Effects of transcutaneous CO_2 application in a rat fracture model of disuse osteoporosis

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INTRODUCTION: Transcutaneous CO_2 therapy has been shown to accelerate fracture healing and osteogenesis by enhancing endochondral ossification and angiogenesis [1]. To date, however, its efficacy for fractures with disuse osteoporosis has not been investigated. This study aimed to elucidate the therapeutic effect of transcutaneous CO_2 application on fracture healing in a fracture model of disuse osteoporosis.
METHODS: This study was approved by our institution's animal ethics committee.
Animal experiments: Eleven-week-old male Sprague Dawley rats (SLC Japan, Shizuoka, Japan) were randomly assigned to three groups. In the Control group, following 3 weeks of normal ambulation, a Kirschner-wire was inserted into the right femur of the rats, followed by a closed fracture created using a three-point bending apparatus with a drop weight [2] and then normal physical weight-bearing ambulation was allowed. In the hindlimb suspension (HS) group, the rats underwent tail suspension for 3 weeks at a 30-degree head-down tilt, such that the hind limbs did not touch the ground [3]; the fracture was then created and the rats allowed normal physical weight-bearing reambulation. In the HS + CO_2 group, the rats were subjected to tail suspension using the same protocol as in the HS group, followed by fracture of the right femur and the rats were then allowed normal physical weight-bearing reambulation with CO_2 application.
Transcutaneous application of CO_2: After sedation with a minimum dose of isoflurane, the fractured limb was shaved, and a hydrogel (META MEDILAB Inc., Osaka, Japan) was applied to enhance transcutaneous absorption of CO_2. Both limbs were sealed in polyethylene bags filled with 100% CO_2 for 20 minutes a day. This treatment was performed 5 days per week. The Control and HS groups received sham treatment in which CO_2 was replaced with air.
Radiographic assessment of fracture repair: Radiographs of the fractured limbs were obtained at 1, 2, 3, and 4 weeks after the fracture (n = 10 per group). The degree of fracture repair was assessed using the modified radiographic union score for tibial fractures (mRUST) [4]. The mRUST score of 11 or more was considered as bone union. All images were independently evaluated in a blinded manner by three board-certified orthopedic surgeons, each with more than 10 years of clinical experience.
Histological assessment of fracture repair: Fractured femora were harvested at 1, 2, 3, and 4 weeks after the fracture (n = 5 per group). Specimens were stained with Safranin O and Fast Green. Histological evaluation was performed using light microscopy to assess using Allen's grading score [5]. Sections were scored independently, in a blinded fashion, by three observers with expertise in experimental fracture healing.
Assessment of gene expression: Gene expression of Collagen II, Collagen X, Runx2, Osterix, ALP and VEGF were measured at 3 days and at 1, 2, 3, and 4 weeks after the fracture (n = 5 per group) by real-time reverse transcription polymerase chain reaction (RT-PCR). Expression levels were normalized to β-actin and expressed as fold change relative to the 3-day time point using the ΔΔCt method.
Statistical Analysis: The Kruskal-Wallis test and Steel-Dwass post-hoc test were used to compare mRUST scores from radiographic evaluations, Allen's grading score from histological evaluations and gene expression results among groups at each time point. Statistical significance was accepted at $p < 0.05$.
RESULTS: ***Radiographic evaluation of fracture repair:*** The mRUST score was significantly higher in the HS + CO_2 group than in the HS group at weeks 3 and 4 (Fig. 1). The bone union rate was significantly higher in the HS + CO_2 group (90 %) than in the HS group (20 %) at week 4.
Histological evaluation of fracture repair: Quantitative assessment with Allen's grading score demonstrated significantly higher scores in the HS + CO_2 group than in the HS group at week 3 (Fig. 2).
Evaluation of gene expression: Gene-expression profiling demonstrated that Runx2 expression were significantly higher in the HS+CO_2 group than in the HS group at week 1. At week 2, Osterix expression was likewise significantly elevated in the HS+CO_2 group relative to the HS group (Fig. 3).
DISCUSSION: Runx2 and Osterix are hierarchical master regulators of osteoblast differentiation and endochondral ossification [6]: Runx2 commits mesenchymal stem cells to the osteoblastic lineage and facilitates the transition from soft to hard callus, whereas Osterix drives the maturation of pre-osteoblasts and the deposition/mineralization of bone matrix. In the present study, transcutaneous CO_2 treatment up-regulated Runx2 expression at postoperative week 1 and Osterix at week 2 in the HS + CO_2 group. These findings indicate that CO_2 therapy could activate the osteogenic program early after fracture, first promoting MSC commitment via Runx2 and subsequently accelerating maturation and new bone formation through timely induction of Osterix. As a consequence, these changes could be considered to have contributed to the enhanced fracture healing observed in the radiographic and histological evaluations.
SIGNIFICANCE: Transcutaneous CO_2 therapy may have the potential to enhance osteoblast differentiation and osteogenesis through early up-regulation of Runx2 and Osterix, thereby representing a promising strategy to accelerate fracture healing in fractures of disuse osteoporosis.
REFERENCES: [1] Koga T, et al. J Bone Joint Surg Am. 2014. [2] Bonnarens F, et al. J Orthop Res. 1984. [3] Morey-Holton ER, et al. Bone. 1998. [4] Litrenta J, et al. J Orthop Trauma. 2015. [5] Allen HL, et al. Acta Orthop Scand. 1980. [6] Oda T, et al. BMJ Open Diabetes Res Care. 2020.

Fig. 1. mRUST score at each time point

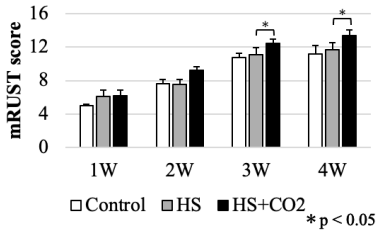

Fig. 2. Allen's grading score at each time point

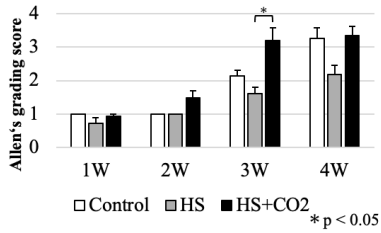

Fig. 3. Evaluation of gene expression at each time point