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Research Paper
Distraction Osteogenesis

The expression of endogenous hydrogen sulfide signal during distraction osteogenesis in a rabbit model

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Abstract. The hydrogen sulfide (H_2S) signal system plays an important role in bone metabolism. However, the role of endogenous H_2S during distraction osteogenesis (DO) remains unclear. Sixty-two male New Zealand White rabbits were subjected to right mandibular DO. Before distraction, the animals were divided randomly into two groups: group A, 0.5 mm twice/day for 10 days; group B, 1.25 mm twice/day for 4 days. Plasma and distraction gap tissue were harvested to determine the H_2S signal. The osteogenesis effect was also evaluated. The newly regenerated bone in group A presented a higher level of mineralization and biomechanical strength than that in group B. The bone mineralization density in group A was 1.95-fold that in group B ($P = 0.028$), while the biomechanical strength in group A was 1.26-fold that in group B ($P = 0.042$) at the end of the experiment. The H_2S signal was detected during the whole process of DO. The relative plasma H_2S concentrations in group A were noticeably higher than those in group B at the middle of distraction ($P < 0.001$), at the end of the distraction ($P = 0.034$), and 2 weeks after the end of distraction ($P = 0.002$). The results suggest that the endogenous H_2S signal system plays a major role during DO.

Key words: hydrogen sulfide; distraction osteogenesis; rabbit; mandible; CSE; CBS; 3-MST.

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First reported by Codivilla in 1905 and then standardized by Ilizarov for the extension of malformed limbs, distraction osteogenesis (DO) is a promising surgical tool with wide ranging clinical applications in limb lengthening, the correction of maxillofacial deformity, and the reconstruction of large bone defects[1–3]. The mechanisms influencing bone generation during DO consist of a complex pattern of cellular and molecular processes, which are different from those occurring during bone fracture healing and still remain to be elucidated[4].

Due to their particular physical and chemical properties, small gaseous transmitters have not usually been adopted as the research subject in studies on DO. Hydrogen sulfide (H_2S) gas has traditionally been considered a metabolic poison in contaminated environments[5]. It has been investigated extensively and is regarded as the third endogenous gaseous transmitter after nitric oxide and carbon monoxide.

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H_2S is synthesized endogenously by cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST)[5,6]. Regarding their localizations, CBS is expressed predominantly in the nervous system, whereas CSE is expressed predominantly in vascular tissue. 3-MST is expressed in all tissues[7]. Plasma membranes are permeable to H_2S, as the solubility of H2S in lipophilic solvents is five-fold greater than in water. Therefore, the gas can easily diffuse through cells and reach the intracellular compartments. As a signaling molecule, endogenous H_2S is extensively required in many physiological and pathological processes[7,8].

A previous study has reported that a physiological level of H_2S is necessary to maintain bone marrow mesenchymal stem cell (BMMSC) self-renewal and osteoblast differentiation[9]. In a previous preliminary animal study, the general administration of GYY4137, a new H_2S donor, was proven to increase bone mineral density (BMD) and improve mechanical properties of bone in a rabbit unilateral mandibular distraction model[10]. However, there is little reported research on endogenous H_2S signal expression during DO. In this animal study, the expression of endogenous H_2S signal during DO was investigated in a rabbit unilateral mandibular distraction model.

Materials and methods

Animals

A total of 62 male New Zealand White rabbits ranging from 3000 g to 3500 g in weight were included in this study. All animal experiments were performed in accordance with the Animal Care and Use Guidelines of the South China University. Anesthesia for all experimental procedures was achieved by intravenous administration of ketamine hydrochloride (20 mg/kg; Sigma, St. Louis, MO, USA) and xylazine (5 mg/kg; Sigma). For pain relief, buprenorphine (0.03 mg/kg; Sigma) was administered subcutaneously twice daily during the distraction period. The animals were kept in separate cages with food and water under standardized environmental conditions with a 12-h light/dark cycle.

Distraction osteogenesis protocol

A 3-cm longitudinal incision was made along the inferior border of the right mandible, following which the platysma and the periosteum were reflected from the mandible laterally. An osteotomy line was made vertically between the first premolar and the mental foramen of the right mandible. Next, a custom-made external titanium distractor was adapted with five titanium miniscrews (2 mm diameter × 8 mm length), which were placed across both cortices. The mandible was osteotomized with a fissure bur under copious sterile saline irrigation. Finally, the wound was closed in layers with 3–0 Vicryl sutures. The animals were then assigned randomly to one of two groups. The distraction protocol was started 5 days after the osteotomy at a rate of 0.5 mm twice a day (8 am and 8 pm) for 10 days (group A, $n = 31$), or 1.25 mm twice a day (8 am and 8 pm) for 4 days (group B, $n = 31$). The distractor was left in place for a consolidation period of at most 8 weeks.

Measurement of serum H_2S

Venous plasma samples were harvested from the auricular veins of all animals at eight time points: before the operation, 5 days after the operation, at the middle time point of distraction, at the end of distraction, and at 2 weeks, 4 weeks, 6 weeks, and 8 weeks after the end of distraction; eight samples from each group were selected at random for the determination of the relative concentration of plasma H_2S. The assay mixture (500 μl) contained tissue homogenate (460 μl), l-cysteine (10 mol/l), and pyridoxal 5′-phosphate (2 mmol/l). After incubation at 37 °C for 30 min, zinc acetate (1% w/v, 250 μl) was added to trap the H_2S generated, followed by trichloroacetic acid (10% w/v, 25 μl) to terminate the reaction. *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mmol/l, 133 μl) and $FeCl_3$ (30 mmol/l, 133 μl) were added and the mixture was centrifuged at 12,000 *g*. Absorbance was determined 10 min later[11]. The absorbance of NaHS solution (2.5–200 mol/l) was determined spectrophotometrically at 670 nm to establish the standard curve.

Detecting the H_2S enzyme signal in the distraction gap tissue

At the middle of the distraction period, the end of distraction, and 4 weeks and 8 weeks after the end of distraction, six rabbits were selected randomly from each group and killed with an overdose of anesthetic. The distracted callus was immediately harvested to detect the expression of H_2S enzyme mRNA and protein.

Total RNA was isolated from the powdered distracted callus by Trizol extraction (Invitrogen, Carlsbad, CA, USA) in liquid nitrogen, and cDNA was prepared using a PrimeScript RT reagent kit (TaKaRa, Dalian, China) in accordance with the manufacturer's instructions. SYBR Green quantitative real-time reverse transcription PCR analyses were performed on an ABI 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Specific primers were CSE sense 5′-TGGGCTTAGTGTCTGTTAATTCC-3′, CSE antisense 5′-TTGGTGTGGGATAAGAGACAGG-3′, CBS sense 5′-CTGTGAAGGGCTATCGCTGC-3′, CBS antisense 5′-CTGGCATTGCGGTACTGGTC-3′, 3-MST sense 5′-TGGGCTTAGTGTCTGTTAATTCC-3′, 3-MST antisense 5′-ACTGTGACAGTATTTCAGGGTAG-3′. The PCR program was initiated with 10 s at 95 °C before 40 thermal cycles, each of 5 s at 95 °C and 31 s at 60 °C. All of these experiments were performed in triplicate. The cycle threshold (Ct) value for each reaction was calculated from the amplification curve to determine the relative gene expression using the comparative cycle threshold method. Sample values were normalized with the Ct value of the housekeeping gene β-actin (β-actin sense 5′-CTCCCAGCACACTTAACTTAGC-3′, β-actin antisense 5′-AAAGCCACAAGAAACACTCAGG-3′).

The total protein from the distracted tissue was harvested using a commercially available kit (Sigma, Saint Louis, MO, USA). Total protein (30 μg) was separated on a sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) by electrophoresis. After blocking the non-specific binding, the membranes were incubated overnight at 4 °C with primary antibodies: CSE (1:750; Proteintech, Chicago, IL, USA), CBS (1:500; Proteintech, Chicago, IL, USA), 3-MST (1:750; Sigma Aldrich, St. Louis, MO, USA), bone morphogenetic protein (BMP)-2 (1:750; Abcam, Hong Kong), and vascular endothelial growth factor (VEGF) (1:1000; Abcam, Hong Kong). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and the signals detected by chemiluminescence detection (Bio-Rad, Hercules, CA, USA). β-actin (1:500; Abcam, Hong Kong) was used as the internal control.

Bone mineral density analysis

Six rabbits from each group were selected randomly for the determination of the BMD

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