DNA and 0.3% Triton-X 100.
68 The suspension was then washed with balanced salt solution (BSS - 137 mM NaCl, 5.4
69 mM KCl, 10 mM Tris-HCl; pH 7.6) and centrifuged (10,000 g, 10 min) at 4°C, and the
70 pellet was resuspended in a final volume of 100 μl BSS for subcutaneous injection. The
71 suspension was stored at 4°C until use. HVJ-E vector is commercially available from
72 Ishihara Sangyo Co. Ltd. (Osaka, Japan).

73 The rabbits were anesthetized with a subcutaneous injection of ketamine hydrochloride
74 (35 mg/kg body weight; Sankyo Pharmaceutical, Tokyo, Japan), xylazine (5 mg/kg body
75 weight; Bayer, Tokyo, Japan), and an intravenous injection of pentobarbital sodium (40-
76 50 mg/kg body weight; Abbott Laboratories, North Chicago, IL). A longitudinal skin
77 incision was made on the anteromedial aspect of the right tibia, and the periosteum was
78 carefully stripped of the surrounding soft tissue and fascia. Four half pins 2 mm in
79 diameter (Stryker, Geneva, Switzerland) were inserted into the medial aspect of the tibia,
80 perpendicular to its axis, and a unilateral external fixator of our design was applied. To
81 simulate a fracture of the rabbit tibia,[33, 40, 41] we created a 3-mm gap between the bone
82 fragments with a bone saw (Stryker, Geneva, Switzerland). This gap was not critical gap.

83 Althogh this model was not a fracture in essence, it was an osteotomy, healing process of
84 both models were same.[33, 40,41]

85 At postoperative Week 2, after induction of anesthesia with ketamine and xylazine, HVJ-
86 E (100 μL) containing either hHGF or pcDNA 3.1(-) plasmid DNA (n = 25 for each
87 group) was percutaneously injected into the fracture gap with a 29-gauge needle (Terumo,
88 Atsugi, Japan) under an image intensifier. The reason we chose postoperative Week 2
89 was that there were many cells to introduce the gene at the fracture gap.

90 On Day 7 after percutaneous injection of HVJ-E containing either hHGF or pcDNA 3.1 (-
91) plasmid DNA (n = 3 for each group), the rabbits were euthanized and the tissue at the
92 fracture gap and the surrounding muscle was harvested and prepared for reverse
93 transcription-polymerase chain reaction (RT-PCR) to detect hHGF mRNA. The reason
94 why we chose 7 days after delivery was that the maximum amount of mRNA from
95 introduced gene was peaked at 5-7 days after the injection.Total RNA was isolated using
96 acid guanidinium thiocyanate-phenol-chloroform and ethanol precipitation. RT-PCR was
97 performed using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied
98 Biosystems, Alameda, CA) with primers specific for hHGF and rabbit GAPDH. The
99 primer pairs for hHGF (sense primer, 5'-ACCCAAGCTGGCTAGCGT-3'; anti-sense
100 primer, 5'-AGTGCTGGATCTATTTGATTAGG-3') and rabbit GAPDH (sense primer,
101 5'-GCGCCTGGTCACCAGGGCTGCTT-3'; anti-sense primer, 5'-
102 TGCCGAAGTGGTCGTGGATGACCT-3') [44] were used to amplify hHGF and rabbit
103 GAPDH. PCR reactions of 40 cycles with annealing temperature of 62°C for 1 minute for
104 hHGF, and 26 cycles with annealing temperature 63°C for 1 minute for rabbit GAPDH

105 were performed. The PCR products (hHGF, 260 bp; GAPDH, 465 bp) were separated by
106 electrophoresis in a 3% agarose gel and stained with ethidium bromide.

107 At postoperative weeks 3 (for the confirmation of the protein expression) and 8 (for the
108 period of the protein expression), two tibias, respectively, from each group were used for
109 immunohistochemistry. Paraffin sections were treated with anti-hHGF monoclonal
110 antibody (R&D Systems Inc., Minneapolis, MN; dilution 1:100), and peroxidase-
111 conjugated goat antimouse immunoglobulin (EnVision, DAKO, Carpinteria, CA) was
112 used as the secondary antibody. To develop the color, a DAB kit (EnVision, DAKO) was
113 used. The sections were counterstained with Mayer's hematoxylin.

114 To monitor bone formation, the fracture gaps of the hHGF group were compared to those
115 of the control vector group by radiography at each postoperative time point (Fig 1A-B).
116 The fracture gap was evaluated by comparing bone density of the hHGF and control
117 groups (n = 20 for each group) on anteroposterior and medial-lateral radiographs with an
118 aluminum step wedge (10 steps, 1 mm/step) on the same film. Radiographs were
119 obtained weekly under anesthesia for 8 weeks after the operation. We (HT) evaluated the
120 quantity of callus over the entire 3-mm gap between the proximal and distal native bone
121 using Scion Image Beta-3b software for Windows (Scion Corporation, Frederick, MD).
122 Briefly, the bone density of the gap was measured by interpreting the entire image in
123 units of thickness of the aluminum plate by comparing it with the gradient of luminosity
124 obtained from the aluminum wedge. The bone density is reported in units of aluminum
125 thickness (mm Al).

126 For the quantitative evaluation of the healing process, 10 rabbits in each group were
127 euthanized by an intravenous dose of sodium pentobarbital 8 weeks after surgery. After
128 the soft tissues were dissected from the tibia and the external fixator was removed. The
129 tibias were stored in gauze soaked in 0.9% saline solution at -20°C and thawed at room
130 temperature before pQCT and mechanical analysis. A quantitative determination of callus
131 development was performed with pQCT (XCT-Research SA+, Stratec, Pforzheim,
132 Germany). A 3-mm gap bone section was analyzed with three consecutive transverse
133 pQCT scans 0.77 mm thick, 2.5 mm apart, and with a pixel size of 0.1×0.1 mm. The
134 XCT Series software package (Rev. 6.00B; Stratec, Pforzheim, Germany) was used to
135 calculate the mineral content (mg/mm), mineralized callus area (mm^2), and bone mineral
136 density (mg/cm^3) at the gap level. In order to assess progression of remodeling, areas of
137 higher bone density (> 690 mg/cm^3) within the callus were measured and separated from
138 areas of low-mineral density. The mineral content and the area of this high-mineral-
139 density callus were calculated. The threshold of 690 mg/cm^3 was selected because it
140 corresponds to the lower level of cortical bone. Furthermore, electronic sections through
141 the long axis of each tibia were created on 3-D reconstructed images.

142 Both tibiae of each rabbit were mechanically tested using an electromechanical testing
143 machine (model MZ-500D; Maruto Machine, Inc., Tokyo, Japan). A three-point bending
144 test was performed at a rate of 2.5 mm/min with 100 kgf of weight axial load. The central
145 loading point was adjusted toward the fracture gap. The lower loading points were
146 separated 30 mm from each other. Failure load values and load-displacement curves were
147 obtained for all samples. The stiffness of each unilateral tibia was calculated as the slope
148 of the linear segment on the load displacement curve. Mechanical data ratios of the

149 mechanical data of the fractured unilateral tibia to the intact tibia (percent failure load and
150 percent stiffness) were calculated.

151 Ten rabbits in each group were euthanized 8 weeks after surgery, and the histology of the
152 fracture gap was studied. Heparinized physiologic saline was perfused through both
153 femoral arteries followed by perfusion with 4% paraformaldehyde solution in a
154 phosphate buffer (pH 7.4). The tibias were fixed for 24 hours in the same solution. The
155 tibias were then decalcified with 10% EDTA solution and embedded in paraffin. The
156 specimens were sectioned at a 5-μm thickness parallel to the bone axis and stained with
157 hematoxylin and eosin.

158 Differences in the bone density, mineral contents, mineralized area, bone
159 mineral density, failure load, and stiffness between control vector group and
160 HGF group were determined by a student's t test.

161 **RESULTS**

162 The administration of HVJ-E/hHGF or HVJ-E/pcDNA to the rabbits produced no
163 obvious adverse effects such as sudden death or abnormal weight loss during the 8 weeks
164 of the experiment.

165 In radiographs, bony callus appeared on the lateral side at postoperative Week 2 in the
166 control vector group and bridged the fracture gap at postoperative Week 3. After 3 weeks,
167 the size of the bridging callus was reduced and the callus became gradually calcified at
168 the fracture gap. At postoperative Week 8, however, corticalization was not sufficient to
169 complete remodeling. The hHGF group demonstrated a similar course to the control

170 vector group until postoperative Week 3. After that time point, the callus at the fracture
171 gap became calcified to a greater extent and more rapidly than that of the control vector
172 group. Furthermore, remodeling, especially corticalization and formation of the
173 medullary canal, progressed in the hHGF group. At postoperative Week 8, remodeling in
174 hHGF group was complete, and the fracture gap looked homogeneous when compared to
175 the host bone.

176 The bone density in the hHGF group was greatercompared to the control vector group at
177 postoperative weeks 4 to 8 ($p= 0.3675$; week 2, $p= 0.0099$; week 4, $p= 0.0002$; week 6,
178 $p= 0.0093$; week 8) (Table 1) (Fig 2). Mineral content in the hHGF group was greater (p
179 $= 0.006$) at postoperative Week 8 compared to the control vector group (control vector
180 group, 18.7 ± 4.7 mg/mm; hHGF group, 25.7 ± 5.4 mg/mm;) (Fig 3A). Mineralized
181 callus area in the hHGF group was also greater ($p = 0.015$) at postoperative Week 8
182 compared to the control vector group (control vector group, 19.4 ± 4.9 mm^2; hHGF group,
183 26.2 ± 6.4 mm^2) (Fig 3B). In addition, bone mineral density in the hHGF group tended to
184 be greater at postoperative Week 8 compared to the control vector group, however, there
185 was no difference ($p = 0.059$) between the two groups (control vector group, 933.5 ± 67.3
186 mg/cm^3; hHGF group, 987.7 ± 52.7 mg/cm^3) (Fig 3C). 3D-CT reconstructed images and
187 axial images of specimens obtained 8 weeks after surgery were created (Fig 4A-B). The
188 fracture gaps in the control vector specimen were not bridged completely with a partial
189 defect of the cortical bone. However, no gap was observed in the fracture gap of the
190 hHGF group (Fig 4A). In the axial view, the fracture gaps in the hHGF group had a
191 circular, thick cortical bone. In contrast, the cortex in the control vector group had a
192 partial defect of circular cortical bone (Fig 4B).

193 The mean ratio of failure loads of the hHGF group was greater ($p = 0.037$) than that of
194 the control vector group ($89.4 \pm 17.5\%$ versus $76.3 \pm 9.1\%$, respectively) (Fig 5A). The
195 hHGF-treated tibiae were relatively stiffer ($p = 0.001$) than those of the control group
196 when both were compared to their contralateral controls ($69.7 \pm 6.5\%$ versus $57.5 \pm$
197 6.3%) (Fig 5B).

198 At postoperative Week 8 in the hHGF group, the fracture gaps were completely
199 remodeled with a firm cortex and a reconstructed medullary canal almost identical to that
200 of normal bone. In contrast, a medullary canal was not observed in the control vector
201 group. In place of a medullary canal, mature cartilage and new trabecular bone were
202 present in the middle of the fracture gap, which was not remodeled sufficiently (Fig 6). In
203 RT-PCR, expression of hHGF mRNA was detected only in the callus of hHGF group and
204 in the surrounding muscle of neither group (Fig 7). Thus HVJ-E/hHGF was transfected
205 locally into the fracture gap sufficiently to express mRNA. By immunohistochemistry,
206 We observed expression of hHGF in immature cells, fibroblasts, osteoblasts, and
207 osteocytes (Fig 8A). No immunohistochemical staining was observed in the surrounding
208 muscle of the hHGF group. No hHGF-positive cells were observed in specimens from the
209 control vector group. This indicates the HVJ-E/hHGF was transfected locally into the
210 fracture gap at the level of protein. Human HGF expression was still observed at this time
211 point, however, when expression at 8 weeks was compared to that at 3 weeks, hHGF
212 protein decreased by the endpoint of treatment (Fig 8B).

213 **DISCUSSION**

214 HGF was originally identified from rat and human hepatocyte. Multiple studies
215 subsequently confirmed the role of HGF in enhancing hepatocyte function. After that,
216 HGF was recognized as a powerful and versatile factor with angiogenesis, mitogen,
217 morphogen, and antiapoptotic activity in various tissues. The question arose as to whether
218 HGF influences bone healing. We therefore hypothesized gene transfer of hHGF directly
219 into fracture gaps would promote bone healing in rabbit. This study had two major
220 limitations. First, we followed up only eight weeks. Although this time period was chosen
221 because the control model showed bone union and sufficient corticalization in radiograph
222 at postoperatibe Week 8, our mechanical test data did not reach intact level. Different
223 results may occur at later stages of healing. However, this study demonstrates bone
224 healing differences resulting from HGF, especially at early stage. Second, we did not
225 confirm the duration of hHGF gene expression. Because, on clinical setteing, gene
226 therapy always accompanies the problem of its safety, we should ascertain its safety until
227 at least the end of expression. We gave the priority to economic and practically reasons.

228 Our data suggest percutaneous injection of HVJ-E/hHGF into tibial fracture gaps
229 effectively promotes bone repair. Consequently, the treatment time for fractures could be
230 shortened when the hHGF gene is administered during the early stages of fracture repair.
231 These data indicate the high potential of hHGF gene therapy using the HVJ-E vectors for
232 treatment of bone fractures.

233 Some osteogenetic factors have been used as therapeutic molecules for fracture healing
234 and promote fracture repair. HGF, however, has not been used for this purpose to date.
235 Many papers have been published describing therapeutic uses of HGF for various
236 diseases such as limb ischemia,[28, 49, 50] myocardial ischemia,[5] brain ischemia,[47, 52] hearing

237 impairment,[39] nerve injury,[27] and spinal cord injury.[45] Notably, HGF plasmid delivery for
238 peripheral limb ischemia is now in clinical trials.[34]

239 Recently, it was demonstrated that HGF along with vitamin D promoted growth and
240 differentiation of human mesenchymal cells into osteogenic cells.[12] Later, it was reported
241 HGF enhanced osteoblast differentiation in vitro.[22] It has also been reported HGF
242 contributes to fracture repair by inducing the expression of BMP receptors during the
243 early phase of fracture repair.[24] Our data support the previous results that the use of HGF
244 for fracture repair will induce the expression of BMPR, which will differentiate
245 mesenchymal cells into osteoblasts and osteoblasts into ossification. Based on these
246 observations, the use of HGF together with other factors such as BMP or vitamin D could
247 enhance fracture healing more rapidly. This possibility should be investigated in future
248 studies.

249 Gene delivery to bone has been accomplished by several vectors, including adenovirus,
250 retrovirus, adeno-associated virus (AAV), lentivirus, and herpes simplex virus (HSV). In
251 this study, we employed the HVJ-E vector system as the delivery method for bone
252 fracture gaps. HVJ-E is a novel vector system that converts inactivated HVJ into a gene
253 transfer vector by introducing plasmid DNA directly into inactivated HVJ particles after
254 treatment with a mild detergent and centrifugation in the presence of plasmid DNA.[26]
255 Previous studies demonstrated the successful delivery of DNA to cultured cells and
256 animal tissues such as the inner ear,[39] liver, skin, uterus, lung, eye, tumor tissues,[26] and
257 brain.[46] HVJ-E is a nonviral vector that is generally less efficient than other viral delivery
258 vehicles. However, it is inexpensive, safe, nonimmunogenic, and easy to handle.[19]

259 In principle, gene delivery is performed in two ways, in vivo or ex vivo.[8, 9, 14] In-vivo
260 gene delivery is a direct approach, where the vector is injected directly into the specified
261 target tissue. Ex vivo is an indirect approach, where the therapeutic gene is delivered
262 outside the body to various cells grown in culture prior to implantation into the body. In-
263 vivo gene delivery involves directly delivering the gene into a specific anatomic gap. The
264 advantages of this method are that it is a simple technique that favors its transfer into a
265 clinical application, and that it has the potential for lower costs. The disadvantages are the
266 difficulties in targeting specific cells for transduction and in achieving high transduction
267 efficiency.[14] Ex-vivo gene transfer is considered safer because transfected cells are
268 introduced into the body, and safety tests are possible before introduction. However, it is
269 technically more complex and more expensive.

270 We used gene delivery rather than protein delivery. Gene therapy has potential
271 advantages over protein delivery due to: (1) long-term expression of the protein from the
272 delivered gene, (2) high local concentration of protein expression, (3) low cost of
273 manufacture, (4) reduced systemic effects, and (5) longer shelf life and easier storage of
274 vectors.[31, 32, 38, 48] Ido[23] reported recombinant hHGF administered intravenously was
275 rapidly decreased in serum with a short half-life of 2.4 min. We detected expression of
276 hHGF 6 weeks after the injection of plasmid. Therefore, gene transfer of HGF plasmid is
277 expected to generate much longer expression of HGF than direct administration of HGF
278 protein. Yoshimura[52] also demonstrated gene transfer of HGF plasmid markedly
279 increased cerebral blood flow in the ischemic brain, whereas a single injection of
280 recombinant HGF failed to do so.

281 There are multiple studies that relate to gene therapy for bone defects and fractures.[6, 7, 8,
282 15, 18, 43] However, therapeutic gene therapy for bone regeneration with a nonviral vector
283 has never been demonstrated. Egermann et al[15] reported inflammation due to an immune
284 reaction to adenovirus vectors caused severe retardation of bone formation. Similar
285 results occurred with injection of Ad-BMP-2 into muscles of immunocompetent rats,
286 causing poor bone formation and an inflammatory response at the injection gap.[1]

287 Furthermore, a small number of studies address direct percutaneous injection of a gene to
288 a fracture gap.[7, 8, 43] Gene transfer by direct percutaneous injection leading to endogenous
289 bioactive protein expression offers the potential advantage of simple direct delivery
290 without the requirement for a carrier or surgery and could be used to treat closed fractures
291 in clinical cases. Rundle[43] also performed percutaneous injection of the BMP-4 gene with
292 a retroviral vector into the subperiosteum, which required the deposit of all vector within
293 the periosteum while avoiding the muscle. Therefore it was technically difficult and not
294 practical. Percutaneous injections of gene to muscle are widely used to promote bone
295 repair due to the high efficiency and ease of transfection and longer duration of gene
296 expression.[1, 11, 35] With intramuscular injections, however, the area of gene expression is
297 uncertain, which raises questions about the effect of gene expression at the fracture gap.
298 Furthermore, the immune response differs between intraosseous and intramuscular gaps,
299 as the muscle has a stronger immunologic reaction.[6]

300 Our data suggests HGF promotes fracture healing in rabbit tibia and in-vivo gene therapy
301 using HVJ-E/hHGF effectively enhances bone formation in fracture healing. Utilization
302 of these methods could shorten treatment time, resulting in improved physical and mental

303 wellbeing of patients. In the future, the safety evaluation of this gene therapy technique

304 and the mechanisms whereby HGF promotes bone healing should be assessed further.

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Legends

Fig 1A-B. (A) A series of anteroposterior radiographs taken of the hHGF group (H) and control groups (C) from each single animal show faster callus formation at the 3-mm gap in the hHGF group. **(B)** A series of mediolateral radiographs taken of the hHGF group (H) and control groups (C) from each single animal show faster remodeling at the 3-mm gap in the hHGF group.

Fig 2. The effect of hHGF plasmid on bone healing was determined by bone density of the 3-mm gap. The results of the bone density are given in units of aluminum thickness

Fig 3A-C. (A) At postoperative Week 8 the mineral content (mean ± SD, $p = 0.0061$) was higher in the hHGF group (H) than in the control vector group (C). **(B)** Mineralized callus area at postoperative Week 8 (mean ± SD, $p = 0.015$) was higher in the hHGF group (H) than in the control vector group (C). **(C)** Bone mineral density 8 weeks after the operation (mean ± SD) in the hHGF group tended to be greater compared to the control vector group (C). There was no difference ($p = 0.059$) between the two groups.

Fig 4A-B. (A) A reconstruction image of whole tibia at postoperative Week 8 shows complete bridging in the hHGF group (H), and not in the control vector group (C) with a partial defect in cortical bone. **(B)** An axial image at the gap level 8 weeks after the operation shows thick, circular cortical bone in the hHGF group (H), and not in the control vector group (C) with a partial defect of cortical bone.

Fig 5A-B. (A) Mean percentage of failure load at postoperative Week 8 (mean ± SD, p = 0.0375) was higher in the hHGF group (H) than in the control vector group (C). **(B)** Mean percentage stiffness at postoperative Week 8 (mean ± SDM,p = 0.0011) was stronger in the hHGF group (H) than in the control vector group (C).

Fig 6. Representative longitudinal histologic sections of the 3-mm gap at postoperative Week 8 are shown. The arrows indicate the original 3-mm defect region (top row). Magnified histology of 3-mm gap (bottom row). The gap in the control vector group (C) had a trabecular bone in the middle of the fracture gap, meanwhile in the hHGF group (H), a firm cortex and a reconstructed medullary canal were observed. (Stain, Hematoxilin-Eosin stain; original magnification, ×1 for left side and ×40 for right side)

Fig. 7. RT-PCR analysis demonstrated the expression of hHGF mRNA in the injected callus and the surrounding muscle of both groups. hHGF mRNA was specifically detected in the callus of the hHGF group (H), but was not detected in the callus and muscle of the control vector group, or in muscle of the hHGF group.

Fig. 8A-B. (A) Immunohistochemistry of hHGF at the fracture gap at postoperative Week 3 (1 week after the injection of plasmid)(Stain, Immunohistochemistry of hHGF ; original magnification, ×400). In the hHGF group (H), hHGF expression was markedly observed in immature cells, fibroblasts, osteoblasts, and osteocytes. No immunohistochemical staining was observed in specimens from the control vector group (C). **(B)** Immunohistochemistry of hHGF of the fracture gap at postoperative Week 3 or 8

(1 and 5 weeks after the injection of plasmid) (Stain, Immunohistochemistry of hHGF ; original magnification, ×400). hHGF expression was still observed at postoperative Week 8; however, it decreased compared to that of postoperative Week 3.

Fig. 1A

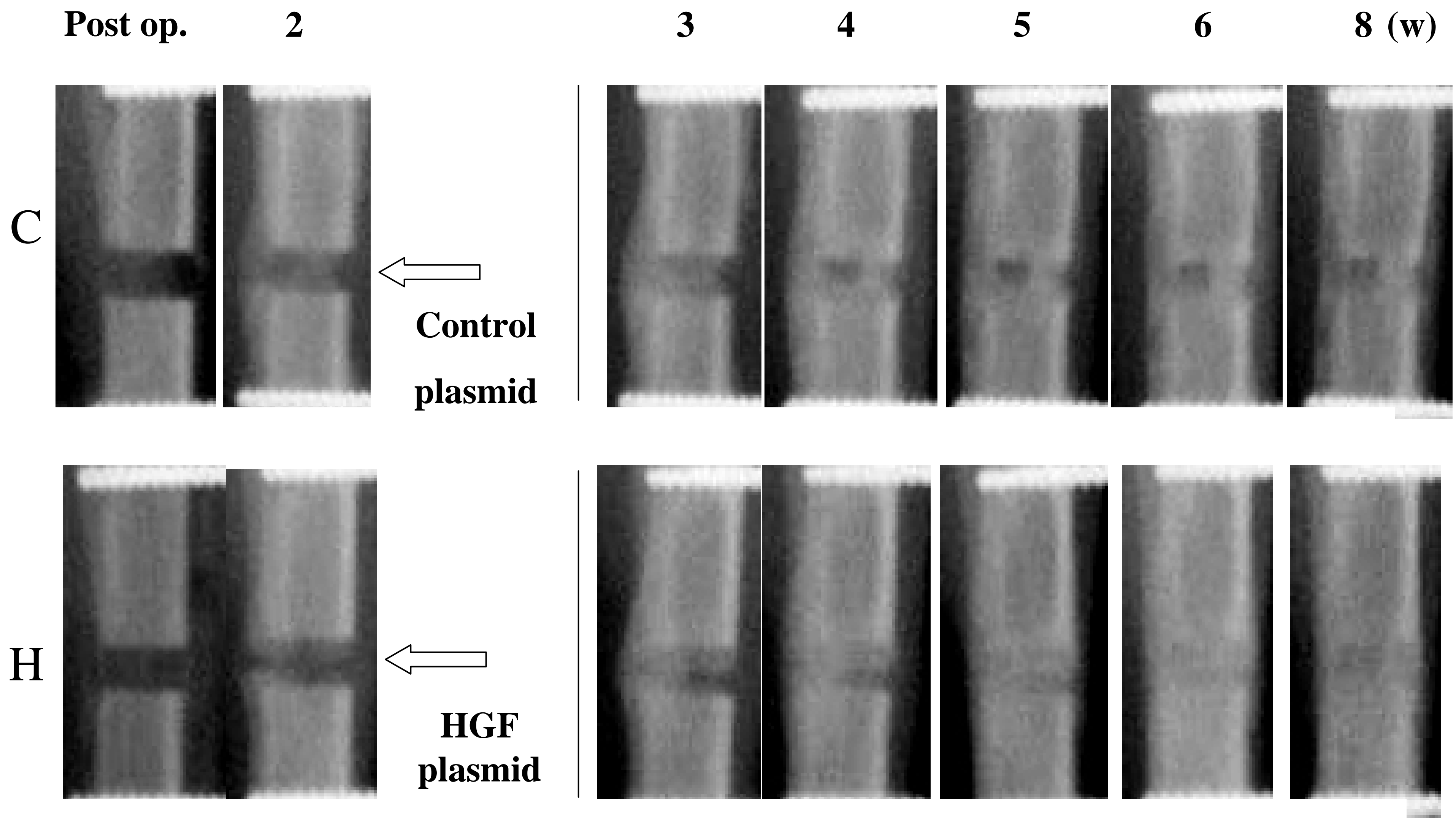


Fig. 1B

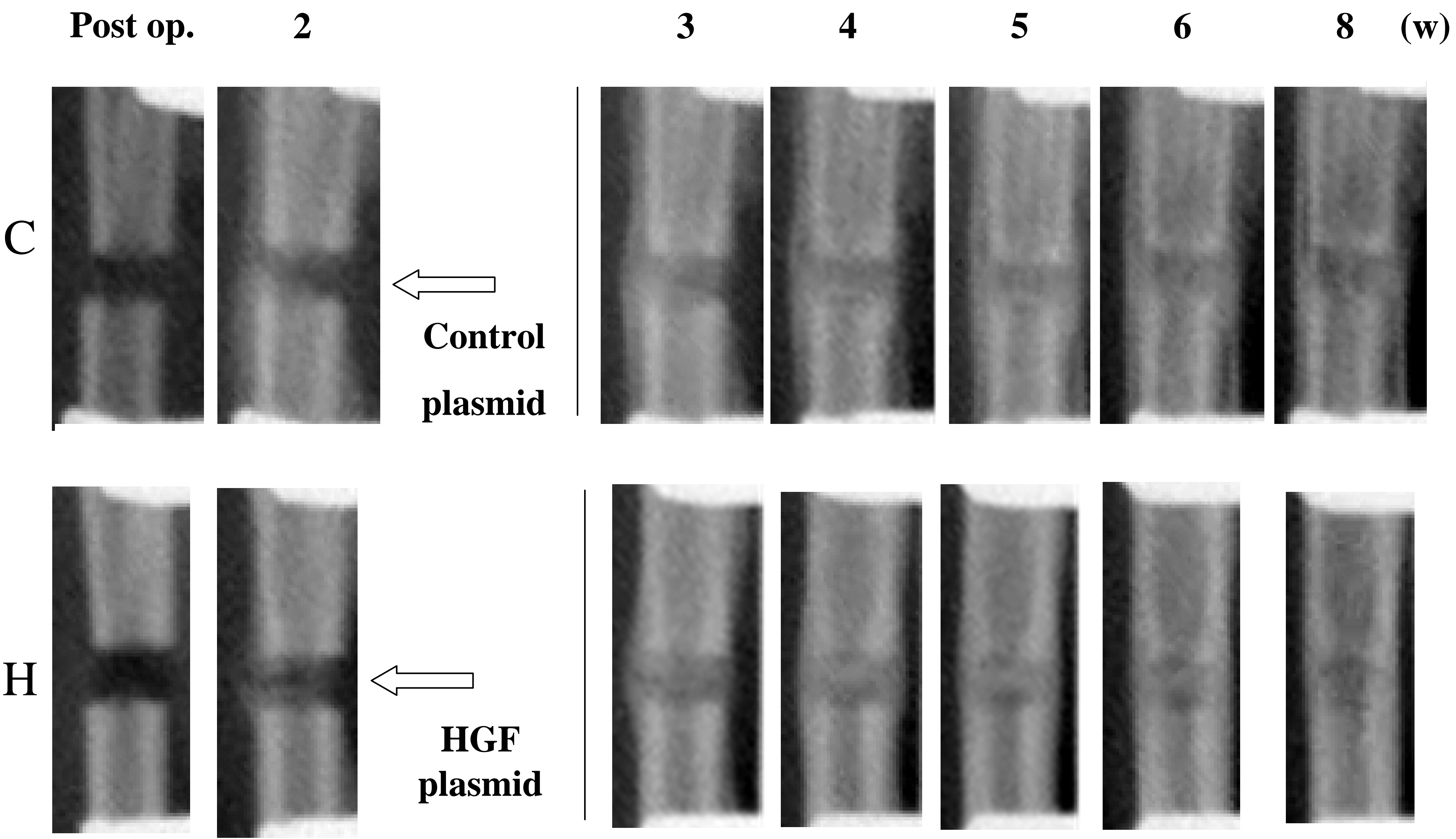


Fig. 2

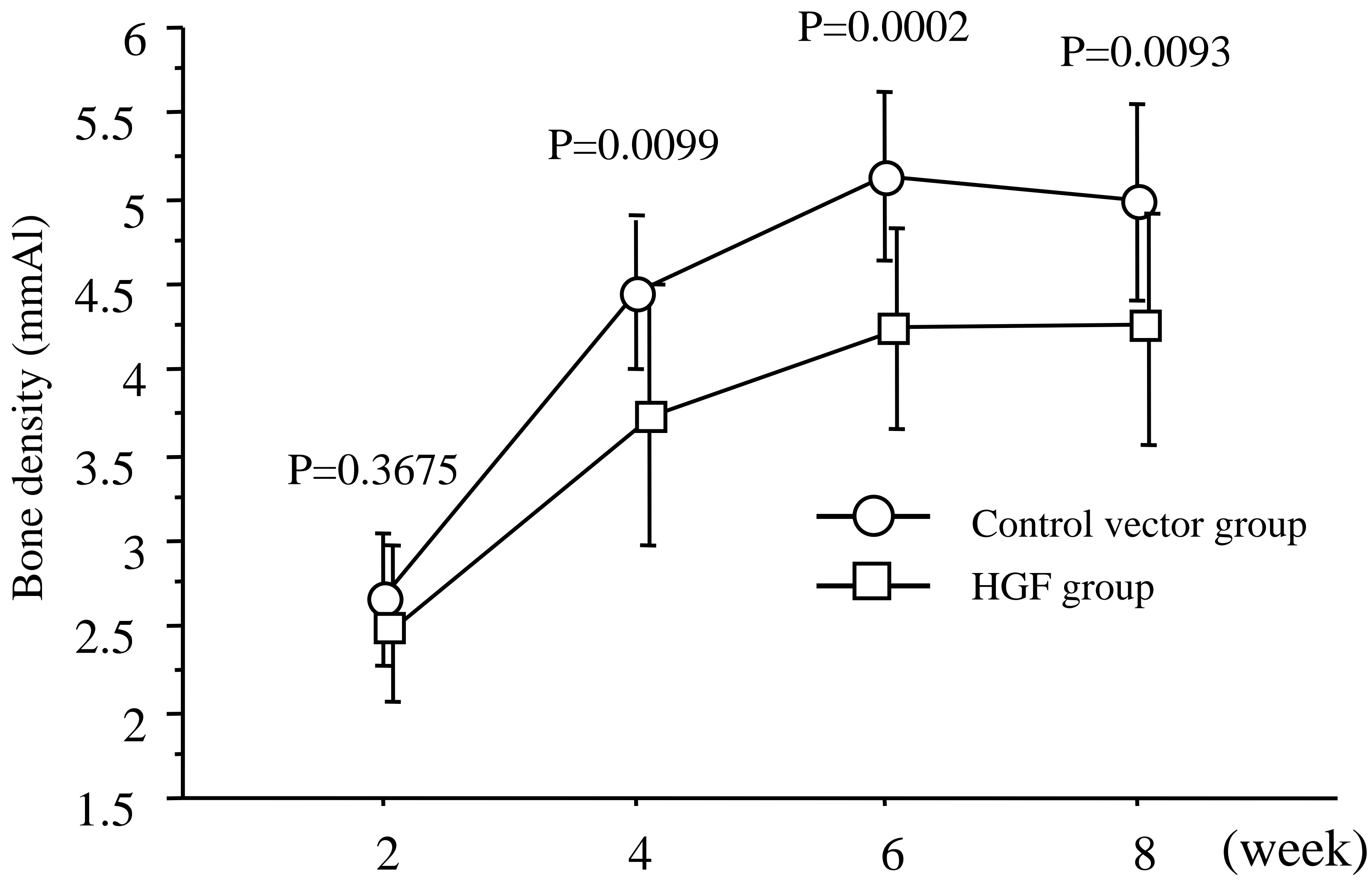
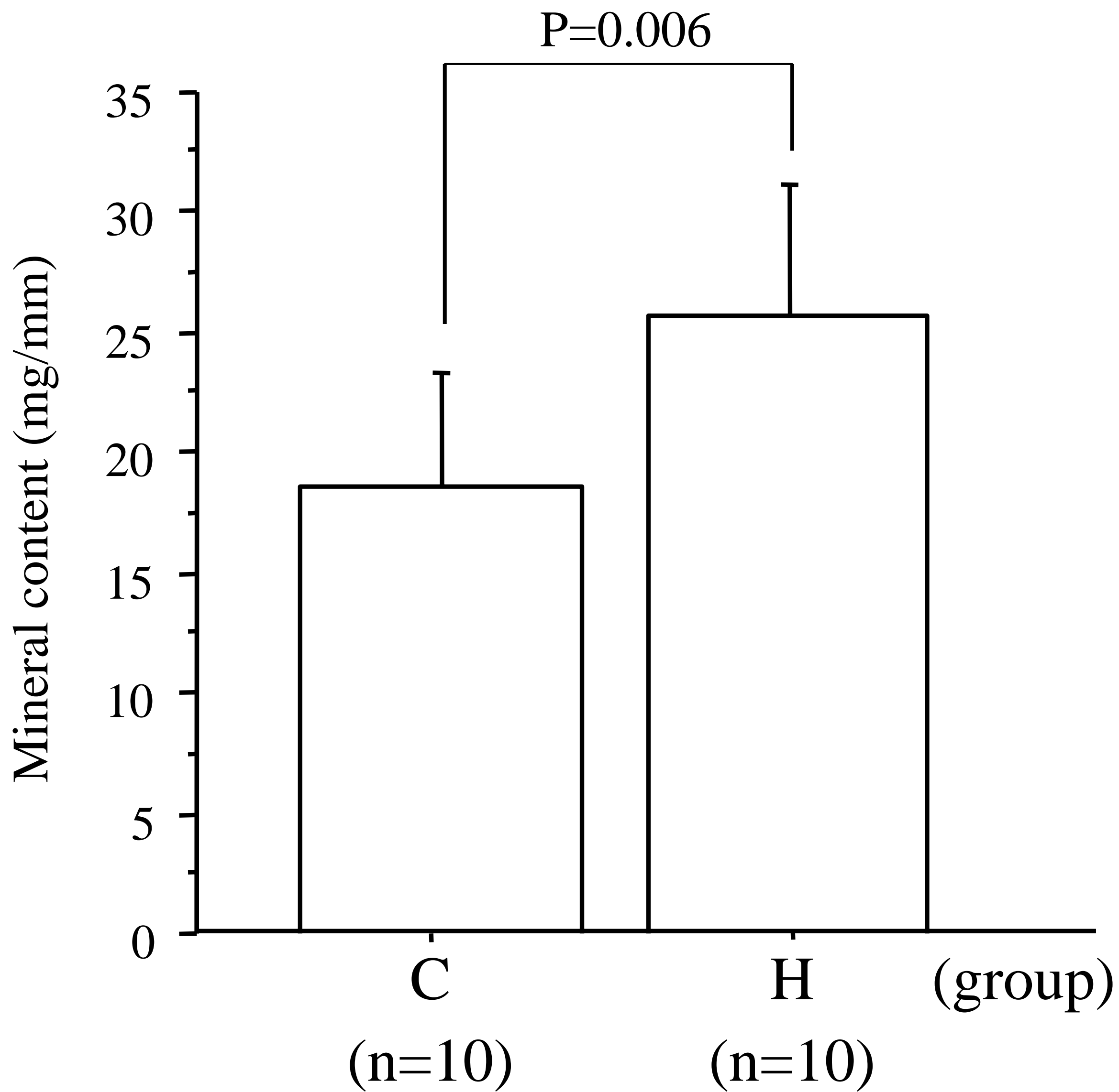


Fig. 3A



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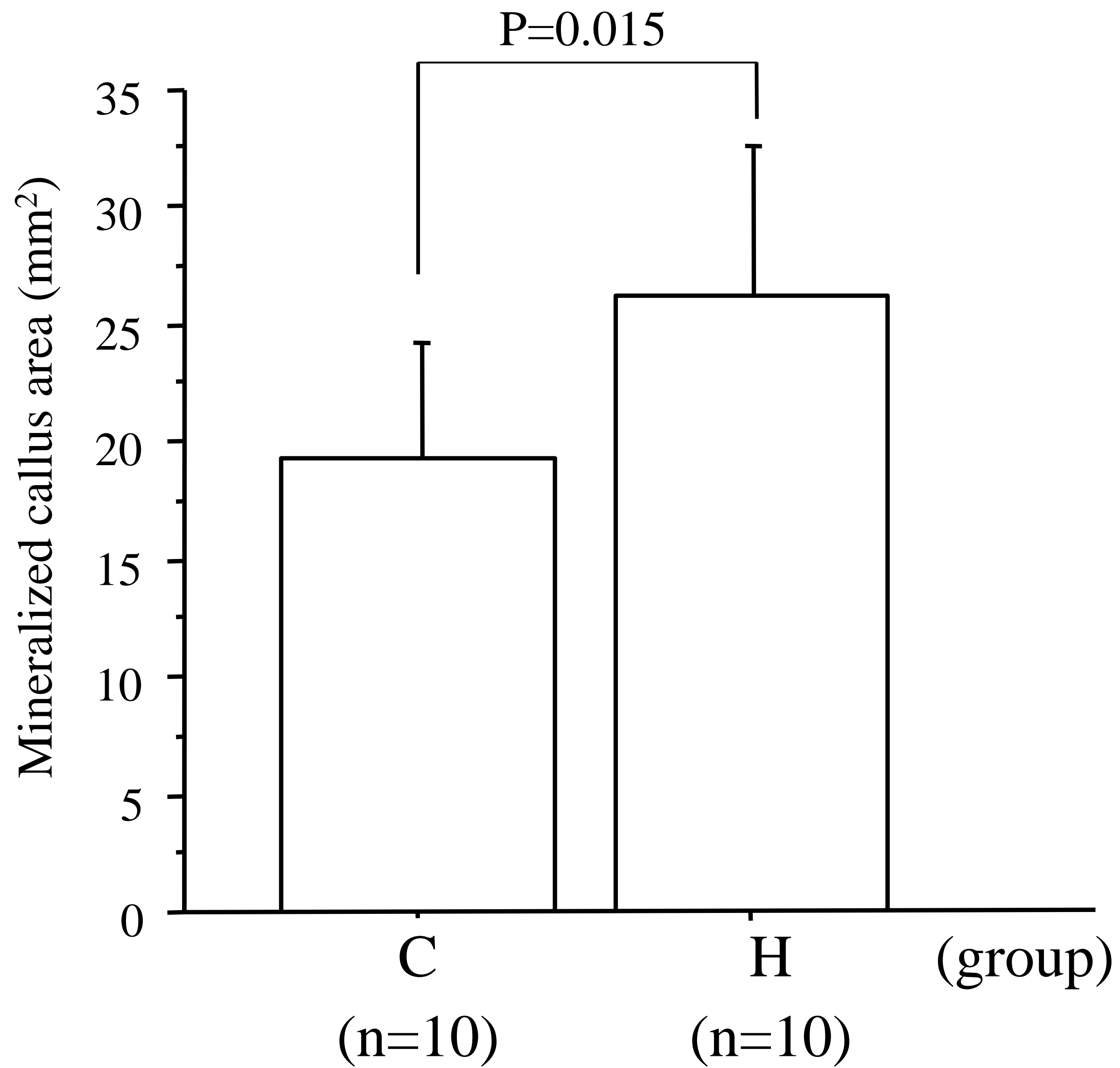

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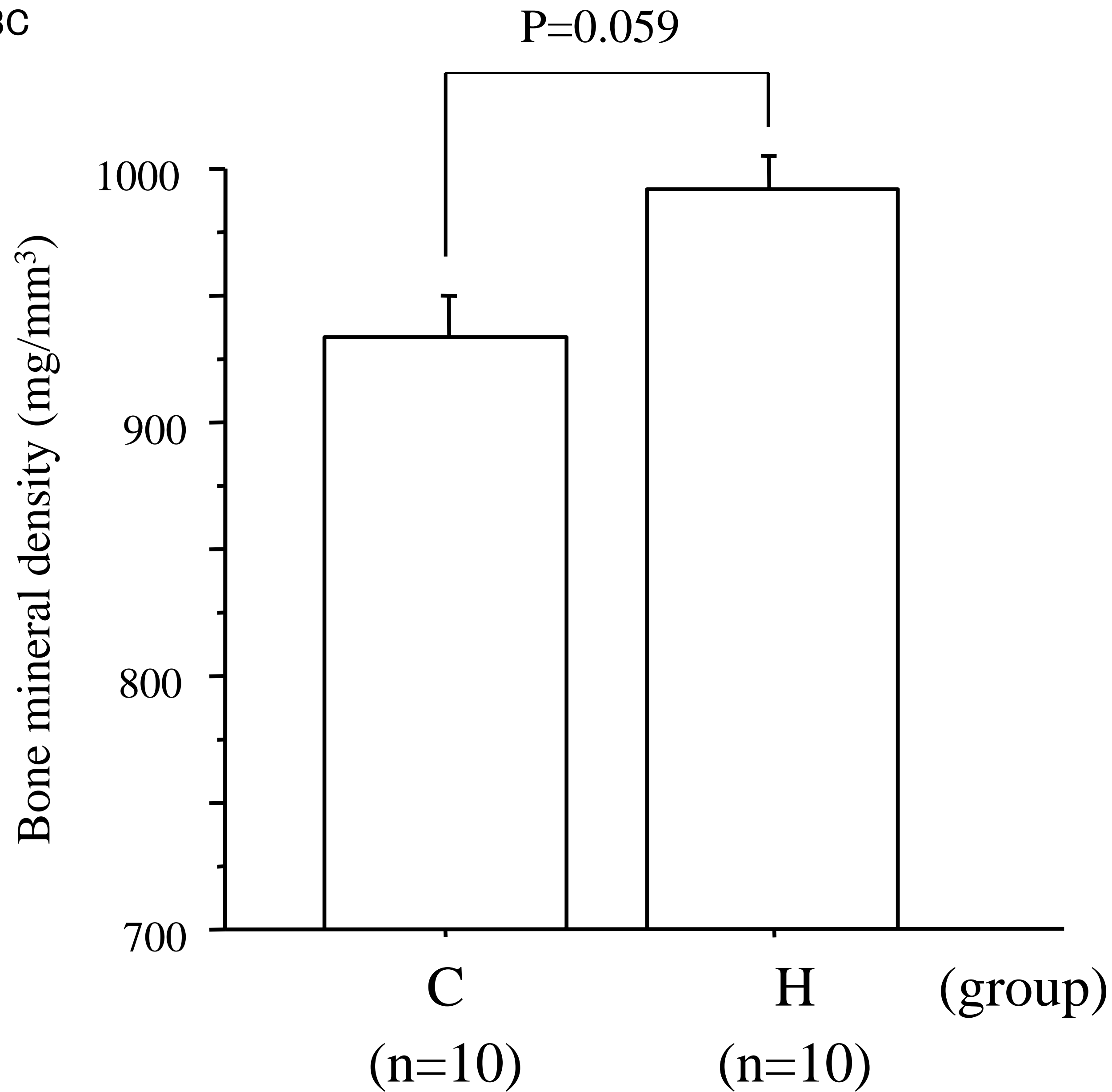

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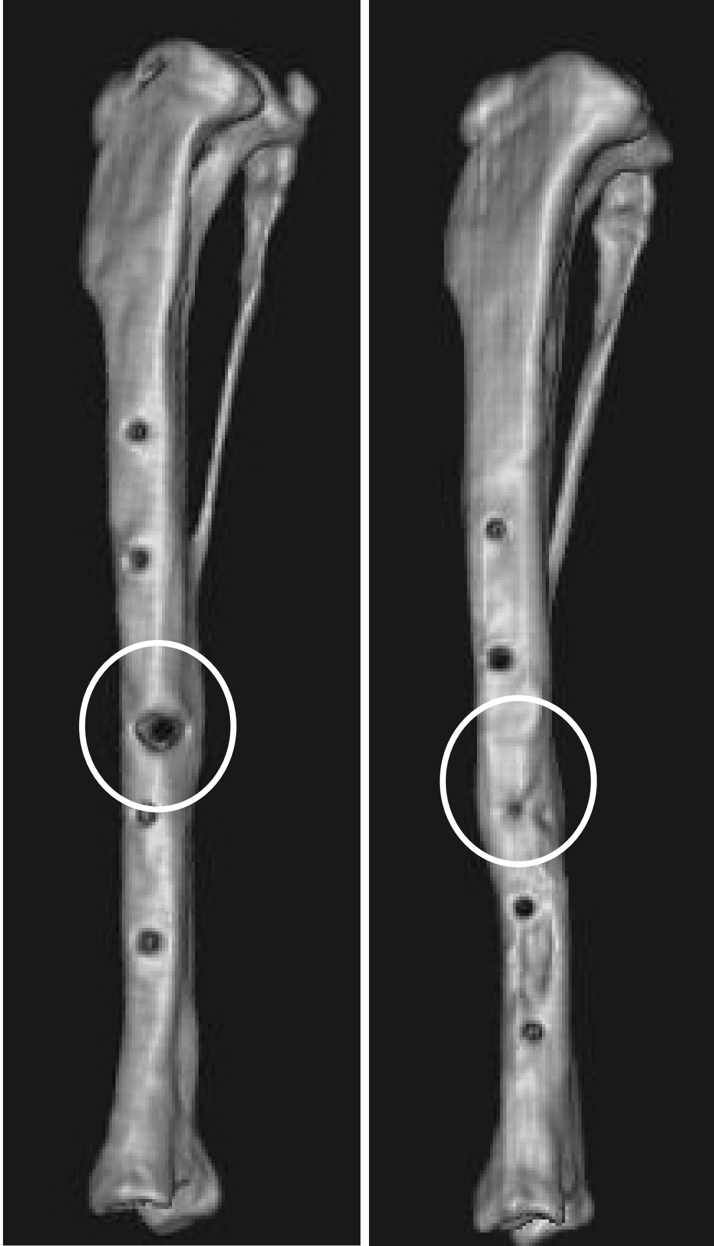
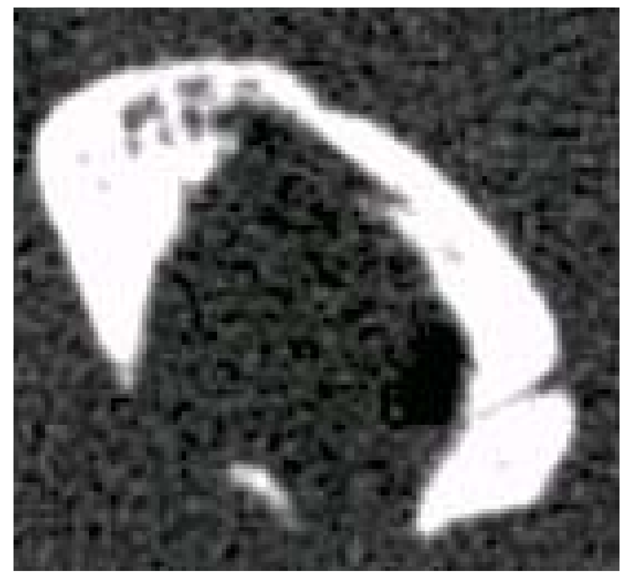


C H

Fig. 4B



C

H

Fig. 5A

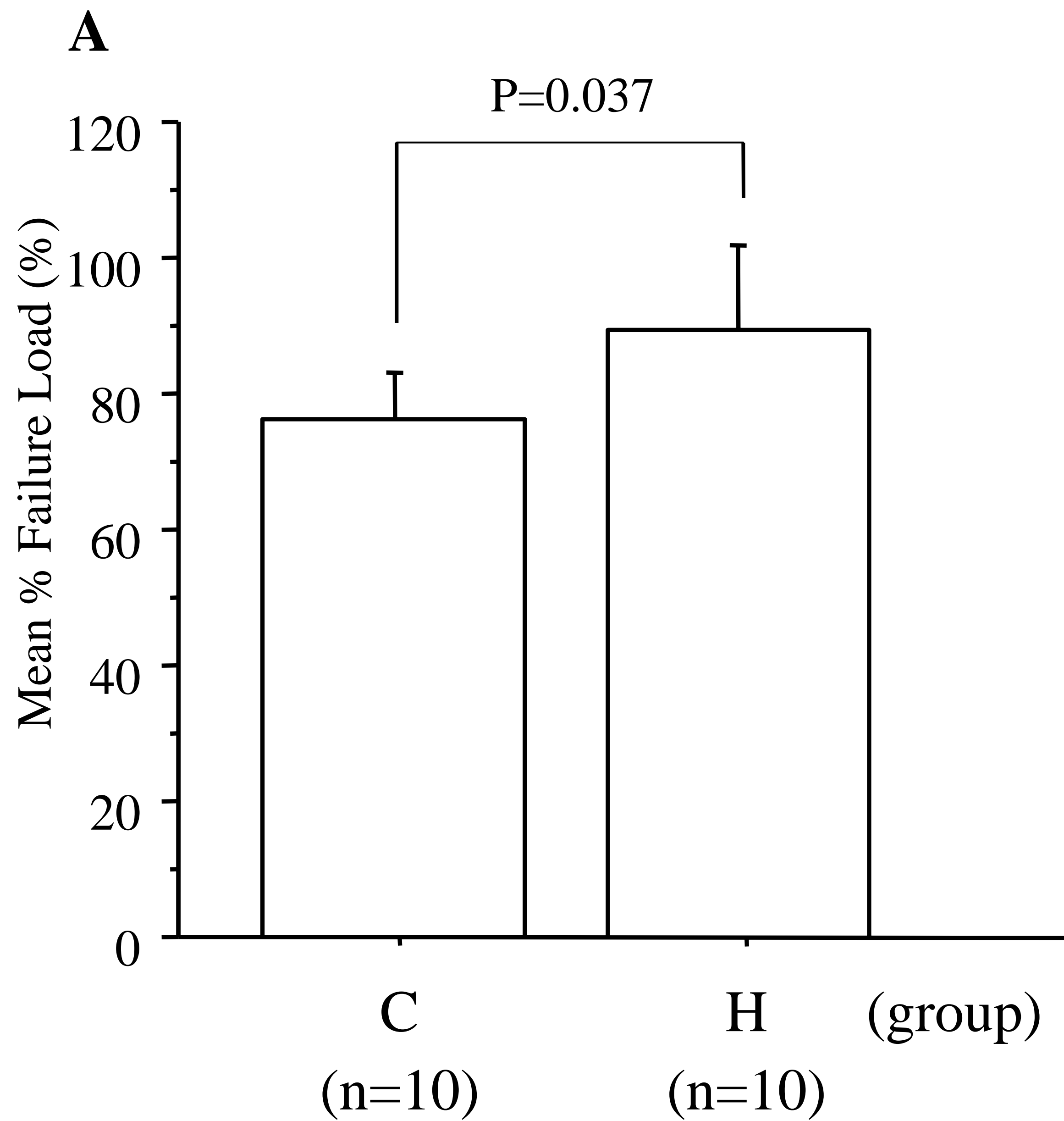


Fig. 5B

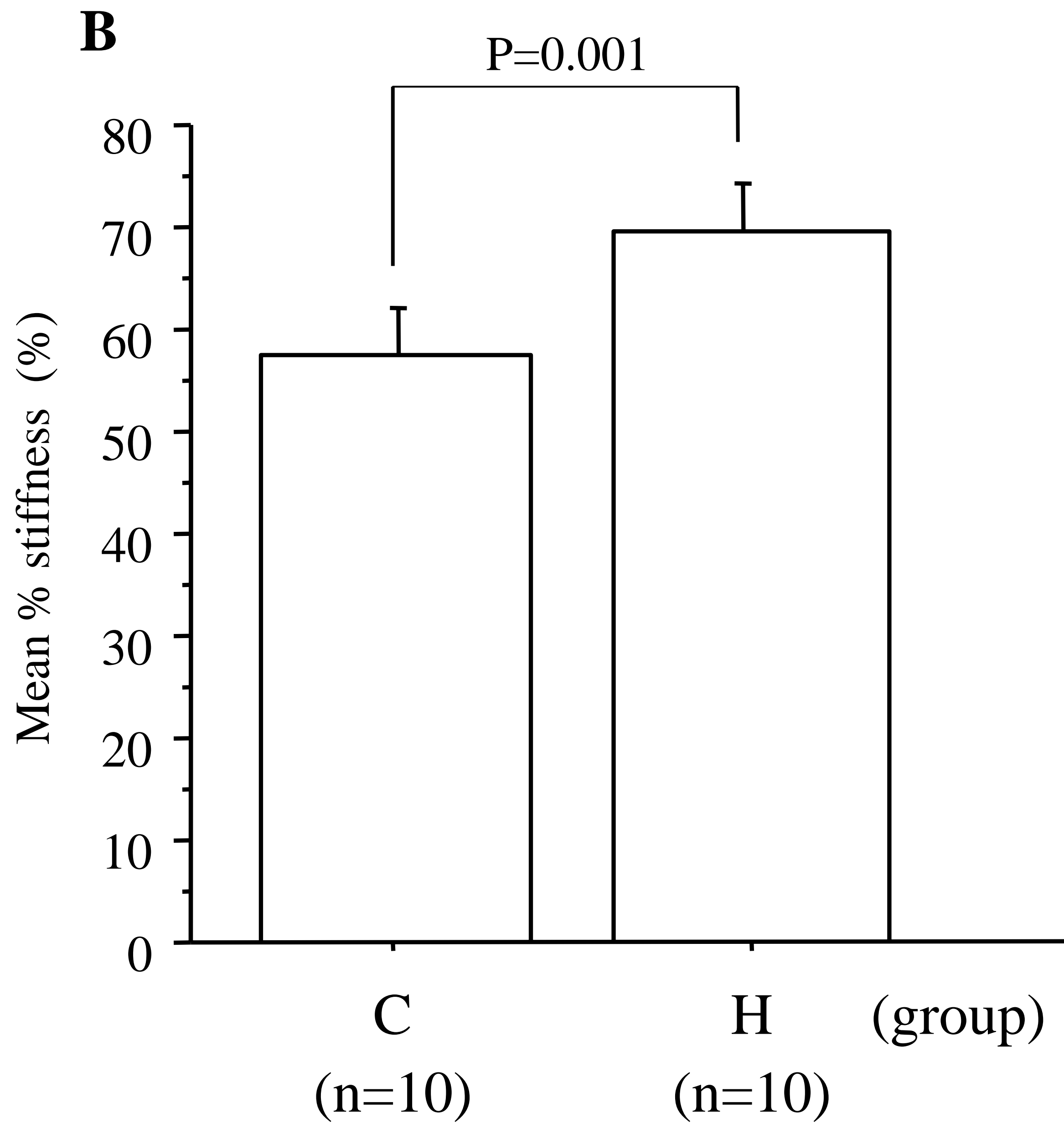
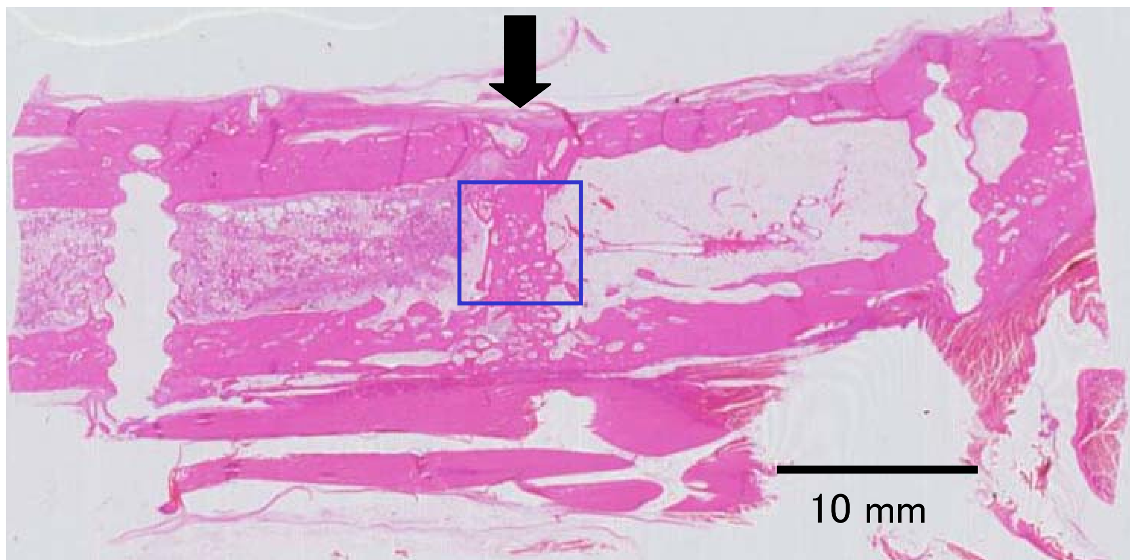
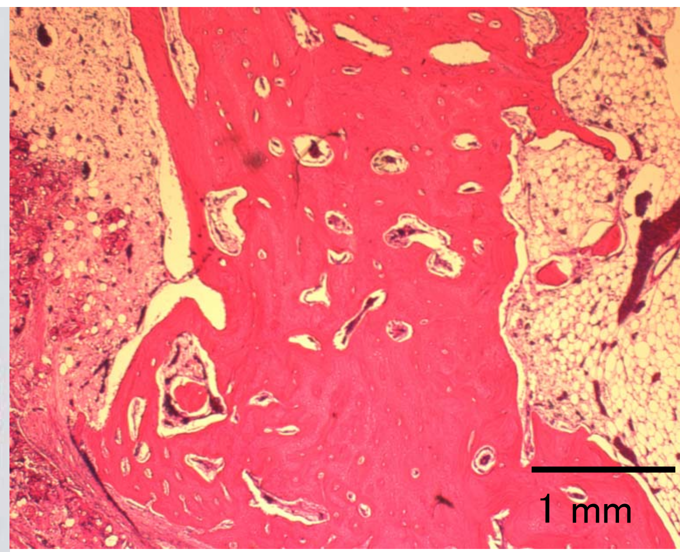


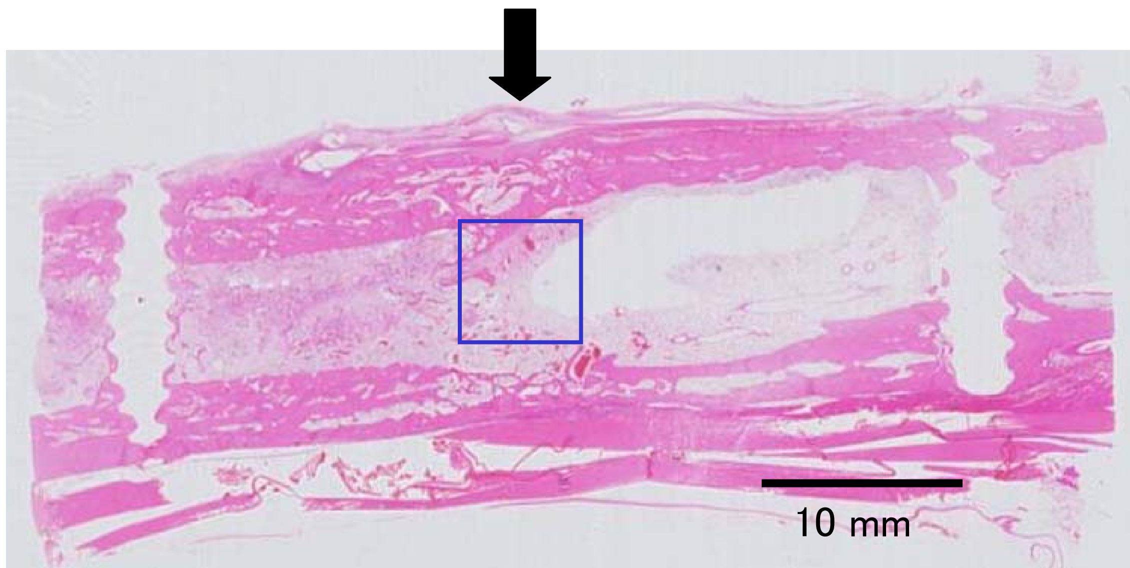
Fig. 6

C





H



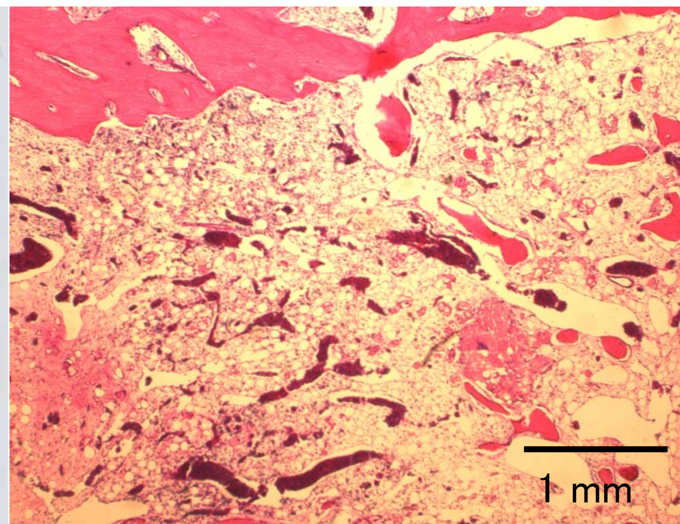


Fig. 7

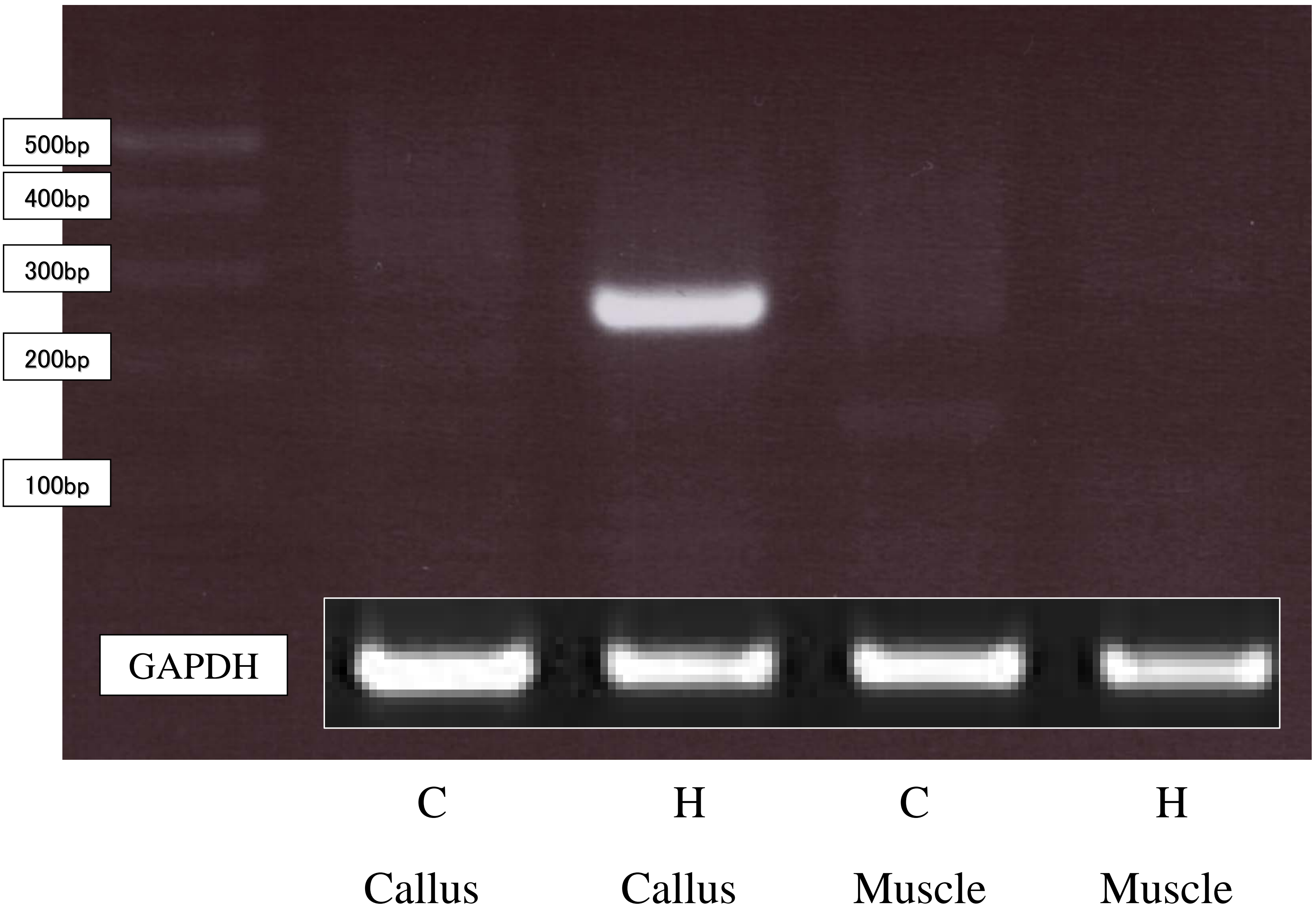


Fig. 8A

immature cell

fibroblast

osteoblast

osteocyte

osteoid

C

H

Fig. 8B

3w

(1w. after injection)

8w

(5w. after injection)