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Effect of compression on mandibular fracture haematoma-derived cells

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Abstract

Mechanical stress induces a variety of biochemical and morphological reactions in bone cell biology. This study aimed to investigate appropriate pressures of osteogenesis on the biological responses of 3-dimensional cultured human mandibular fracture haematoma-derived cells by compressive loading. Six patients with mandibular fractures who underwent open reduction and internal fixation were included in the study. During the operation, fracture haematomas that formed fibrin clots were manually removed before irrigation. First, pressures were applied to human mandibular fracture haematoma-derived cell-seeded collagen sponges. The sponges were subjected to mechanical compression using loading equipment applied at no compression, 0.5, or 1 mm. Compressive loading was applied to the samples prior to compression for 0, 6, 12, or 24 hours. Collagen sponge samples were collected for quantification of mRNA using several parameters including alkaline phosphatase (ALP), osteopontin (OPN), osterix (OSX), runt-related gene 2 (RUNX2), protein level, and immunocytochemistry (anti-sclerostin). Among these the 0.5 mm compression group compared with the control and 1.0 mm compression groups upregulated mRNA expression of OPN and OSX after 24 hours. Additionally, compared with the control group, a significantly higher OSX gene expression was observed in both the 0.5 mm and 1.0 mm groups after 6, 12, and 24 hours of compression ($p < 0.05$). However, no significant differences were observed regarding ALP and RUNX2 expression. These results indicated increased stimulation of osteogenesis of the mandibular fracture-line gap in the 0.5 mm compression group compared with the control and 1.0 mm compression groups.

Keywords: Maxillo-mandibular surgery; Mandibular injuries; Mechanical stress; Primary cell culture; Alkaline phosphatase; Osteogenesis

Introduction

Mandibular fractures are repaired via intramembranous ossification without cartilage formation.[1] Through this process, a fracture haematoma consisting of immune and chondroprogenitor and osteoprogenitor cells, which initiate fracture healing after trauma, forms between the bony fragments.[2] Surgical management of mandibular fracture involves removal of the haematoma, which results in a prolonged healing process.[3] Hasegawa et al demonstrated the osteogenic differentiation capacity of mandibular fracture haematoma-derived cells (MHCs) in 2D cultures.[4]

The best method of fixing mandibular fractures remains controversial.[5] Over several decades the type of osteosynthesis has changed from wire (non-rigid fixation) and miniplates (semi-rigid fixation) to compression plates (rigid fixation) and compression screws (lag screw technique). Each technique offers unique advantages and disadvantages. The advantages of miniplates are easy handling avoidance of an external incision, and elimination of potential nerve damage. However, they have limitations. Their small size reduces rigidity and may lead to torsion or movement of the fracture-line gap under loading. Compression plating has the added advantage that fracture segments of cortical bone, which are brought into direct contact and fixed with absolute stability, will heal without formation of an external callus. However, complete primary bony healing is rarely achieved with rigid fixation. Its strengths have been evaluated using a universal testing machine in animals, and in humans different

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study designs have been used to evaluate clinical outcomes.[5,6] Many previous studies of mechanical stress have also described applied loading with a special compressive device,[7] although the appropriate distance of compression on the fracture-line gap is unclear in the actual surgery. To our knowledge, no report investigating the molecular mechanisms of mechanical stress in vitro has been conducted; this could improve the treatment of mandibular fractures by researching the appropriate distance of compression on the fracture-line gap. This study therefore aimed to investigate the appropriate distance of compression on the biological responses of 3-dimensional cultured human MHCs by compressive loading.

Material and methods

This study was conducted in compliance with the World Medical Association Declaration of Helsinki. All procedures in the research were approved by the Ethics Committee of Kobe University Hospital (approval number: No.1246), and informed consent was obtained from each patient.

The study included patients with mandibular fractures who underwent open reduction and internal fixation at the authors' institution between July 2016 and February 2018. Patients who had open reduction and internal fixation for mandibular fractures and no history of maxillofacial surgery, were included. Patients who had been under anticoagulant, steroid, or non-steroidal anti-inflammatory medication within three months of injury, and those with systemic comorbidities including diabetes, rheumatoid arthritis, or conditions that may cause immunosuppression, were excluded. In the study period, six patients fulfilled the inclusion criteria (3 men and 3 women, median age (range) 26.5 (8–54) years). The fracture involved the mandibular angle in three and mandibular body in the remaining three. After a mean (range) of 1.5 (1–5) days, specimens of mandibular fracture haematomas were obtained. MHC isolation and culture were performed as previously described.[4] The median (range) wet weight of the obtained haematomas was 2.93 (0.69–8.46) g.

Briefly, haematoma at the fracture site was harvested and minced into small pieces with growth medium (α-modified minimum essential medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mmol/l L-glutamine (Gibco), and antibiotics (penicillin G, 100 units/ml; streptomycin, 100 μg/ml, Sigma-Aldrich)) in a culture dish. The cultures were incubated at 37 °C with 5% humidified carbon dioxide. Medium exchange was performed weekly and passaged at a cellular confluency of 80%–90%.

A porous atelocollagen sponge (AteloCell®, MIGHTY, KOKEN) with a diameter of 5 mm and thickness of 3 mm was used as a scaffold. The shape of the scaffold was cylindrical. The collagen scaffold, which originated from type I collagen extracted from bovine dermis, was freeze-dried then cross-linked and sterilised. The pore size was designed to be 30–200 nm and the pores were inter-connected. It was produced as previously described.[8] Trypsinised cells (1×10^6/pellet) collected by centrifugal force were suspended in the growth medium, and the cell suspension (50 μl) seeded on to a collagen scaffold. The collagen sponge samples were precultured for seven days in a growth medium, then cultured for another seven days in an osteogenic medium. Osteogenic medium with dexamethasone (10^{-8} M; Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 50 mg/ml ascorbic acid, was added. The culture medium was replaced twice a week. Collagen sponge samples were subjected to sustained compressive loading at 37 °C under 5% humidified carbon dioxide using loading equipment. The loading equipment compressed the samples in a six-well plate with a 2 or 2.5 mm thick plastic plate with a hole 10 mm in diameter in the centre, a no-hole plastic plate, and glass cylinder, stabilised using a rubber band (Fig. 1). The collagen sponges were subjected to mechanical compression using the loading equipment applied at 0 (no compression), 0.5 mm, or 1 mm. Pressure was applied to the samples prior to compression (0), and for 6, 12, or 24 hours according to previous reports.[7,9,10] The sample without treatment served as the baseline for analysis.

Cell proliferation assay

Cell proliferation was measured by the WST test (Roche Diagnostics GmbH) for non-radioactive quantification of cell proliferation via measurement of the formazan product in living cells. Briefly, the collagen sponges were washed in phosphate buffered saline (PBS) prior to running the assay, to remove the medium. They were then placed into a new 12-well plate and treated with 1 ml of medium containing 100 μl WST-1 reagent/well, then incubated for 1.5 hours. After incubation, the medium was collected and analysed with a spectrophotometer at an absorbance of 450 nm on a microtiter plate reader (Bio–Rad). The same volume of culture medium and cell proliferation reagent WST-1 was used in this study as a background control for the plate reader. The proliferative activity of each sample was shown as a relative value of absorbance compared to the baseline sample (cell viability = (absorbance of sample − absorbance of blank)/(absorbance of the no compression control at each time point-absorbance of blank)).

Histological observation

On each slide the cell-seeded porous collagen sponges were fixed in 4% neutral buffered formalin and processed for histological analysis. The paraffin-embedded tissue blocks were sliced into 6 μm thick sections for subsequent histological examination. Quantification of apoptosis was conducted with the In situ Apoptosis Detection Kit (TAKARA Bio Inc). Following TUNEL staining according to the manufacturer's instructions, the nucleus was stained with DAPI (Nacalai Tesque Inc).

For immunohistochemical evaluation, slides were deparaffinised with xylene and rehydrated in a graded alcohol series. Heat-mediated antigen retrieval was performed

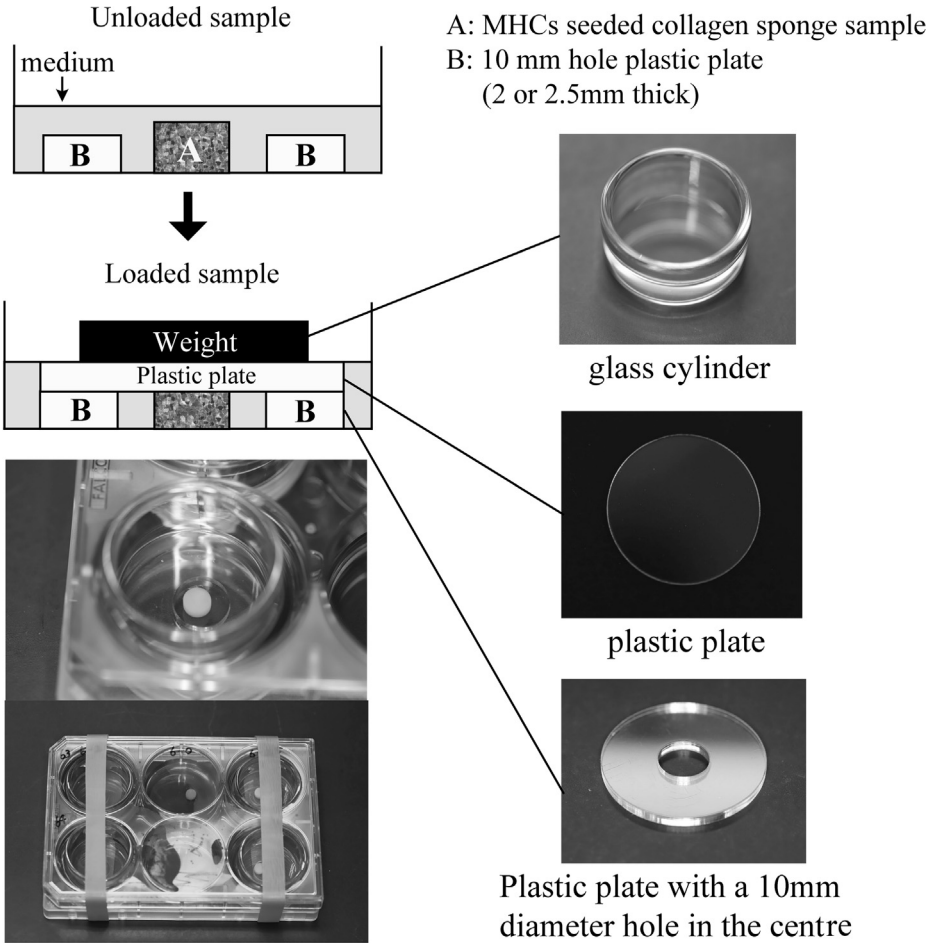

Fig. 1. Sponge compression loading system. 3D cultured constructs produced from mandibular fracture haematoma-derived cells and seeded on to a porous collagen disc. The loading equipment compressed the samples in a 6-well plate with 2 or 2.5 mm thick plastic plates with a 10 mm diameter hole in the centre, no-hole plastic plate, and glass cylinder, stabilised using a rubber band.

with Tris/EDTA buffer pH 9.0, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2, and samples were incubated overnight at 4 with the following primary antibody in Can Get Signal® immunostain solution A (Toyobo): polyclonal goat anti-sclerostin (1:100 dilution, ab194940, Abcam). Following treatment, sections were incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG polyclonal antibody (Nichirei Biosciences Inc) for two hours at room temperature. The signal was developed as a brown stain using the peroxidase substrate 3 and 3-diaminobenzidine (Nichirei Biosciences Inc), and sections were counterstained with haematoxylin.

Evaluation of stained slides

The samples were observed with the BZ-X700 All-In-One fluorescence microscope (Keyence). The number of TUNEL was counted in three fields in the central area of the collagen sponge (magnification ×10), and the proportion of positive cells to total cells was calculated using ImageJ (National Institutes of Health (NIH)). The number of sclerostin-positive cells was counted within three fields at a magnification of ×200 and the median was used for statistical analysis. Quantitative analyses of the sclerostin-positive regions, which we referred to as the immunopositive regions, were performed using Hybrid Cell Count Module/BZ-H3C software (Keyence).

Quantitative real-time RT-PCR

After compression, total RNA was extracted from the embedded cells, and gene expression was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR). Briefly, the collagen sponge was homogenised using 1 ml of TRIzol reagent (Invitrogen) at room temperature, and total RNA was extracted. RNA was further cleaned using the RNeasy Mini Kit (Qiagen). The cDNA was synthesised (160 ng of total RNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The mRNA expression of β-actin, alkaline phosphatase (ALP), osteopontin (OPN), osterix (OSX), and runt-related gene 2 (RUNX2) were analysed using quantitative PCR (qPCR). Among these, β-actin was used as the reference gene. RT-PCR was performed using an ABI PRISM® 7700 sequence

detection system and SYBR Green reagents (both from Applied Biosystems) following the recommended protocols. The primer sequences are shown in Table 1. All primers were obtained from Invitrogen.

Alkaline phosphatase (ALP) activity assay

ALP activity of the extracted samples was assayed. The collagen sponge was washed twice with PBS, homogenised using 1 ml PBS, and sonicated with the Microson™ Ultrasonic Cell Disruptor XL2000 (Misonix) on ice. Release of p-nitrophenol from p-nitrophenyl phosphate was then determined using p-nitrophenyl phosphate (AnaSpec). Protein concentration was standardised using a BCA protein assay kit (Pierce, ThermoFisher Scientific) and the results expressed as the amount of p-nitrophenol produced in nmol/min/mg of protein. Expression levels were expressed relative to the unloaded compression (0h) culture levels.

Statistical analysis

Ekuseru-Toukei 2012 (Social Survey Research Information Co Ltd, Tokyo, Japan) was used for statistical analyses. The expression of each target gene normalised to an endogenous reference gene relative to the calibrator was calculated using the delta-delta CT method (Applied Biosystems). The calibrator was a 1× sample, and all other quantities were expressed as an n-fold difference. Non-parametric tests (Kruskal–Wallis test) with multiple comparisons (Steel–Dwass test) were applied to assess differences in the medians between the no compression control and compression groups at each time point for mRNA expression levels of each gene and ALP activity. Data are presented as the median ± standard error (SE). Differences were considered statistically significant at $p < 0.05$.

Results

Quantitative real-time RT-PCR

Figure 2 shows the mRNA expression levels of ALP, OPN, OSX, and RUNX2. After 24 hours of compression, a significantly higher OPN gene expression was observed in the 0.5 mm compression group compared with the 1.0 mm compression ($p = 0.043$) and control ($p = 0.011$) groups. Also, compared with the control group, a significantly higher OSX gene expression was observed in both the 0.5 mm and 1.0 mm groups after 6, 12, and 24 hours of compression (0.5 mm: 6,12, 24 hours, $p = 0.011$, 0.018, 0.011, respectively; 1.0 mm: 6,12, 24 hours, $p = 0.011$, 0.028, 0.011, respectively). Additionally, after 24 hours of compression, OSX gene expression was significantly higher in the 0.5 mm compression group than in the 1.0 mm group ($p = 0.043$). However, no significant differences were observed regarding ALP and RUNX2 expression.

ALP activity assay

Figure 3 shows that no significant effect was observed in ALP activity.

WST-1 assay and TUNEL staining

WST-1 assay and TUNEL staining were used to investigate the proliferative and apoptotic effects of compressive loading in 3D cultured MHCs. The cell number in the 1.0 mm compression group was significantly higher than in the no compression control group after 12 hours' compression ($p = 0.037$) (Fig. 4). However, no significant differences were observed at other time points. No significant effect was observed in apoptosis.

Immunohistochemical expression of anti-sclerostin

As analysed by immunohistochemistry, the 0.5 mm compression group was significantly higher than the no compression control group after 12 hours' compression ($p = 0.032$) (Fig. 5). There were no significant differences at other time points.

Discussion

Bone fractures are caused by excessive mechanical stress. During the fracture the bone marrow canal is opened and

Table 1
Primer sequences used for the quantification of gene expression.

Target gene	Primer set	Sequences (5′–3′)	Size (bp)
β-actin	Forward	GATGAGATTGGCATGGCTTT	100
	Reverse	CACCTTCACCGTTCCAGTTT	
Alkaline phosphatase (ALP)	Forward	CCCAAAGGCTTCTTCTTG	357
	Reverse	CTGGTAGTTGTTGTGAGC	
Osteopontin (OPN)	Forward	GGCTAAACCCTGACCCATCTC	98
	Reverse	TCATTGCTCTCATCATTGGCT	
Osterix (OSX)	Forward	GTCAAGAGTCTTAGCCAAACTC	124
	Reverse	AAATGATGTGAGGCCAGATGG	
Runt-related gene 2 (RUNX2)	Forward	TCCACACCATTAGGGACCATC	136
	Reverse	TGCTAATGCTTCGTGTTTCCA	

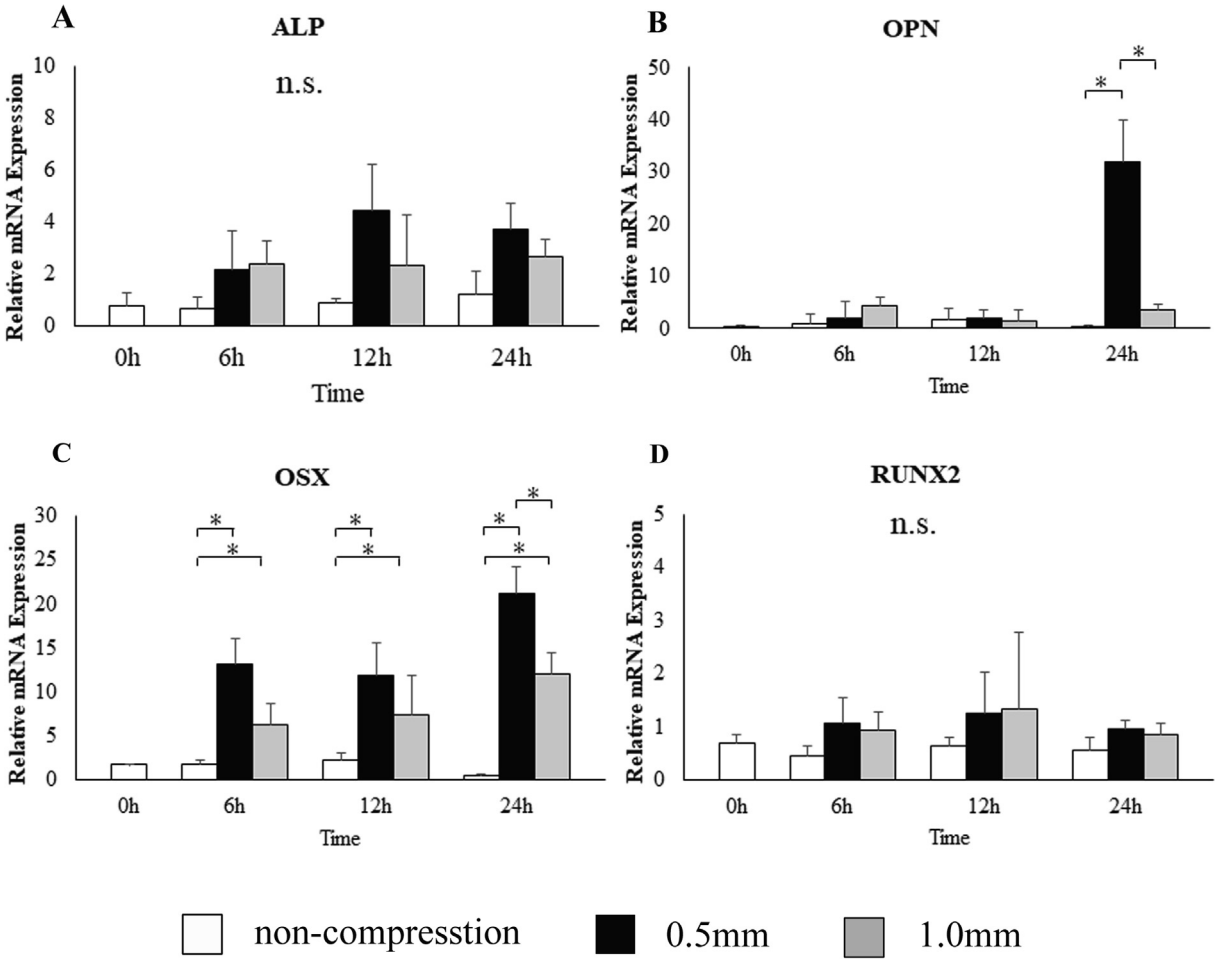

Fig. 2. The mRNA expression levels of (A) ALP, (B) OPN, (C) OSX, (D) RUNX2 assayed using reverse transcription–polymerase chain reaction. Collagen sponges were subjected to mechanical compression using the loading equipment applied at 0 (non-compression), 0.5 mm, or 1 mm. Pressures were applied on samples prior to compression (0) for 6, 12, or 24 hours (*$p < 0.05$).

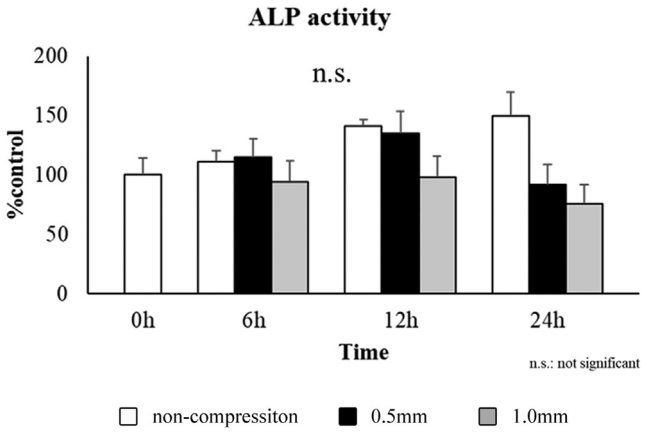

Fig. 3. Alkaline phosphatase (ALP) activity. The results are presented as the relative percentage of the ALP activity compared with that observed prior to compression (considered to be 100%) in each group.

adjacent vessels ruptured. After vessels are ruptured, peripheral blood mixes with the bone marrow in the fracture gap and then coagulates to form a fracture haematoma.[11] Bones possess the unique capability to heal via cellular regeneration and the production of a mineral matrix, and fracture haematomas and local inflammatory processes are thought to initiate healing.[12] As stated previously, isolated cells from MHCs are capable of osteogenic differentiation in 2D cultures, and MHCs have the potential to function as local reservoirs and sources of osteogenic progenitors for intramembranous ossification.[4,13]

Mandibular fractures are one of the most common maxillofacial fractures, and fractures to the body of the mandible account for approximately 21% of cases.[14] Internal fixation using plates has shown the highest success rate with the lowest incidence of complications. The significance of reducing the fracture-line gap in the duration of recovery has recently been established. Reduction using compression plates decreases the distance between the bony fragments without resorption of the fragment ends and intracortical remodelling, and this can promote healing.[15] On the other hand, rigid fixation is not absolutely at the fracture-line gap for bony healing, so stimulation of osteogenesis using a compressive force remains unclear.[16] In the present study, OPN and OSX gene expression in the 0.5 mm compression group was significantly higher than in the no compression control

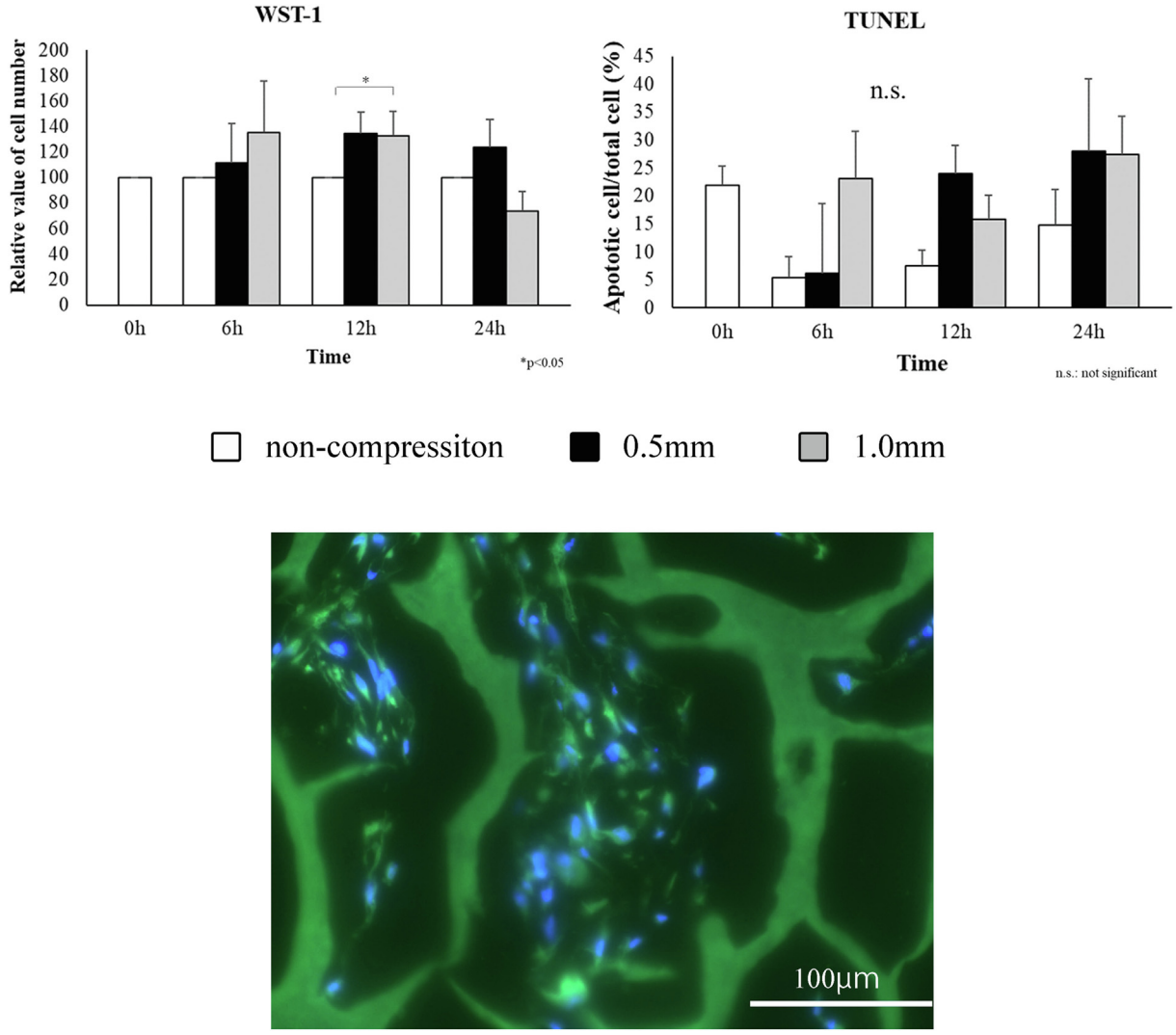

Fig. 4. WST-1 assay and TUNEL staining. The cell number on the 1.0 mm compression group was significantly higher than the non-compression control group after 12 hours' compression (immunostaining for TUNEL-positive cells (green), nucleus stained by DAPI (blue), original magnification ×400).

group. Similarly, OPN and OSX gene expression in the 0.5 mm compression group was significantly higher than in the 1.0 mm compression group after 24 hours of compression. This indicates greater stimulation of osteogenesis in the 0.5 mm compression group than in the other groups. Kerawala et al achieved appropriate compression of 0.67 mm using eccentrically placed screws perpendicular to the bone in monocortical miniplates.[15] Jafarian et al achieved 0.3 mm compression with a divergent placement of screws in monocortical miniplates.[17] Aghdashi et al showed mean fixations of 0.354 mm and 0.582 mm with monocortical miniplates and dynamic compression plates, respectively, after final fixation.[6] Compressing the fracture-line gap using a modified screw insertion through miniplates or dynamic compression plates therefore improves primary bony healing and promotes the healing process.

Mechanical stress can cause a variety of biochemical and morphological reactions in many cell types that have critical regulatory functions in bone cell biology.[18] In addition, some studies have shown that mechanical stresses such as occlusal pressure and orthodontic force affect periodontal tissues during remodelling, repair, and regeneration.[9,19] Sclerostin (SCL), first identified in 2001 by Moester et al, is an inhibitor of bone formation.[20] It is expressed by mature osteocytes, and antagonises the canonical Wnt signalling pathway, thus stimulating osteoblast apoptosis.[21] SCL is therefore recognised as an inhibitor of canonical Wnt signals, a therapeutic target of great potential. A mouse model, in which the Wnt signalling pathway was specifically inhibited in osteocytes, has shown normal bone formation but a significant increase in the number of osteoclasts and bony resorption.[22] Robling et al indicated that mechanical strain has been shown to suppress SCL in long bones.[23] We therefore predicted that MHCs would suppress SCL by compressive loading. The present study, however, found that the 0.5 mm compression group was significantly higher than the no compression control group after 12 hours' compression as analysed by immunohistochemical expression of anti-sclerostin. Interest-

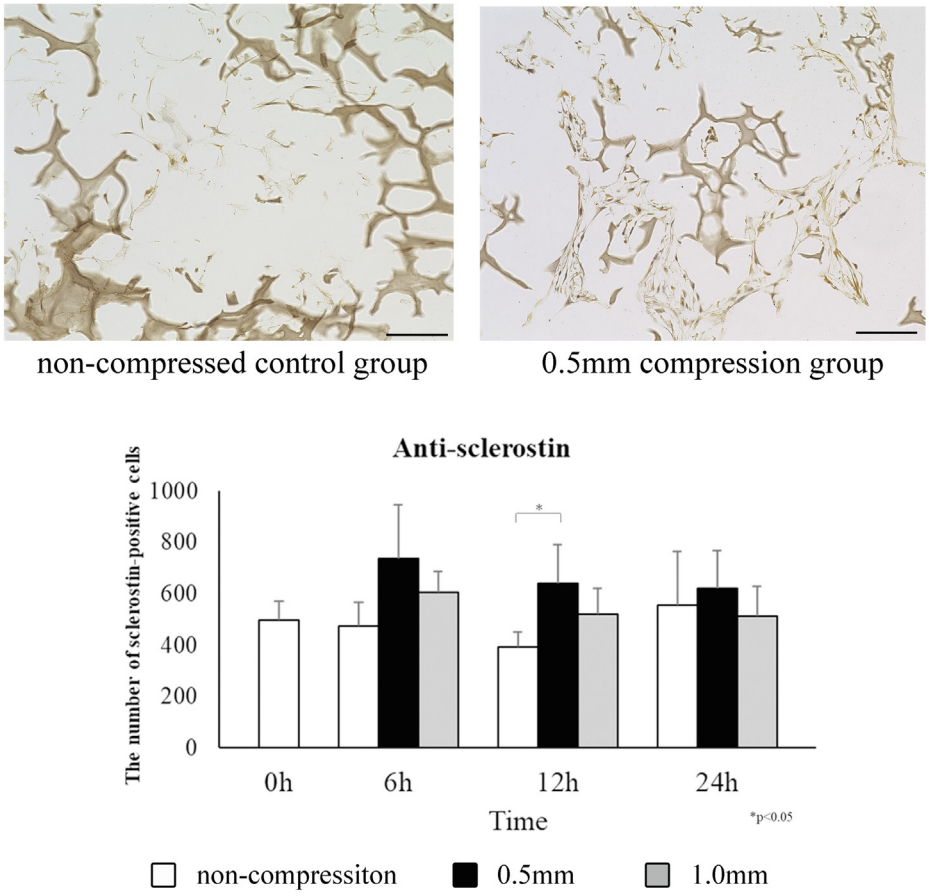

Fig. 5. Immunohistochemical expression of anti-sclerostin after 12 hours' compression (original magnification ×200). Representative sections of the 0 (no compression) group and the 0.5 mm compression group (scale bar = 100 μm). The 0.5 mm compression group was significantly higher than the no compression control group after 12 hours' compression. There were no significant differences at other time points (*$p < 0.05$).

ingly, SCL expression was increased along with osteoblastic differentiation in vitro[24] and was not detected in undifferentiated cells, occurring after the onset of mineralisation.[25] Sutherland et al. suggested that SCL expression was increased in mineralised cultures of human mesenchymal stem cells.[26] The increase in the expression of SCL, under-promoting bone formation conditions that promote bone formation, is seemingly paradoxical; the majority of completed cortical osteons (66%) contained only SCL-positive osteocytes, indicating that SCL secretion occurred after the onset of mineralisation. Similarly, Poole et al reported that SCL secretion by new osteocytes after mineralisation inhibited cortical bone formation and osteon infilling by osteoblast cells.[27] These findings are consistent with the concept of the expression pattern of SCL in mineralised tissues during development. Similar results were obtained in the present study.

Conclusion

In conclusion, greater stimulation of osteogenesis was observed in the 0.5 mm compression group than in the other groups, which can offer clinical benefits. Our study, however, has several limitations. First, our sample size was small, therefore further studies involving a larger sample are warranted to validate our results. Second, the experimental protocol period was short but acceptable when compared to the literature, although a long-term protocol period would be desirable. Third, this study investigated the biological responses of 3D cultured MHCs by sustainable compressive loading but, in clinical practice, bony fragments are subjected to cyclic compressive and tensile forces. Additional studies are needed to confirm this hypothesis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement/confirmation of patients' permission

None.

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