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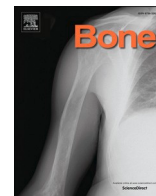
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Full Length Article

Effects of transcutaneous CO₂ application on fracture healing in a rat fracture model of disuse osteoporosis

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ABSTRACT

Transcutaneous carbon dioxide (CO₂) therapy has been shown to accelerate fracture healing and osteogenesis by enhancing endochondral ossification and angiogenesis. We aimed to investigate the effects of this therapy on fracture healing in a rat hindlimb suspension model of disuse osteoporosis. Eleven-week-old male Sprague-Dawley rats were randomly assigned to three groups: 1) Control group (normal ambulation for 3 weeks, followed by femoral fracture with Kirschner-wire fixation); 2) Hindlimb suspension (HS) group (HS; 3 weeks of HS before fracture); 3) HS + CO₂ group (same as HS, followed by transcutaneous CO₂ therapy after fracture). HS was induced by tail suspension at a 30° head-down tilt to prevent hindlimb weight-bearing. Transcutaneous CO₂ therapy involved applying a CO₂-permeable hydrogel and enclosing both hindlimbs in polyethylene bags filled with 100% CO₂ for 20 min/day, 5 days/week. The HS + CO₂ group showed significantly higher modified Radiographic Union Score for Tibial fractures than did the HS group at weeks 3 and 4, as well as a significantly higher radiographic union rate at week 4. They also showed significantly higher Allen's histological grading scores at week 3. Compared with the HS group, the CO₂-treated animals showed significantly higher expression levels of runt-related transcription factor 2 and Osterix at weeks 1 and 2, respectively. These findings suggested that transcutaneous CO₂ therapy promoted osteogenic activation and accelerated radiological and histological fracture healing of disuse osteoporosis, supporting its potential as an adjunctive strategy for fragility fractures.

Abbreviations

CO ₂	carbon dioxide
VEGF	vascular endothelial growth factor
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
HS	hindlimb suspension
HS + CO ₂	hindlimb suspension + carbon dioxide
CT	computed tomography
BV/TV	trabecular bone volume fraction
Tb.N	trabecular number
Tb.Th	trabecular thickness
Tb.Sp	trabecular separation
Av	all bone volume
Cv	cortical bone volume
Ct.Th	cortical thickness
mRUST	modified Radiographic Union Score for Tibial fractures

RT-PCR	real-time reverse transcription polymerase chain reaction
Actb	β-actin
Runx2	runt-related transcription factor 2
ALP	alkaline phosphatase
ICC	intraclass correlation coefficient
ANOVA	analysis of variance
MSCs	mesenchymal stromal cells

1. Introduction

Osteoporosis-related fractures represent a major global health and socioeconomic burden, particularly relevant to population aging. Approximately one in two women and one in four men aged 50 years or older will sustain at least one osteoporotic fracture during their lifetime in the United States [1]. Patients who experience osteoporotic fractures have more than a twofold increase in 1-year mortality compared with individuals without fractures; this excess risk has been reported to

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persist for at least six years [2]. The economic impact is also substantial, with healthcare costs in patients with osteoporotic fractures reported to be approximately threefold higher compared with those in individuals without fractures [3]. Osteoporosis increases fracture risk due to reduced bone mass and deterioration in bone quality and may impair all phases of fracture healing (inflammatory, repair, and remodeling phases) thereby contributing to delayed union and non-union [4,5]. Age-related osteoporosis is a systemic skeletal disorder associated with aging-related alterations in bone turnover, reduced osteogenic capacity, and deterioration in bone quality [6], whereas disuse osteoporosis is primarily induced by mechanical unloading associated with immobilization or loss of weight bearing. In disuse osteoporosis, reduced mechanical loading, impaired angiogenesis, insufficient callus formation [7] and local hypoxia [8] may contribute to delayed fracture healing. Since osteoblast activity, the most important cells for fracture healing, is highly sensitive to oxygen tension, hypoxia might further impair fracture repair by suppressing osteoblast growth, differentiation, and bone-forming capacity [9]. In contrast, age-related impairment of fracture healing has been reported to be associated with cellular senescence, altered inflammatory responses, impaired angiogenesis, and reduced osteoblast function [10]. Accordingly, safe, effective, and low-burden adjunctive therapies are urgently needed for optimizing fracture repair in this high-risk population.

Several pharmacological agents, including active vitamin D analogs, bisphosphonates, and parathyroid hormone analogs, have been investigated as adjuncts in fracture management [11,12]. However, these treatments have substantial limitations in clinical practice, including agent-specific adverse effects, restrictions on indications and treatment duration, cost, adherence challenges, and their limited ability to address disuse-related local circulatory dysfunction [13–16]. In parallel, non-invasive biophysical modalities such as low-intensity pulsed ultrasound and pulsed electromagnetic fields have been explored as potential stimulators of fracture healing [17,18]. More recently, therapeutic strategies aimed to improve local metabolism and oxygenation by enhancing blood flow and promoting angiogenesis have attracted increasing attention [19–23].

Transcutaneous carbon dioxide (CO₂) therapy is a non-invasive therapeutic modality by which CO₂ is absorbed through the skin to induce local vasodilation and enhance microcirculation [24–26]. We have reported the beneficial effects of transcutaneous CO₂ application on skeletal repair, including accelerating fracture healing [27], promoting new bone formation in critical-sized bone defects [28], and suppressing tumor-induced bone destruction [29]. The proposed mechanisms include improved local oxygenation via the Bohr effect; whereby increased CO₂ shifts the oxygen-hemoglobin dissociation curve to the right, thereby enhancing oxygen release to tissues [26]. In addition, transcutaneous CO₂ may enhance angiogenesis and blood flow through up-regulation of vascular endothelial growth factor (VEGF), potentially mediated by peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) [30]. In experimental models of disuse osteoporosis, transcutaneous CO₂ therapy has been shown to enhance local angiogenesis and blood flow, accompanied by increased osteoblastic activity and reduced osteoclastic bone resorption [31]. Despite these beneficial effects, fractures in disuse osteoporosis are prone to impaired healing due to the combined effects of absent mechanical loading and compromised perfusion, which suppress angiogenesis at the fracture site and disrupt bone remodeling homeostasis [7]. Although these suggest that transcutaneous CO₂ therapy could ameliorate the pathological environment associated with disuse-related fractures, its efficacy in this specific context remains uncertain.

On this basis, we hypothesized that transcutaneous CO₂ application would promote healing of fracture with disuse osteoporosis. The aim of this study was to elucidate the effects of transcutaneous CO₂ therapy on fracture healing in a rat model of disuse osteoporosis. Given its non-invasive nature and feasibility for repeated application, this approach might offer practical advantages for managing older adult patients and

those requiring prolonged immobilization after surgery or trauma.

2. Materials and methods

2.1. Animals

All experimental procedures conformed to the ARRIVE guidelines; the study protocol was approved by the Animal Care and Use Committee of the Kobe University Graduate School of Medicine (P230803). Eleven-week-old male Sprague-Dawley rats (SLC Japan, Shizuoka, Japan) were randomly allocated to one of three experimental arms:

- Control group: Rats underwent 3 weeks of unrestricted ambulation, followed by a standardized closed fracture of the right femur and subsequent normal weight-bearing activity.
- Hindlimb suspension (HS) group: Rats were subjected to HS for 3 weeks, after which the right femur was fractured and normal weight-bearing re-ambulation was allowed.
- HS + CO₂ group: After 3 weeks of HS, the right femur was fractured; during the re-ambulation phase, in addition to normal weight bearing, the suspended limbs received transcutaneous CO₂ treatment.

To achieve the target number of animals for analysis after attrition during the HS experiments, a total of 50, 61, and 58 rats were allocated to the Control, HS, and HS + CO₂ groups, respectively. Since HS could not be maintained due to detachment from the HS clip, eleven and eight rats from the HS and HS + CO₂ groups, respectively, were excluded. No animals were excluded due to other adverse effects. After these exclusions, 50 rats remained in each group for analysis. Animals were individually housed in a temperature-controlled room (23.5–25.5 °C) on a 12-h light/dark cycle, with ad libitum access to standard chow and water. At predetermined endpoints, rats were euthanized by an overdose of pentobarbital sodium, from which tissues were harvested for subsequent analyses.

2.2. Hindlimb suspension model

The HS rat model, widely used for investigating disuse osteoporosis, has also been applied to simulate bone loss during spaceflight [32]. In both the HS and HS + CO₂ groups, hindlimb unloading was achieved by tail suspension for 3 weeks. The rats were suspended from the ceiling of the cages by a specialized rat HS clip (Yamashita Technical Research Institute Co., Ltd., Tokushima, Japan) attached to their tails, positioning the animal at approximately 30° head-down tilt. The suspension height was adjusted to prevent the hind limbs from contacting the cage floor but still allowing 360° rotational movement, thereby enabling unrestricted access to food and water [33]. In our previous study using the same hindlimb suspension protocol, micro-computed tomography (CT) demonstrated significant reductions in trabecular bone volume fraction (BV/TV) (%) (Control, 25.11 $\pm$ 1.77; HS, 12.76 $\pm$ 1.67; p = 0.0013), trabecular number (Tb.N) (1/mm) (Control, 3.63 $\pm$ 0.17; HS, 2.14 $\pm$ 0.23; p = 0.0002), and trabecular thickness (Tb.Th) (μ m) (Control, 68.76 $\pm$ 1.86; HS, 58.56 $\pm$ 1.78; p = 0.0270), together with increased trabecular separation (Tb.Sp) (μ m) (Control, 211.08 $\pm$ 16.18; HS, 438.28 $\pm$ 50.75; p = 0.0006), as well as significant cortical bone loss (all bone volume (Av) (mm³) (Control, 14.48 $\pm$ 0.54; HS, 12.60 $\pm$ 0.32; p = 0.0013), cortical bone volume (Cv) (mm³) (Control, 9.44 $\pm$ 0.23; HS, 7.78 $\pm$ 0.15; p < 0.0001), cortical thickness (Ct.Th) (μ m) (Control, 730.78 $\pm$ 14.39; HS, 627.08 $\pm$ 12.57; p = 0.0002)), strongly suggesting that this model could induce disuse osteoporosis [31].

2.3. Femoral fracture in the animal model

A standardized, stabilized, and closed fracture of the right femoral shaft was created, as previously described [34]. In brief, anesthesia was

induced with an intraperitoneal injection of medetomidine (0.15 mg/kg) and midazolam (2 mg/kg). Butorphanol (2.5 mg/kg) was administered for perioperative pain relief. A 1.25 mm diameter Kirschner wire was then introduced retrogradely into the femoral intramedullary canal to provide internal stabilization. A transverse mid-shaft fracture was created using a three-point bending apparatus with a drop weight mechanism, by which a 500 g weight was dropped from a height of 70 cm. This procedure created a simple transverse fracture at the mid-shaft of the femur. The patellar aponeurosis and overlying skin were then closed separately with nylon sutures. Rats were allowed full and unprotected weight bearing post-operatively.

2.4. Transcutaneous application of CO₂

Transcutaneous CO₂ therapy was administered according to a previously described protocol [27]. After light sedation with isoflurane (minimum effective concentration), the hair on the fractured limb was removed; furthermore, a hydrogel (META MEDILAB Inc., Osaka, Japan) was applied to facilitate CO₂ diffusion through the skin. The hydrogel (pH 5.5) comprised carbomer, glycerin, sodium hydroxide, sodium alginate, sodium dihydrogen phosphate, methylparaben, and deionized water. Both hindlimbs were then enclosed in polyethylene bags filled with 100% CO₂ for 20 min. Treatments were administered five times per week. The Control and HS groups underwent similar sham procedure with the bags filled with air instead of CO₂.

2.5. Radiographic assessment of fracture repair

Radiographs of the fractured femora were taken at 1, 2, 3, and 4 weeks post-fracture ($n = 10$ per group). Fracture healing was assessed by the modified Radiographic Union Score for Tibial fractures (mRUST), which assigns 1–4 points to the anterior, posterior, medial, and lateral cortices on orthogonal projections, yielding a total score of 4–16 that reflects callus maturation and fracture-line obliteration [35]. Originally developed for human tibial fractures, the mRUST system has been validated in animal models and in other long bones [36]. Radiographic union was defined a priori as an mRUST ≥ 11 [35]. All images were independently evaluated by three board-certified orthopaedic surgeons in a blinded manner, each with more than 10 years of clinical experience. The median score assigned by the three evaluators was calculated for each cortex; the final mRUST was the sum of these median values.

2.6. Histological assessment of fracture repair

Fractured femora were harvested from rats at 1, 2, 3, and 4 weeks post-fracture ($n = 5$ per group). Specimens were fixed in 4% paraformaldehyde for 24 h at room temperature, decalcified in 10% ethylenediaminetetraacetic acid until complete demineralization, and processed routinely for paraffin embedding. Sagittal sections (6 μ m) were cut, deparaffinized in xylene, rehydrated through graded ethanol, and stained with Safranin O and Fast Green. Histological evaluation was performed using light microscopy to assess callus composition and cartilage formation. Fracture healing was assessed by Allen's five-point grading system (grade 0: pseudoarthrosis; grade 1: partial cartilaginous union; grade 2: complete cartilaginous union; grade 3: partial bony union; and grade 4: complete bony union) [37]. Sections were independently scored, in a blinded fashion, by three experts in experimental fracture healing.

2.7. Assessment of gene expression

Quantitative gene-expression profiling was performed at 3 days and 1, 2, 3, and 4 weeks post-fracture ($n = 5$ per group) using real-time reverse transcription polymerase chain reaction (RT-PCR). At day 3 and week 1, when a distinct fracture callus was not yet macroscopically evident, samples were harvested from the fracture ends and tissues

around fracture-site. At later time points, the callus tissue at the fracture site was harvested after removing the surrounding soft tissues. Peri-fracture tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with a T18 ULTRA-TURRAX homogenizer (IKA-Werke, Staufen, Germany). Total RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform procedure and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with on-column DNase digestion (RNase-Free DNase Set; Qiagen) to remove genomic DNA. First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantification of target transcripts was performed in duplicate using the ABI PRISM 7700 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Expression levels were normalized to β -actin (Actb) as the housekeeping gene and expressed as fold change relative to the 3-day time point, using the $\Delta\Delta$ Ct method [38]. The target genes quantified were Collagen II, Collagen X, runt-related transcription factor 2 (Runx2), Osterix, alkaline phosphatase (ALP), and VEGF. The corresponding primer sequences are presented in Table 1.

2.8. Statistical analysis

Fisher's exact test and Bonferroni post-hoc test were used for comparing the between-group fracture union rate based on the radiographic evaluations at each time point. Inter-observer variability for mRUST and Allen's grading score was assessed by the intraclass correlation coefficient (ICC). A two-way random-effects model with absolute agreement was used; ICC values were calculated because the score of three independent observers was used for analysis. The mRUST derived from radiographic evaluations and Allen's grading score based on histological evaluations were evaluated by aligned rank transform analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for post-hoc analyses. Gene expression data were evaluated by ordinary two-way ANOVA, followed by Sidak's multiple comparisons test for post-hoc analyses. A p value < 0.05 was considered statistically significant. Data are presented in columns with individual data points and error bars indicate mean and standard error.

3. Results

3.1. Radiographic evaluation

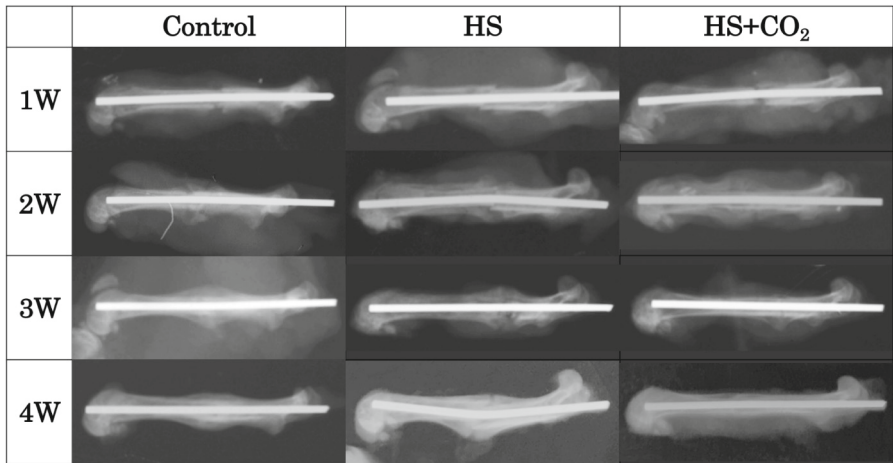
Fig. 1A shows representative radiographs for each group across the four-week observation period. At post-operative week 1, no callus formation was observed in any group. By week 2, callus formation was evident in all groups, with bridging callus appearing in a subset of specimens in the Control and HS + CO₂ groups; however, radiographic union was not observed in any sample. At week 3, complete cortical

Table 1
Details of the primers used for amplification.

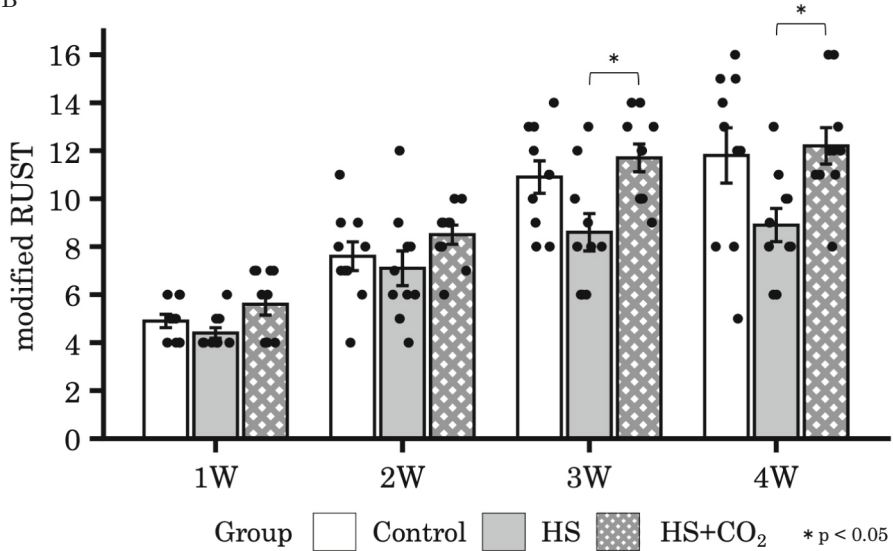
Gene	Primer sequences (5' to 3') (forward/reverse)
Actb	CTAAGGCCAACCGTGAAAAG AACACAGCCTGGATGGCTAC
Collagen II	GGGCTCCAGAACATCACCTACCA TCGGCCCTCATCTCCACATCATG
Collagen X	GGCAGCAGCACTATGACCCAA ACAGGCCTACCCAAACGTGAGTCC
Runx2	GCGTCAACACCATCATTCTG CAGACCAGCAGCACTCCATC
Osterix	AGCTCTTCTGACTGCTGCCTA TGGGTGCGTGTATGTTTGCT
ALP	TCCCAAAGGCTTCTTCTTGC ATGGCCTCATCCATCTCCAC
VEGF	TGCACCTGGACCCCTGGCTTTAC CGGCAGTAGCTTCGCTGGTAG

Actb, β -actin; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; VEGF, vascular endothelial growth factor.

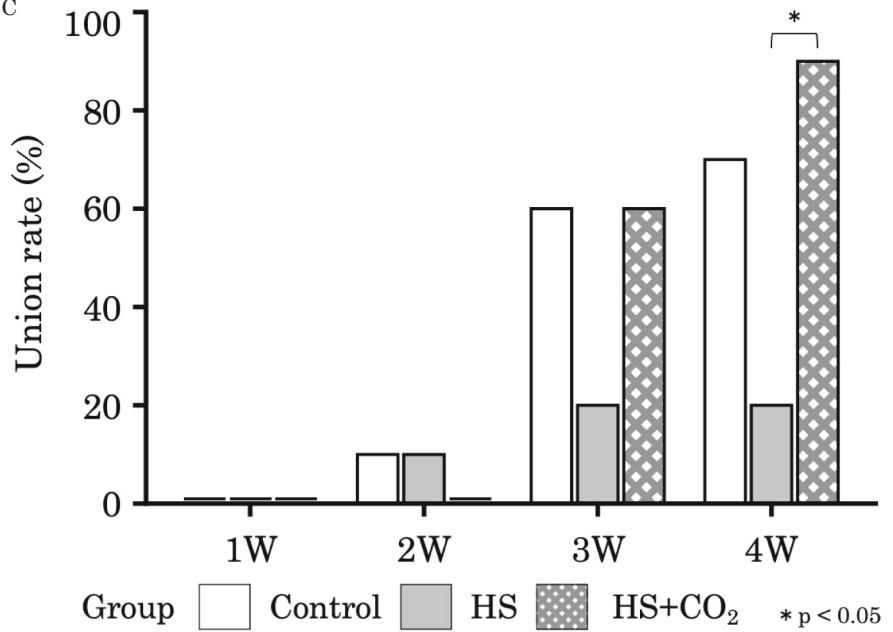
A



B



C



(caption on next page)

Fig. 1. Radiographic assessments. (A) Representative lateral radiographs demonstrating the progression of fracture healing in each group at the indicated time points. (B) The Modified Radiographic Union Score for Tibial fractures (mRUST) for each group at the indicated time points ($n = 10$ per group). The mRUST was evaluated by aligned rank transform analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for post-hoc analyses. Aligned rank transform ANOVA of the mRUST demonstrated significant main effects of the treatment group ($p < 0.001$) and time point ($p < 0.001$), whereas the treatment group $\times$ time point interaction was not significant. The mRUST was significantly higher in the HS + CO₂ group than in the HS group at weeks 3 and 4. Error bars indicate standard error. * indicates $p < 0.05$. (C) Radiographic fracture union rates for each group at the indicated time points ($n = 10$ per group). Comparisons were performed using Fisher's exact test and Bonferroni post-hoc test. The radiographic union rate at week 4 was significantly greater in the HS + CO₂ group than in the HS group. * indicates $p < 0.05$.

HS, hindlimb suspension group; HS + CO₂, hindlimb suspension + carbon dioxide group.

bridging was achieved in several specimens in the Control and HS + CO₂ groups, whereas most HS specimens exhibited insufficient callus formation to achieve bridging. By week 4, most femora in the Control and HS + CO₂ groups met the criteria for radiographic union, while specimens in the HS group continued to demonstrate inadequate bridging.

Aligned rank transform ANOVA of the mRUST demonstrated significant main effects of the treatment group ($p < 0.001$) and time point ($p < 0.001$), whereas the treatment group $\times$ time point interaction was not significant. Inter-observer variability for the mRUST was good, with an ICC of 0.85. Using the post-hoc analysis, the mRUST was significantly higher in the HS + CO₂ group than in the HS group at week 3 (HS, 8.60 ± 0.78 ; HS + CO₂, 11.70 ± 0.58 ; $p = 0.025$) and week 4 (HS, 8.90 ± 0.69 ; HS + CO₂, 12.20 ± 0.76 ; $p = 0.022$), as shown in Fig. 1B. No inter-group differences were found at weeks 1 or 2. Consistent with these findings, the radiographic union rate at week 4 was significantly greater in the HS + CO₂ group compared with that in the HS group (week 4: HS, 20%; HS + CO₂, 90%; $p = 0.016$), as shown in Fig. 1C. No differences were found among the groups at earlier time points.

3.2. Histological evaluation of fracture repair

Fig. 2A shows representative Safranin O and Fast Green-stained sections for each group over the four-week healing period. Aligned rank transform ANOVA of Allen's grading score demonstrated significant main effects of the treatment group ($p < 0.001$) and time point ($p < 0.001$), as well as a significant treatment group $\times$ time point interaction ($p < 0.01$). Inter-observer variability for Allen's grading score was excellent, with an ICC of 0.93. At week 1, cartilage formation was minimal in all groups. By week 2, cartilaginous callus was observed in all groups. The cartilaginous layer tended to be thicker and more extensive in the Control and HS + CO₂ groups than in the HS group; however, this trend was not accompanied by significant differences in Allen's grading score. At week 3, the HS + CO₂ group exhibited woven bone with minimal residual cartilage, whereas both the Control and HS groups retained more cartilaginous tissue. Consistent with this earlier transition to bony callus, the Allen's grading score was significantly higher in the HS + CO₂ group than in the HS group (week 3: HS, 1.60 ± 0.20 ; HS + CO₂, 3.20 ± 0.37 ; $p = 0.044$), as shown in Fig. 2B. By week 4, bony callus formation was observed in all groups; however, varying degrees of residual cartilage remained. No between-group differences in Allen's grading score were found at this time point.

3.3. Evaluation of gene expression

The gene expression levels in the tissues surrounding the fracture site, evaluated by real-time RT-PCR, are shown in Fig. 3.

Regarding genes associated with chondrocyte differentiation, ordinary two-way ANOVA showed no significant main effects of the treatment group or time point and no significant treatment group $\times$ time point interaction for Collagen II expression. However, Sidak's multiple comparisons test showed that Collagen II expression was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 2 (HS + CO₂, 27.50 ± 60.07 ; Control, 3.73 ± 1.21 ; $p = 0.019$; HS, 19.29 ± 10.56 ; $p = 0.031$) without significant differences among the groups at other time points. Regarding Collagen X expression, ordinary two-way ANOVA showed no significant main effect of the treatment

group; however, a significant main effect of time point was found ($p < 0.001$) and a significant treatment group $\times$ time point interaction ($p = 0.044$). Sidak's multiple comparisons test showed that Collagen X expression was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 2 (HS + CO₂, 136.87 ± 71.66 ; Control, 12.69 ± 3.92 ; $p < 0.001$; HS, 57.48 ± 37.99 ; $p = 0.038$) without significant differences among the groups at other time points.

Regarding genes associated with osteoblast differentiation, ordinary two-way ANOVA showed no significant main effect of the treatment group for Runx2 expression, whereas a significant main effect of time point ($p = 0.026$) and a significant treatment group $\times$ time point interaction ($p < 0.001$) were found. Sidak's multiple comparisons test showed that Runx2 expression was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 1 (HS + CO₂, 40.78 ± 17.46 ; Control, 0.69 ± 0.12 ; $p < 0.001$; HS, 0.91 ± 0.20 ; $p < 0.001$) and higher in the Control group than that in the HS and HS + CO₂ groups at week 4 (Control, 2.71 ± 5.62 ; HS, 1.12 ± 0.13 ; $p = 0.027$; HS + CO₂, 1.30 ± 0.11 ; $p = 0.028$), whereas no significant differences were found among the groups at other time points. Considering Osterix expression, ordinary two-way ANOVA showed no significant main effects of the treatment group or time point; however, a significant treatment group $\times$ time point interaction ($p = 0.043$) was found. Sidak's multiple comparisons test showed that Osterix expression was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 2 (HS + CO₂, 13.28 ± 7.67 ; Control, 0.14 ± 0.046 ; $p = 0.005$; HS, 0.085 ± 0.070 ; $p = 0.005$), whereas no significant differences were found among the groups at other time points. Ordinary two-way ANOVA showed no significant main effects of the treatment group or time point and no significant treatment group $\times$ time point interaction for ALP expression. However, Sidak's multiple comparisons test showed that ALP level was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 2 (HS + CO₂, 2.75 ± 2.33 ; Control, 0.51 ± 0.056 ; $p = 0.001$; HS, 0.53 ± 0.11 ; $p = 0.001$), whereas no significant differences were found at other time points.

Regarding angiogenesis-related gene expression, ordinary two-way ANOVA showed no significant main effect of the treatment group and no significant treatment group $\times$ time point interaction for VEGF expression, whereas a significant main effect of time point was found ($p = 0.014$). Sidak's multiple comparisons test showed that VEGF expression was significantly higher in the HS + CO₂ group than that in the Control and HS groups at week 2 (HS + CO₂, 2.21 ± 1.26 ; Control, 0.41 ± 0.075 ; $p < 0.001$; HS, 0.83 ± 0.15 ; $p = 0.003$), whereas no significant differences were found among the groups at other time points.

4. Discussion

In this study, we investigated the effects of transcutaneous CO₂ therapy on fracture healing in a rat femoral fracture model of disuse osteoporosis, using radiological, histological, and genetic assessments. To our knowledge, this is the first study to assess transcutaneous CO₂ therapy for fragility fractures associated with disuse osteoporosis. Previous studies have shown beneficial effects of transcutaneous CO₂ application on fracture repair in standard and diabetic rat fracture models [27,47], as well as preventive effects on disuse osteoporosis in a hindlimb suspension model [31]. However its effects on fracture healing under disuse osteoporotic conditions have not been previously assessed.

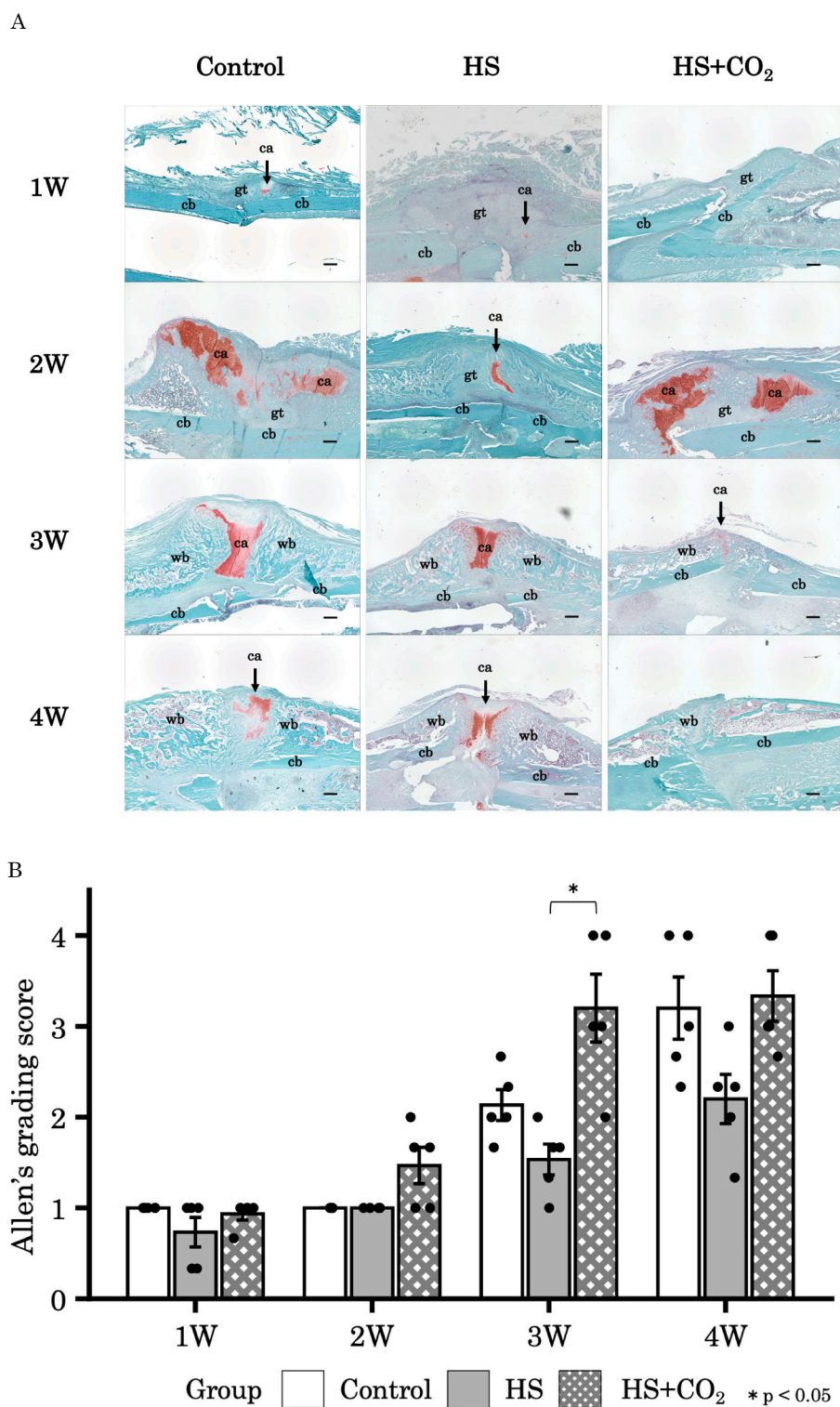


Fig. 2. Histological assessments of fracture repair. (A) Representative Safranin O/ Fast Green-stained sections for each group over the 4-week healing period. ca, cartilage; cb, cortical bone; gt, granulation tissue; wb, woven bone; scale bar = 500 μ m. (B) Allen's grading score for each group at the indicated time points ($n = 5$ per group). The Allen's grading score was evaluated by aligned rank transform analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for post-hoc analyses. Aligned rank transform ANOVA of Allen's grading score demonstrated significant main effects of the treatment group ($p < 0.001$) and time point ($p < 0.001$), as well as a significant treatment group $\times$ time point interaction ($p < 0.01$). The Allen's grading score at week 3 was significantly higher in the HS + CO₂ group than that in the HS group. Error bars indicate standard error. * indicates $p < 0.05$. HS, hindlimb suspension group; HS + CO₂, hindlimb suspension + carbon dioxide group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

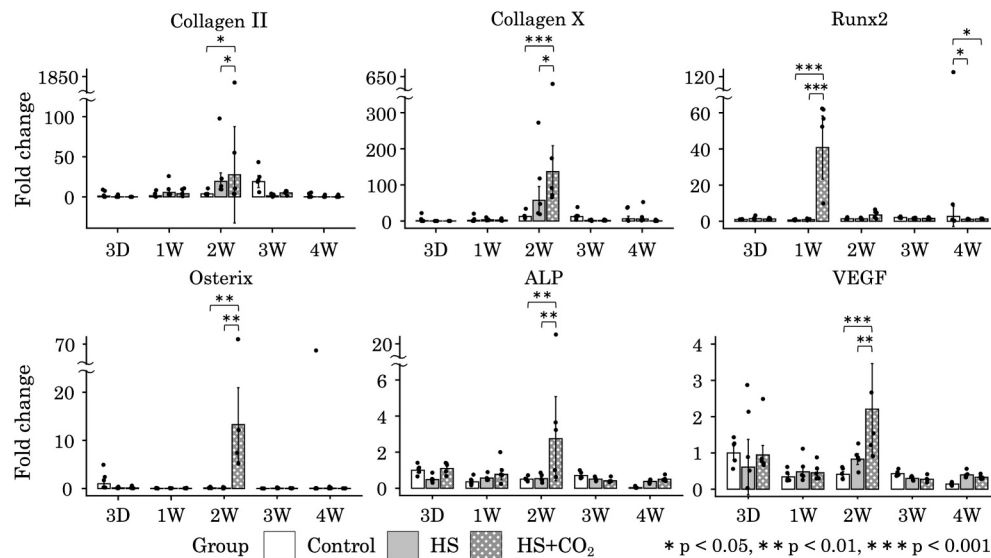


Fig. 3. Gene expression evaluated by real-time RT-PCR ($n = 5$ per group). Expression levels were normalized to β -actin (Actb) and expressed as fold change relative to the Control group sample at 3-day time point. Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; VEGF, vascular endothelial growth factor. Gene expression data were evaluated by ordinary two-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for post-hoc analyses. Regarding Runx2 expression, ordinary two-way ANOVA showed no significant main effect of the treatment group, whereas a significant main effect of time point ($p = 0.026$) and treatment group $\times$ time point interaction ($p < 0.001$) were found. The expression of Runx2 was significantly higher in the HS + CO₂ group than that in both the Control and HS groups at week 1. Considering Osterix expression, ordinary two-way ANOVA showed no significant main effects of the treatment group or time point; however, a significant treatment group $\times$ time point interaction ($p = 0.043$). The expression of Osterix was significantly higher in the HS + CO₂ group than that in both the Control and HS groups at week 2. Error bars indicate standard error. * indicates $p < 0.05$. ** indicates $p < 0.01$. *** indicates $p < 0.001$. HS, hindlimb suspension group; HS + CO₂, hindlimb suspension + carbon dioxide group; RT-PCR, real-time reverse transcription polymerase chain reaction.

The HS + CO₂ group showed significantly higher early expression of Runx2, Osterix and Collagen X than did the HS group, accompanied by more advanced radiological and histological union at later stages. As these findings indicate, transcutaneous CO₂ therapy might offer a potential strategy for enhancing repair of fragility fractures associated with disuse osteoporosis.

The HS model is well-established for studying disuse osteoporosis and characterized by reduced mechanical loading that suppresses bone formation and enhances bone resorption [39,40]. Mechanical unloading decreases osteoblast differentiation and callus formation by reducing the expression of osteoblast-related transcription factors such as Runx2 and Osterix, while augmenting osteoclast activity [7]. In addition, diminished bone blood flow, vascular remodeling with reperfusion injury, and osteocytic hypoxia impair local oxygenation and have been reported to delay fracture healing under such conditions [8,41,42]. Thus, the HS fracture model reproduces a state in which reduced mechanical stimulation is combined with decreased local blood flow and oxygen delivery, creating an environment prone to delayed endochondral ossification and impaired fracture repair.

In this study, the HS group showed a delayed progression trend toward union compared with the Control group, with insufficient bridging callus in most specimens at week 3 and persistently inadequate bridging in many cases at week 4.

Transcutaneous CO₂ therapy has been reported to improve local blood flow and oxygenation by promoting oxygen release from hemoglobin via the Bohr effect and by inducing vasodilatation and enhancing microcirculation [26,30,43–46]. In rat femoral fracture models and diabetic fracture models, CO₂ therapy has been shown to accelerate fracture healing through increased blood flow, stimulation of angiogenesis, and augmentation of endochondral ossification [27,47]. In the HS model, this therapy has also been reported to prevent disuse osteoporosis and muscle atrophy by promoting local angiogenesis and increasing blood flow [31]. Collectively, these findings suggested that CO₂ therapy might correct disturbances in microcirculation and tissue oxygenation induced by mechanical unloading and exert beneficial

effects on disuse-related abnormalities in bone metabolism.

Fracture healing proceeds through sequential inflammatory, soft callus, and hard callus formation phases, during which mesenchymal stromal cells (MSCs) recruited by cytokines and growth factors differentiate along both chondrogenic and osteoblastic lineages. Regarding secondary fracture healing, cartilage formation is an essential intermediate step; the cartilaginous callus is subsequently replaced by bone through endochondral ossification [48]. In addition, the local mechanical environment at the fracture site, particularly the magnitude and mode of interfragmentary strain, influences whether mesenchymal tissues preferentially form bone, cartilage, or fibrous tissue [49,50]. Runx2 and Osterix are transcription factors that hierarchically regulate this differentiation process. Runx2 functions as a master regulator of osteogenesis by committing MSCs to osteoblastic lineage; however, its expression is tightly controlled in a stage-dependent manner, and downregulation is required during the terminal stages of osteoblast maturation [51–56]. Runx2 also contributes to chondrocyte maturation, including the transition toward hypertrophic chondrocytes [57,58]. In regard to hypertrophic chondrocytes, Runx2 has also been implicated in the regulation of RANKL and OPG expression, thereby influencing osteoclastogenesis, cartilage resorption, and progression of endochondral ossification and remodeling [59]. Osterix acts downstream of Runx2 and promotes the progression of pre-osteoblasts to mature osteoblasts responsible for bone matrix production and mineralization. In mouse fracture models, Osterix-positive cells appear within osteogenic regions of the callus from 7 to 14 days after fracture [60,61], whereas Osterix-deficient embryos fail to form bone and remain cartilaginous [60–62]. Thus, Osterix is regarded as an essential factor for the final stages of osteoblast differentiation and bone formation.

With respect to the chondrogenic phase of fracture healing, Collagen II is a major component of the cartilage extracellular matrix, while Collagen X is characteristically expressed by hypertrophic/terminally differentiated chondrocytes and associated with matrix remodeling and calcification during endochondral ossification [63,64]. In addition, SOX9 is a master regulator of chondrogenic differentiation; the Sox

family transcription factors are involved in the chondrogenic phase of fracture repair [65,66]. Although not studied in the current study, assessment of cartilage-related transcription factors such as SOX9 might have provided further mechanistic insight into whether transcutaneous CO₂ application influenced chondrogenic differentiation itself, maintenance of the cartilaginous callus, or the subsequent progression toward hypertrophic cartilage and endochondral ossification [65,66]. Cartilage formation is closely associated with low-oxygen conditions. In addition, hypoxia-inducible factor (HIF) signaling, particularly HIF-1 α , plays an important role in chondrocyte survival, cartilage metabolism, and endochondral skeletal development [67–69]. HIF signaling has also been implicated in bone formation and fracture repair through its interactions with chondrogenesis, angiogenesis, and subsequent ossification [67,68,70]. In the present study, the greater cartilage formation observed histologically in the HS + CO₂ group at week 2, followed by less residual cartilage and more woven bone at week 3, is consistent with more efficient progression from cartilaginous callus formation to endochondral ossification. Together with the higher Collagen X expression at week 2 in the HS + CO₂ group, these findings suggest that transcutaneous CO₂ therapy might have promoted maturation toward hypertrophic cartilage and subsequent transition to bony callus under unloading-impaired conditions.

In the present study, increased expressions of Runx2 at week 1 and Osterix at week 2 in the HS + CO₂ group suggested that transcutaneous CO₂ therapy improved the microcirculation and oxygen supply at the fracture site [26,30], thereby promoting angiogenesis and endochondral ossification. In bone, the vascular network and bone formation are closely coupled; furthermore, angiogenesis progresses in parallel with osteogenesis activation [71]. Consistent with this coupling, our temporal analysis showed that Runx2 expression was first upregulated at week 1 in the HS + CO₂ group, followed by increased expression of its downstream effector Osterix at week 2. This expression pattern corresponds to stepwise activation of the osteoblastic lineage and endochondral ossification under improved hemodynamic and oxygenation conditions. Through these mechanisms, transcutaneous CO₂ therapy possibly facilitated the progression of endochondral ossification and transition to hard callus, ultimately contributing to the observed improvements in radiographic and histological fracture union.

Although previous studies have suggested that transcutaneous CO₂ application could enhance angiogenesis and blood flow [30], the VEGF results should be cautiously interpreted in this study. VEGF expression showed a significant main effect of time point, indicating that angiogenesis-related signaling changed during the healing process. However, neither the treatment group effect nor the treatment group $\times$ time point interaction was significant. Although post hoc analysis showed higher VEGF expression in the HS + CO₂ group at week 2, this finding should be regarded as exploratory rather than definitive evidence that CO₂ therapy directly increased VEGF expression in this model. The radiological and histological improvements observed in the HS + CO₂ group might therefore reflect improved local microcirculation and oxygenation through mechanisms not fully reflected by VEGF expression at the selected sampling points.

The findings of this study, together with those of our previous study [31], suggested that transcutaneous CO₂ therapy might ameliorate disuse osteoporosis and its sequelae, including muscle atrophy, loss of bone mineral density, and delayed fracture healing. In the context of an aging society, this approach might provide a novel adjunctive therapy that could complement surgical treatment and rehabilitation for osteoporosis-related problems, including fragility fractures and disuse. From a clinical perspective, transcutaneous CO₂ therapy might also have potential as an adjunctive treatment of fractures in patients with other conditions associated with impaired bone healing, including hormone-related osteoporosis. The primary pathophysiological features of postmenopausal osteoporosis include abnormal bone remodeling with increased bone resorption relative to bone formation, reduced bone mass, and impaired bone quality secondary to estrogen deficiency

[72,73]. Whether transcutaneous CO₂ therapy could directly reverse these systemic abnormalities remains unclear. However, it may improve the local reparative environment at the fracture site by enhancing microcirculation, activating VEGF and PGC-1 α signaling, promoting osteogenic pathways, and attenuating local inflammation and bone resorption, thereby facilitating fracture healing [27,30,31]. Furthermore, in more problematic fractures, such as distal tibial shaft fractures, which are prone to delayed union or non-union [74], both mechanical and biological factors are often compromised, including fracture instability [75], soft-tissue injury, and impaired local blood flow [76]. Although transcutaneous CO₂ therapy might improve the local biological environment through enhanced microcirculation, angiogenesis, and osteogenic activity [30,31], it is unlikely to correct mechanical factors such as fracture instability. Therefore, the extent to which this therapy could influence the healing process in such fractures remains uncertain. Further studies are needed to determine its therapeutic value in these clinical settings.

This study has some limitations. First, the HS model used in this study reproduced disuse osteoporosis driven by mechanical unloading in young male rats, which could differ in its pathophysiological basis from postmenopausal osteoporosis, where estrogen deficiency is the primary cause. Although the HS model reflects bone loss associated with loss of mechanical loading, such as during space flight or prolonged bed rest, the standard preclinical model of postmenopausal osteoporosis is the ovariectomized rat, which exhibits high-turnover bone loss secondary to estrogen deficiency. In addition, only male rats were used in this study. Considering that sex-related differences in bone biology and fracture healing have been reported [77,78], caution is warranted in generalizing the present findings as they cannot be directly extrapolated to postmenopausal patients.

Second, the duration of HS presents limitations. In this study, HS was applied for 3 weeks before fracture creation, after which re-ambulation was allowed. Prolonged HS beyond 3 weeks causes epidermal breakdown of the tail, and continued suspension after fracture creation would possibly exacerbate tail injury. Although maintaining HS after fracture would more closely model a sustained disuse state, this scenario was not assessed in the present study.

Third, the degree of osteoporosis was not directly quantified in the present study, because micro-CT analysis was not performed. However, it is supposed that the severity of bone loss could be inferred from our previous study using the same hindlimb suspension protocol [31].

In conclusion, transcutaneous CO₂ therapy was associated with stepwise activation of the osteoblastic lineage, reflected by early increases in Runx2, Osterix, and Collagen X expression, and appeared to promote progression of endochondral ossification and bone formation, leading to improved radiological and histological fracture union. Considering previous evidence that this therapy improves microcirculation and oxygenation, these effects possibly reflect a more favorable biological environment at the fracture site. Taken together, our findings suggested that transcutaneous CO₂ therapy might represent a promising therapeutic strategy for enhancing fracture healing in disuse osteoporosis. Clinically, transcutaneous CO₂ therapy might be particularly relevant as an adjunctive treatment of fragility fractures in older adults with reduced activities of daily living, prolonged immobilization, or low activity levels.

CRedit authorship contribution statement

Hyuma Kondo: Writing – original draft, Visualization, Investigation, Formal analysis. **Tomoaki Fukui:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Kenichi Sawauchi:** Writing – review & editing. **Yohei Kumabe:** Validation. **Ryota Nishida:** Validation. **Jonathan:** Investigation. **Genta Fukumoto:** Validation. **Ryosuke Kuroda:** Supervision. **Keisuke Oe:** Project administration.

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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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Data availability

Data will be made available on request.

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