Effect of $CoCl_2$ on fracture repair in a rat model of bone fracture

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Abstract. Low oxygen availability is known to activate the hypoxia-inducible factor-1α (HIF-1α) pathway, which is involved in the impairment of fracture healing. However, the role of low oxygen in fracture healing remains to be fully elucidated. In the present study, rats were divided into two groups and treated with $CoCl_2$ or saline, respectively. Rats with tibial fractures were sacrificed at 14, 28 and 42 days subsequent to fracture. Autoradiography was performed to measure healing of the bone tissue. In addition, the effects of cobalt chloride ($CoCl_2$) on the expression of two major angiogenic mediators, HIF-1α and vascular endothelial growth factor (VEGF), as well as the osteoblast markers runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OC) were determined at mRNA and protein levels by reverse transcription-quantitative polymerase chain reaction, western blot analysis and immunohistochemistry. Systemic administration of $CoCl_2$ (15 mg/kg/day intraperitoneally) significantly promoted fracture healing and mechanical strength. The present study demonstrated that in rats treated with $CoCl_2$, the expression of HIF-1α, VEGF, Runx2, ALP and OC was significantly increased at mRNA and protein levels, and that $CoCl_2$ treatment enhances fracture repair *in vivo*.

Introduction

Traumatic fractures are the most common type of injury in daily life. In the majority of clinical cases, the most simple fractures heal with minimal intervention; however, in severe fractures and in certain patient populations, including diabetics and patients with splintered fractures, impaired fracture healing and bone defects occur (1,2). In spite of numerous advances in every discipline of medicine, patients with complex bone injuries of the upper and lower extremities and are required to undergo prolonged reconstructive procedures for retrieval of their limb functions (2,3).

It has been reported that the hypoxia-inducible factor (HIF) pathway is the central pathway for sensing and responding to alterations in local oxygen levels in a wide variety of organisms (4). Activation of the HIF-1α pathway can act as a critical mediator of neoangiogenesis, which is required for skeletal regeneration; thus, it is suggested that the application of HIF activators may be used as therapies to improve bone healing (4). An increasing number of studies suggested that hypoxia may be a powerful stimulus for bone cells via the mediation of angiogenesis [vascular endothelial growth factor (VEGF)], cellular metabolism (glucose transporter) and the recruitment of mesenchymal cells (MSCs) to areas of matrix damage (5-7). A more thorough understanding of hypoxia in bone healing will lead to the elucidation of cellular and molecular mechanisms that may aid in the development of protective therapies. $CoCl_2$, a mimic of hypoxia, directly enhances HIF-1α stabilization and downstream target genes by inhibiting prolyl hydroxylase enzymes (8). An improved understanding of the alterations in gene expression that occur during fracture healing induced by $CoCl_2$ *in vivo* may lead to improvements in treatment.

In the present study, it was hypothesized that tibiae treated with $CoCl_2$ may exhibit accelerated fracture healing. To determine whether systemic application of $CoCl_2$ enhances the rates of fracture healing in a pre-clinical model, the effects of $CoCl_2$ on callus formation and fracture healing using radiographic evaluation as well as callus mechanical strength and integrity using three-point bending and key gene expression were examined *in vivo*.

Materials and methods

Animal care. The present study was performed in accordance with the regulations of Xuanwu Hospital Affiliated to Capital Medical University (Beijing, China) and approved by the local animal research committee. All Sprague-Dawley rats were obtained from the Laboratory Animal Center of Capital Medical University.

Animal experiments. Sprague-Dawley (SD) rats, 6 weeks of age, were maintained under humidity (50-60%) and temperature (23-25˚C) controlled conditions with a 12-h light/dark cycle at the Central Animal Facility, Capital Medical University (Beijing, China). The animals were allowed 1 week

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acclimatization to local vivarium conditions and had free access to untreated tap water and standard rat chow. A total of 48 female SD rats were randomly assigned to two groups: i) Control animals treated with saline (n=24) and ii) animals with 15 mg/kg $CoCl_2$ treatment per day, administered by intraperitoneal injection (n=24) prior to fracture. Subsequent to anesthesia with intraperitoneal ketamine hydrochloride (80 mg/kg body weight) and xylazine (10 mg/kg body weight) both purchased from Fujian Furuta Pharmaceutical Co., Ltd. (Fujian, China), surgery was performed with a fracture device, as described previously (9). In brief, fractures were created at the tibial tuberosity using a blunt guillotine driven by a drop weight, and a steel K-wire was inserted into the medullary canal. Radiographs were captured immediately to confirm the extent of fractures.

Subsequent to capturing the radiographs, the rats were sacrificed by cervical dislocation at 7, 28 and 42 days following fracture (n=8 for each time-point). The K-wire was removed, the tibiae were dissected, and the fracture zone was prepared for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and immunohistochemistry.

Radiological analysis. Radiographic analysis was performed to assess healing parameters using a Faxitron x-ray machine (MX-20 Specimen Radiography System; Faxitron Bioptics, LLC, Tucson, AZ, USA). Radiographs were captured at multiple time-points post-fracture (7, 28 and 42 days) and were assessed blindly by three independent investigators using the scoring scale described previously (10). The scale was obtained according to rebridgement (no rebridgement, partial or complete) and the results were expressed as a percentage alteration from saline-treated groups (saline-treated =100%).

RNA extraction and RT-qPCR. Total RNA was extracted from fresh bone tissues of each group using the TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA quantity and quality was determined by using the NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA (500 ng) was reverse-transcribed using the ReverTra Ace following the manufacturer's instructions (Toyobo Co., Ltd., Osaka, Japan). RT-qPCR was performed to measure mRNA expression levels relative to β-actin (ACTB) mRNA expression with the iCycle iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green Master Mix (Toyobo, Co., Ltd.). The primers used were as follows: HIF-1α, 5'-CCC CTACTATGTCGCTTTCTTGG-3' (forward) and 5'-GGT TTCTGCTGCCTTGTATGG-3' (reverse); VEGF, 5'-CGA CAAGGCAGACTATTCAACG-3' (forward) and 5'-GGC ACGATTTAAGAGGGGAAT-3' (reverse); runt-related transcription factor 2 (Runx2), 5'-CCCACGAATGCACTA TCCAG-3' (forward) and 5'-GGCTTCCATCAGCGTCAA CA-3' (reverse); ALP, 5'-GGACGGTGAACGGGAGAA C-3' (forward) and 5'-CCCTCAGAACAGGGTGCGTAG-3' (reverse); osteocalcin (OC), 5'-CGGACCACATTGGCTTCC AG-3' (forward) and 5'-GCTGTGCCGTCCATACTTTCG-3' (reverse); and ACTB, 5'-CCGTAAAGACCTCTATGCCAA CA-3' (forward) and 5'-CGGACTCATCGTACTCCTGCT-3' (reverse). Primers were synthesized by Sangon Biotech (Shanghai, China). The PCR thermal cycling conditions were as follows: 95˚C for 5 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All experiments were performed in triplicate and were repeated a minimum of three times. The qRT-PCR results were expressed relative to gene expression levels at the threshold cycle (Ct) and were related to the control.

Immunohistochemistry. Sections were prepared and processed using standard techniques following a previously described method (11). In brief, tissues generated from the fracture site were cut and mounted on slides, and following de-paraffinization and hydration, antigen retrieval was performed by incubating with 10 mmol/l sodium citrate (pH 6.0) and followed by 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxide. The slides were incubated with primary antibodies in the blocking solution in a humidified chamber at 4˚C overnight. To determine the expression of activated forms of VEGF, ALP and OC proteins, rabbit polyclonal VEGF antibody (cat. no. ABS82, 1:100, EMD Millipore, Temecula, California, USA), rabbit polyclonal ALP antibody (cat. no. ab84401, 1:100, Abcam, Cambridge, MA, USA) and mouse monoclonal OC antibody (cat. no. ab13418, 1:100, Abcam) were used. Finally, the sections were incubated with the secondary antibody for 10 min. Subsequent to washing with phosphate-buffered saline, the sections were then incubated with streptavidin-peroxidase conjugate for 10 min. The final staining was performed in diaminobenzidine tetrahydrochloride (Ventana Medical Systems, Inc., Tucson, AZ, USA) solution and were then counterstained with hematoxylin, dehydrated and mounted. Negative controls included replacement of the primary antibody with normal polyclonal mouse immunoglobulin G of the same concentration.

The score was assessed by two independent observers, under a light microscope (Olympus BX61; Olympus, Melville, NY, USA). The percentages of stained cells and staining intensity were taken into account in order to obtain the score. Staining intensity was scored as follows: 0, no staining; 1, weak intensity; 2, moderate intensity; and 3, high intensity. The number of positive cells was evaluated as follows: 0 (negative), <10% positive cells; 1 (weak), <30% positive cells; 2 (moderate), <50% positive cells; and 3 (strong), >70% positive cells.

Biomechanical analysis. Hydrated tibiae were assessed in regard to torsion using previously published methods (12). The rat tibiae from each group were tested to failure by three-point bending using a material testing system (ELF 3400; EnduraTEC, Minnetonka, MN, USA). Biomechanical parameters, including breaking force (maximum load), stiffness (average slope of linear portion of the curve before yielding) and work-to-fracture (bend strain at maximum and bend strength at maximum) were calculated from the force displacement data.

Western blot analysis. Radioimmunoprecipitation assay buffer containing protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) was used to prepare tissue lysates with 1% SDS. Protein quantification was performed using a Bicinchoninic

Acid Protein Assay kit (Thermo Fisher Scientific). Total proteins (50 μg) were resolved on 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated overnight at 4˚C with primary rabbit polyclonal antibodies against HIF-1α (cat. no. PA1-16601, Thermo Fisher Scientific), VEGF (cat. no. ABS82; EMD Millipore), ALP (cat. no. ab84401; Abcam), Runx2 (cat. no. H00000860-M04; Abnova, Taipei, Taiwan), OC (cat. no. ab13418; Abcam) and mouse monoclonal β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). All antibodies were diluted 1:1,000 in Tris-buffered saline. Blots were washed in TBST and labeled with the horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc, Danvers, MA, USA). Bands and band intensity were detected and calculated using chemiluminescence (Thermo Fisher Scientific) and Image Quant LAS4000 (GE Healthcare Life Sciences, Little Chalfont). The protein expression levels were expressed relative to β-actin levels.

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical differences between groups were evaluated using Student's two-tailed t-test and $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). All experiments were repeated a minimum of three times, independently.

Results

$CoCl_2$ accelerates the formation and remodeling of bone during fracture repair. Previous studies have demonstrated that hypoxia affects the fracture healing by MSCs (5,13); however, the underlying cellular and molecular mechanisms have remained to be fully elucidated. To further examine the effects of hypoxia during fracture repair, callus formation was performed for the radiographic examination on days 7, 28 and 42 in all animals. Treatment of rats with $CoCl_2$, a hypoxia mimic, markedly strengthened new bone formation during the course of fracture repair (Fig. 1). Of note, fractured tibiae exhibited enhanced repair at 7 days compared with vehicle-treated animals and displayed near-complete healing of the $CoCl_2$-treated tibiae at 42 days, whereas incomplete bridging of cortical bone was clearly visible in the vehicle-treated animals. These results indicated that $CoCl_2$ may serve an important role in fracture healing.

In order to confirm this observation, re-bridgement of the cortices and acceleration of healing was analyzed using a grading scale (10). Radiological evaluation of animals treated with $CoCl_2$ at 7, 28 and 42 days suggested a significant increase in the healing rate (Fig. 2). These results indicated that $CoCl_2$ may promote fracture repair.

The HIF-1 pathway is functional and mediates hypoxia-induced gene expression during fracture repair under hypoxia. Hypoxia is one of the most important pathological features of numerous diseases and it is well known that $CoCl_2$ is able to mimic the effects of HIF-1α. The mRNA levels of

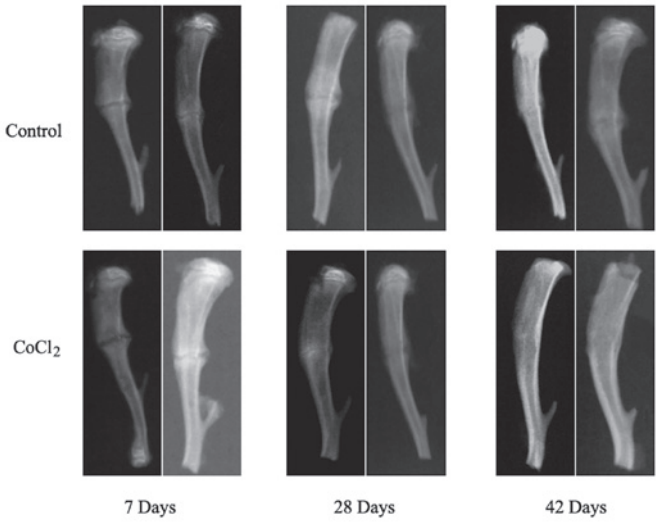

Figure 1. Radiographic images of the fractured callus at 7, 28 and 42 days following the procedure. Two representative images of each group (n=8) are shown.

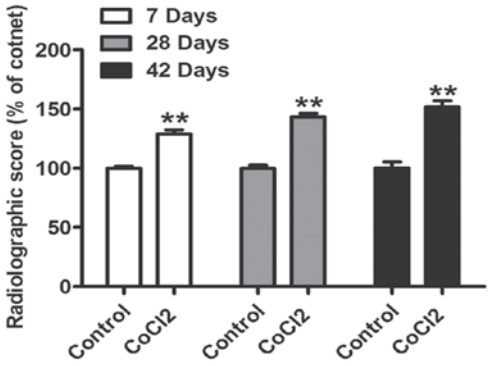

Figure 2. Radiological score obtained at 7, 28 and 42 days for each group using a grading scale. Values are expressed as the percentage of the vehicle-treated group [mean ± standard deviation (n=8)]. $^{**}P<0.01$ vs. rats treated with saline.

HIF-1α were analyzed using RT-qPCR. As presented in Fig. 3, rats with $CoCl_2$ exhibited a significantly increased HIF-1α expression. In addition, it was observed that the protein levels of HIF-1α were increased at the measured time-points (Fig. 4). To further evaluate whether HIF-1α had a direct functional role in this process, the expression of downstream genes of HIF-1α was investigated using RT-qPCR and western blot analysis. The results also indicated that $CoCl_2$ significantly increased VEGF, Runx2, ALP and OC mRNA and protein levels (Figs. 3 and 4). These results suggested that the effect of $CoCl_2$ on fracture healing partly involved the activation of the HIF-1α signaling pathway.

Immunohistochemical analysis. Runx2 is considered to be an osteoblast-specific transcriptional factor and is involved in chondrocyte maturation and osteoblast differentiation (14,15). Osteoblast-specific expression of genes, including ALP and OC, is an important characteristic of bone healing (16). Therefore, the expression of VEGF, ALP and OC was assessed in bone tissues by immunohistochemistry at 42 days following fracture. An increase in the expression of VEGF, ALP and

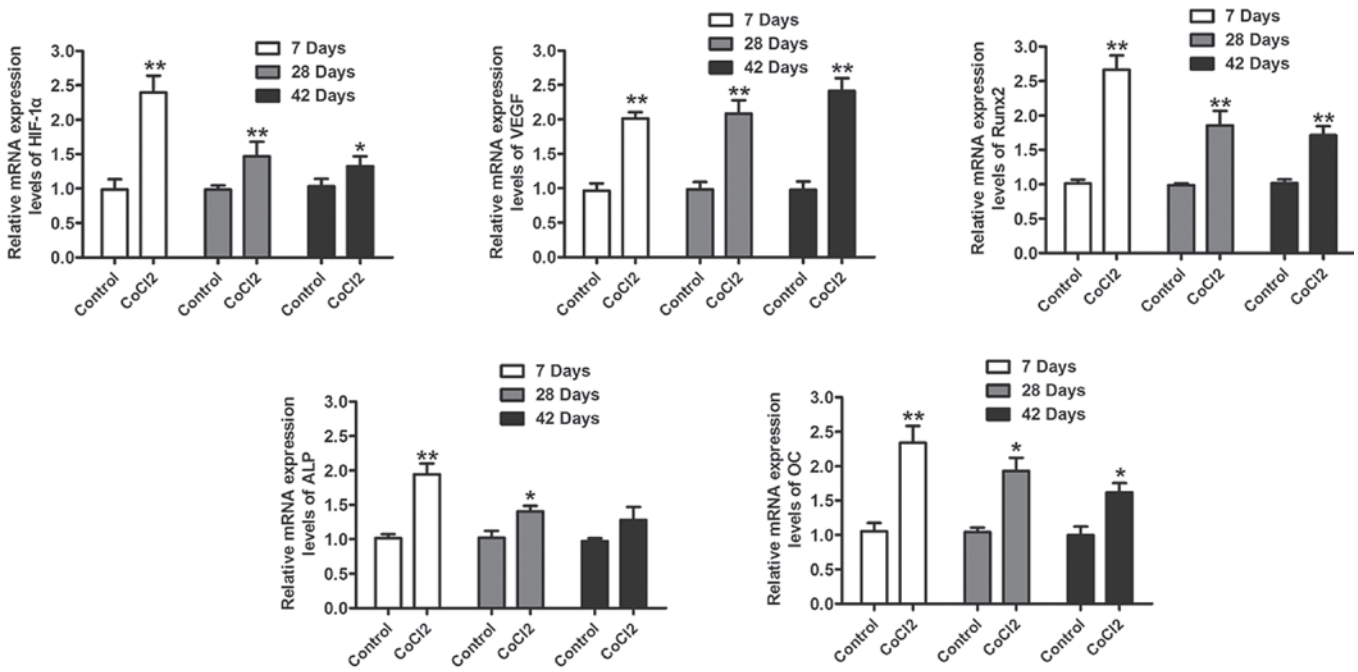

Figure 3. Relative mRNA expression levels of HIF-1α, VEGF, osteoblast-specific transcriptional factor Runx2 and bone-associated molecules ALP and OC were determined by quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation. *P<0.05; **P<0.01 vs. rats treated with saline at indicated time. HIF-α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin.

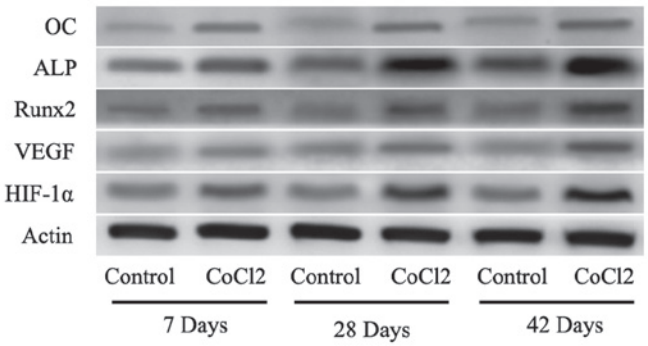

Figure 4. Bone lysates were subjected to immunuoblotting with HIF-1α, VEGF, Runx2, ALP and OC or β-actin antibodies. HIF-α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin.

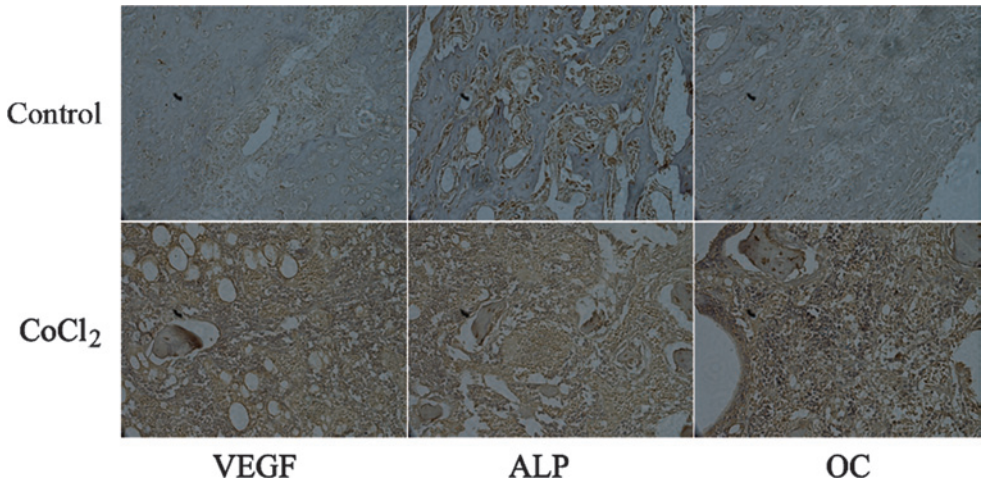

Figure 5. Representative images of immunohistochemical staining for VEGF, ALP and OC in bone tissues at 42 days subsequent to fracture (magnification, x100). VEGF, vascular endothelial growth factor; ALP, alkaline phosphatase; OC, osteocalcin.

Table I. Biomechanical testing.

	Control group		$CoCl_2$ group	
Parameter	28 days	42 days	28 days	42 days
Maximum force (N)	68.9±3.4	116.5±6.7	82.9±5.4[a]	181.3±12.7[b]
Stiffness (N/mm)	193.8±13.6	307.2±24.6	237.1±16.2[a]	462.5±20.8[b]
Work to fracture (N-mm)	18.7±0.6	32.8±2.7	22.4±1.8	41.6±3.4[a]

Biomechanical testing evaluated at the indicated time-points by three-point bending. [a]P<0.05; [b]P<0.01, vs. control. Values are expressed as the mean ± standard deviation.

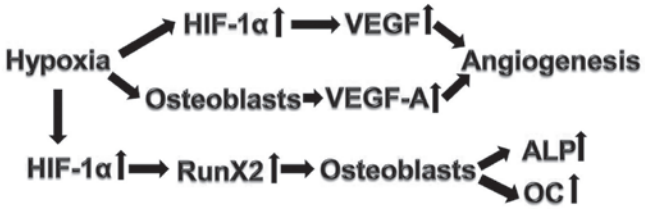

Figure 6. Schematic representation of the molecular mechanism of the effect of $CoCl_2$ on fracture healing. HIF-α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin.

OC was observed in the groups treated with $CoCl_2$ compared with that in the vehicle-treated animals (Fig. 5). These results further suggested that $CoCl_2$ induces fracture healing through the activation of HIF-1α and its target genes.

Biomechanical analysis: Three-point bending. To further assess the functional features of fracture healing, the influence of $CoCl_2$ on the mechanical properties of tibiae was evaluated in rats. All specimens were tested according to the guidelines of the American Society for Testing and Materials for the uniaxial strength testing. The tibiae of the animals that had been administered $CoCl_2$ were significantly stronger than those of the control animals (Table I). Of note, the force required to break the bone and the structural stiffness of the fractured tibia was 55.4 and 50.4% greater, respectively, than that of the vehicle-treated controls at 42 days.

Discussion

Normal fracture healing is a complex process involving cellular recruitment, specific gene expression and synthesis of compounds that regenerate native tissue to restore the mechanical integrity, and thus the function, of damaged bone (17-19). Treatments for fracture healing have addressed numerous aspects, including biological, nutritional, physical and genetic factors. Previous studies have demonstrated that hypoxia may induce MSC recruitment to areas of matrix damage by upregulating the osteopontin/CD44 pathway (5). VEGF serves an important role in physiological and pathological neovascularization (angiogenesis) and has been demonstrated to stimulate bone healing in animal models (6). In the present study, it was established that hypoxia is able to promote fracture healing and it was demonstrated that an osteoblast-specific transcriptional factor, Runx2, as well as the osteoblast-specific genes ALP and OC were upregulated. This provides a potential strategy for the regeneration of bone tissue.

As $CoCl_2$ is a well-known mimic of hypoxia, it was hypothesized that this chemical may have a delayed pre-conditioning effect in fracture healing. The fracture models were first identified by radiological evaluation. Consistent with a previous study, administration of $CoCl_2$ was able to mimic the effect of hypoxia and promote fracture healing (5). Subsequently, the expression of HIF-1α was detected at mRNA and protein levels, and it was identified that the expression of HIF-1α was significantly upregulated throughout the process. HIF-1α is known to interact with the core DNA sequence 5'-[AG]CGTG-3' at the hypoxia response element target gene promoters, resulting in the upregulation of numerous hypoxia-sensitive genes, including VEGF (20). Of note, $CoCl_2$ has been observed to enhance the expression of Runx2, an essential osteoblast transcription factor, as well as ALP and OC, which are required for osteogenesis *in vivo* (21-23). In addition to the significant alterations in gene expression observed, the biomechanical properties of the healing construct can be attributed to the density and to the amount of tissue. The results of the biomechanical assessment indicated that $CoCl_2$ was able to significantly increase the maximum load, stiffness and energy absorption. These results indicated that pretreatment with $CoCl_2$ may support the restoration of cartilage and bone during fracture healing *in vivo*.

Previous studies have indicated that MSCs can be recruited by osteocytes under hypoxia, and genetic activation of the HIF-1α pathway increases neoangiogenesis and promotes bone regeneration (13). The present study identified that $CoCl_2$ was able to significantly increase the expression of Runx2, ALP and OC. In spite of inadequate blood supply caused by hypoxia being a major cause of delayed union or non-union during fracture healing (24), an appropriate amount of hypoxia may aid in bone

healing, providing a novel strategy for the treatment of bone fractures. A potential pathway for these processes is proposed in Fig. 6. A mouse fracture model treated with hypoxia results in upregulated HIF-1α, which activates VEGF and Runx2, leading to the osteogenic differentiation and angiogenesis.

In conclusion, the results of the present study demonstrated that $CoCl_2$ enhances fracture healing. It was demonstrated that $CoCl_2$ induces bone and cartilage formation, increases tissue vascularization and may activate the HIF-1α pathway.

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