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Original Article

Effects of acidosis on the osteogenic differentiation of human bone marrow mesenchymal stem cell

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Abstract: Background: Acidosis affects the function of osteoblasts including mineral metabolism. The aim of this study was to investigate the changes of proliferation and differentiation in human bone marrow mesenchymal stem cells (hBMMSCs) induced by simulated respiratory acidosis and the mechanisms underlying it. Materials and methods: A simulated model of hBMMSCs were performing with respiratory acidosis. Protein expression was measured by western blotting. Cell proliferation was measured with MTT assay, and the cell cycle was analyzed by flow cytometry. The cytotoxicity rate was measured using lactate dehydrogenase-Cytotoxicity Assay Kit. Expression levels of runx2, alkaline phosphatase, and type I collagen mRNA were measured by RT-qPCR. Results: Levels of alkaline phosphatase mRNA expression in acidosis medium raised up were less than alkaline phosphatase mRNA in negative controls. Compared to negative controls, exposure to acidic medium suppressed alkaline phosphatase enzyme activity. The toxicity of acid on hBMMSCs was tested for up to 24 h in culture. Comparing with negative controls, acidosis did not result in any significant toxicity to the cells; however, cell proliferation significantly declined. The increase of runx2 messenger RNA (mRNA) expression levels in acidosis was higher than the levels observed in negative controls. Corresponding to runx2, the levels type I collagen mRNA in acidosis appeared to be greater than levels observed in negative controls. Similar to the mRNA, the increase in collagen deposit after normalization by protein concentration was observed in cells cultivated in acidic environment. Conclusion: The results revealed that the adverse effect of acidosis on bone formation was largely due to the impairment of bone matrix mineralization. Acidosis had diverse impact on osteoblastic genes and altered the differentiation of osteoblast, as well as attenuated the function of osteoblast.

Keywords: Acidosis, human bone marrow mesenchymal stem cells (hBMMSCs), differentiation, biomineralization

Introduction

The pH of blood is important for us to our body. The acid-base balance in the body plays marked roles on cellular functions, including the activity of enzymes in bone-related cells, the activity of transcription factors and the structure of other proteins involved in bone metabolism, because of the strict influence of the proton concentrations in tissue fluid on the structure and function of proteins [1]. Previous reports on bone histology in patients with distal renal tubular acidosis revealed reduction in osteoblast number, and suppression of bone formation due to varying extent of impact in bone matrix mineralization, when compared to healthy subjects [2-4]. Although the abnormal bone remodeling may mainly result from an alteration in mineral balance [5], the direct influence of metabolic acidosis on bone cells may also deserve attention. Even though numerous studies have reported the effect of metabolic acidosis on bone metabolism, the underlying mechanisms are not clearly understood.

Stem cell have been widely studied in therapeutic potential to regenerate injured tissue. Bone marrow mesenchymal stem cells (BMMSCs) have the ability to self-renew and can be isolated from bone marrow and cultured under inducing conditions that promote their in vitro differentiate into multifunctional cell including os-

Table 1. Primer and probe sequences

Gene		Primers (5'-3')	Probe (5'-3')
18S rRNA	Forward	CGGCTACCACATCCAAGGAA	TGCTGGCACCAGACTTGCCCTC
	Reverse	GCTGGAATTACCGCGGCT	
Runx2	Forward	GCCTTCAAGGTGGTAGCCC	CCACAGTCCCATCTGGTACCTCTCCG
	Reverse	CGTTACCCGCCATGACAGTA	
Type I collagen	Forward	CAGCCGCTTCACCTACAGC	CCGGTGTGACTCGTGCAGCCATC
	Reverse	TTTTGTATTCAATCACTGTCTTGCC	
Alk Phos	Forward	GACCCTTGACCCCCACAAT	TGGACTACCTACCTATTGGGTCTCTTCGAGCCA
	Reverse	GCTCGTACTGCATGTCCCCT	

teoblasts, chondrocytes, adipocytes and other mesodermal cell types [6]. When required, osteoblasts produced active substances for bone remodeling and repair can originate from mesenchymal stem cells (MSCs) in the bone marrow [7]. Therefore, the adjustments of BMSCs function contribute to replacement of osteoblasts in bone turnover and fracture healing throughout life, and maintain bone homeostasis. In view of their physiological functions in regulating bone remodeling, the investigation of BMMSCs responding to microenvironmental changes has generated considerable interest.

Previous studies with a variety of stem cells appear that acidosis can reversely influence the function of protein and lipid and then affect stem cells proliferation and differentiation [8]. Although numerous studies have revealed acidosis has a direct effect on osteoblasts and possibly interferes with osteoblast differentiation using hydrochloric acid or ammonium chloride to induce metabolic acidosis, the influences of respiratory acidosis on BMMSCs function and the possible mechanisms underlying it need to be explored. The current study was designed to investigate whether the proliferation and osteogenic potential could be changed as a result of simulated respiratory acidosis in BMSCs and further to explore the molecular mechanisms underlying it.

Materials and methods

The study protocol was approved by ethical committee on Research Involving Human Subjects at Affiliated Hospital of Wei Fang Medical University.

Cell culture

Primary human bone marrow mesenchymal stem cells (hBMMSCs) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Shanghai, China) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO_2. We discarded the detached cells every 4 day. These hBMMSCs were trypsinized and passaged at 90% confluence for the next expansion. Acidosis (7.1 ± 0.02) was induced by incubating cells under pressure in 10% CO_2 after 16 h as an actual measurement (n=12). Some of the measurements involved in this characterization included confirmation of ability to undergo adipogenesis and osteogenesis, expression of CD105 and absence of CD45 and CD34 expression.

Studies of osteoblastic gene expression by quantitative real-time reverse transcription-PCR

Total RNA extraction was performed using TRIzol reagent and reverse transcribed to cDNA using a first-strand cDNA synthesis kit according to the manufacturer's instructions. The mRNA expressions of osteoblastic gene, including runx-2, type I collagen, and alkaline phosphatase gene, were determined using quantitative real-time reverse transcription-PCR (RT-qPCR) described by Disthabanchong et al. [5]. Multiplexed PCR reaction was conducted with both target and reference genes (18S ribosomal RNA (rRNA)) synthesised by Shanghai Jima Biotechnology Co., Ltd. (Shanghai, China) in the same reaction. Each sample was analysed in triplicate. The probe for 18S rRNA fluorescently labelled with VIC™ and TAMRA, and the probes for gene of interest were labelled with 6-carboxyfluorescein TAMRA. The nucleotide sequences of primer and probe are listed in **Table 1**. PCR conditions and analysis were consistent with previously published methods [9].

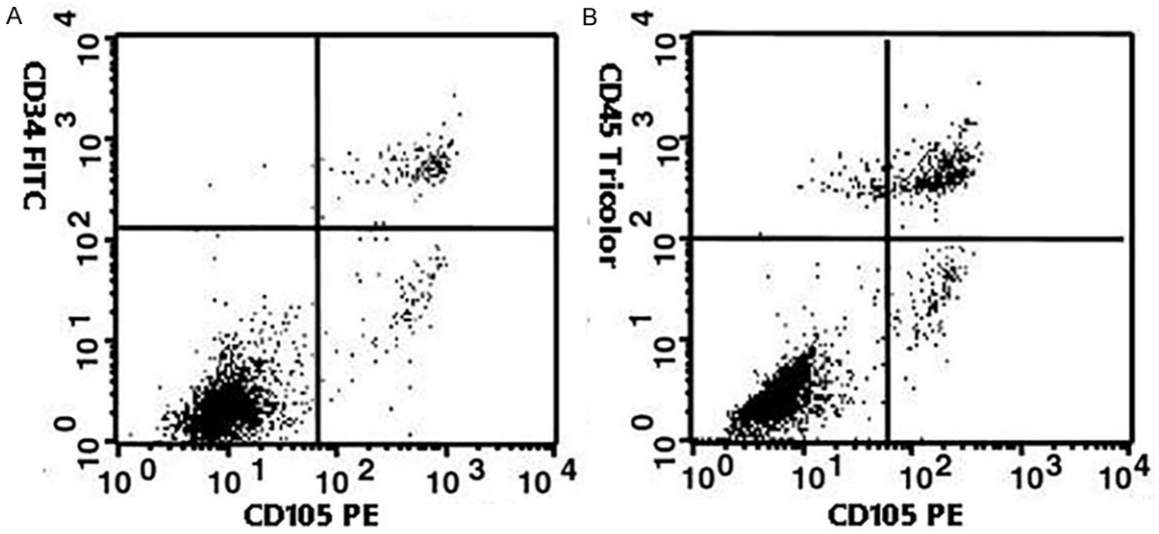

Figure 1. Flow cytometry analysis. Flow cytometry of bone marrow mononuclear cells before plating for (A) CD34, CD105, and (B) CD45, CD105.

Western blot analysis

Western blotting was performed using standard procedures. Runx2 protein were measured, and β-actin was used as an internal control. Briefly, adherent cells lysed with cold cell lysis buffer and Protease Inhibitor Cocktail shared in 1:10 ratio followed by vigorous pipetting and centrifugation. Protein (10-20 mg) was heated for 5 min at 95°C in sample loading buffer and run on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transfer onto nitrocellulose membrane. After blocking with 5% non-fat dry milk, the membrane was probed with following primary antibodies. Membranes were washed and then secondary antibody (1: 2,000) was incubated for 2 h at room temperature and rinsed in TBS-T wash buffer 3 times for 10 min each. Developed films were digitised by scanning, and the optical densities were analysed with the image software.

Alkaline phosphatase enzyme activity assay and quantification of collagen concentration

Alkaline phosphatase enzyme activity was determined using a DEVD-AMC (Sigma A1086) kit with standard procedure provided by Fillmore et al. [10]. The experiments. The staining and quantification of collagen concentration were performed in 25-mm2 tissue culture dishes, as described previously [11]. Cell layers were fixed for 1 h in Bouin's fixative followed by addition of Sirius red F3BA solution (0.1% in saturated picric acid). After 1-h staining, cell layers were washed in running tap water and again in 0.01 NHCl to remove the non-bound dye. For quantification of collagen content, the dye was dissolved in 0.1 N NaOH. The absorbance was determined at 550 nm. The amount of collagen was normalized by the protein concentration using Bradford Reagent. Assays were performed in duplicate.

Cell proliferation and cytotoxicity analysis

Cell viability was measured using a standard MTT assay protocol, as described previously [12]. Cells were seeded into 96-well plates at a density of 3×10^3 cells/well and placed in an incubator until the cells grew to confluence. Subsequently, 0.5 mg/ml MTT (Invitrogen M6494) was added to incubate at 37°C for 2 h. The absorbance value of each well was measured at 550 nm. Cytotoxicity was assessed with lactate dehydrogenase-Cytotoxicity Assay Kit (Biovision, CO, USA) according to the manufacturer's instruction. A standard protocol was introduced by Bushinsky et al. [13]. Adherent cells were incubated in the assay medium containing 1% fetal bovine serum with and without increasing concentration of CO_2 for up to 24 h. Cells incubated with 1% Triton X-100 were added as positive control. Absorbance of lactate dehydrogenase activity released from damaged cells was recorded at 490 nm with reference wavelength of 550 nm. Assays were performed in duplicate.

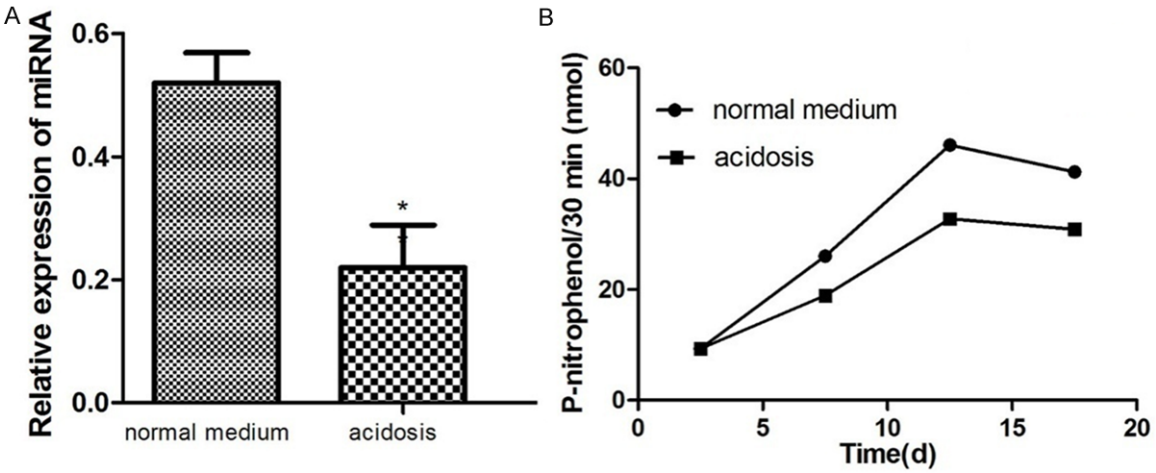

Figure 2. The effect of acidosis on the expression of alkaline phosphatase gene. A: Real-time PCR was used to detect the alkaline phosphatase mRNA expression level. B: Time course of the effect of acidosis on the expression of alkaline phosphatase enzyme activity.

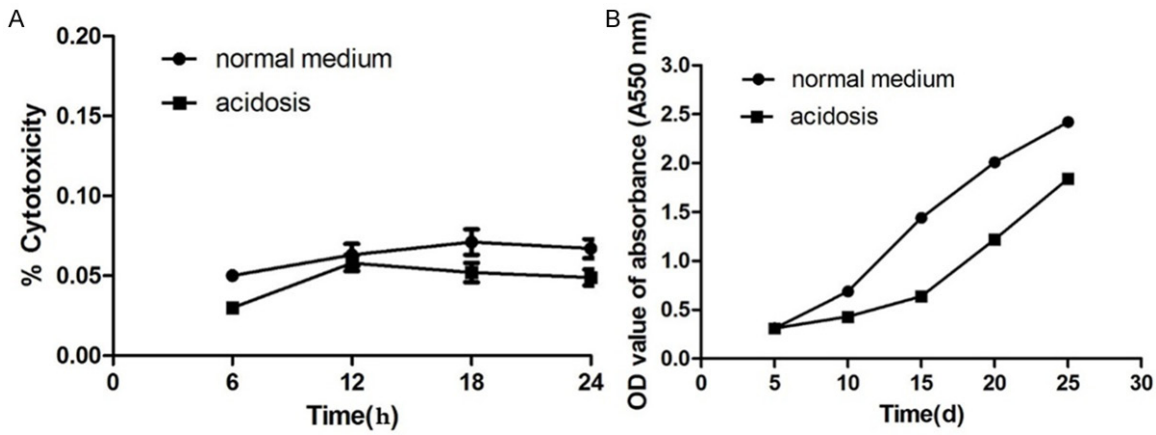

Figure 3. Cell cytotoxicity and proliferation assay. A: Cell cytotoxicity as evaluated by lactate dehydrogenase-cytotoxicity assay. B: Cell proliferation by a standard MTT assay.

Statistical analysis

Values are displayed as mean ± standard error of the mean (SEM). Student *t*-test was performed using Graphpad Prism software to determine statistical significance. Results shown in figures are from combined experiments. Number of experimental replicates is indicated in figure legends. Difference is considered statistically significant if $P < 0.05$.

Results

Effect of acidosis on mineralization

To explore whether simulated respiratory acidosis impaired bone matrix mineralization, alkaline phosphatase enzyme which is necessary for mineralization was detected (**Figure 1**) [8, 14]. Although alkaline phosphatase mRNA expression levels in both two mediums were increased, the levels in acidosis medium raised up were less than alkaline phosphatase mRNA in negative controls. Evidently, respiratory acidosis suppressed alkaline phosphatase mRNA expression (**Figure 2A**). Alkaline phosphatase enzyme activity was also examined. As shown in **Figure 2B**, compared to negative controls, exposure to acidic medium suppressed alkaline phosphatase enzyme activity.

Analysis of cell proliferation and cytotoxicity

Flow cytometry of hBMMSCs revealed the predominant hematopoietic cells ($CD34^{pos}$) and

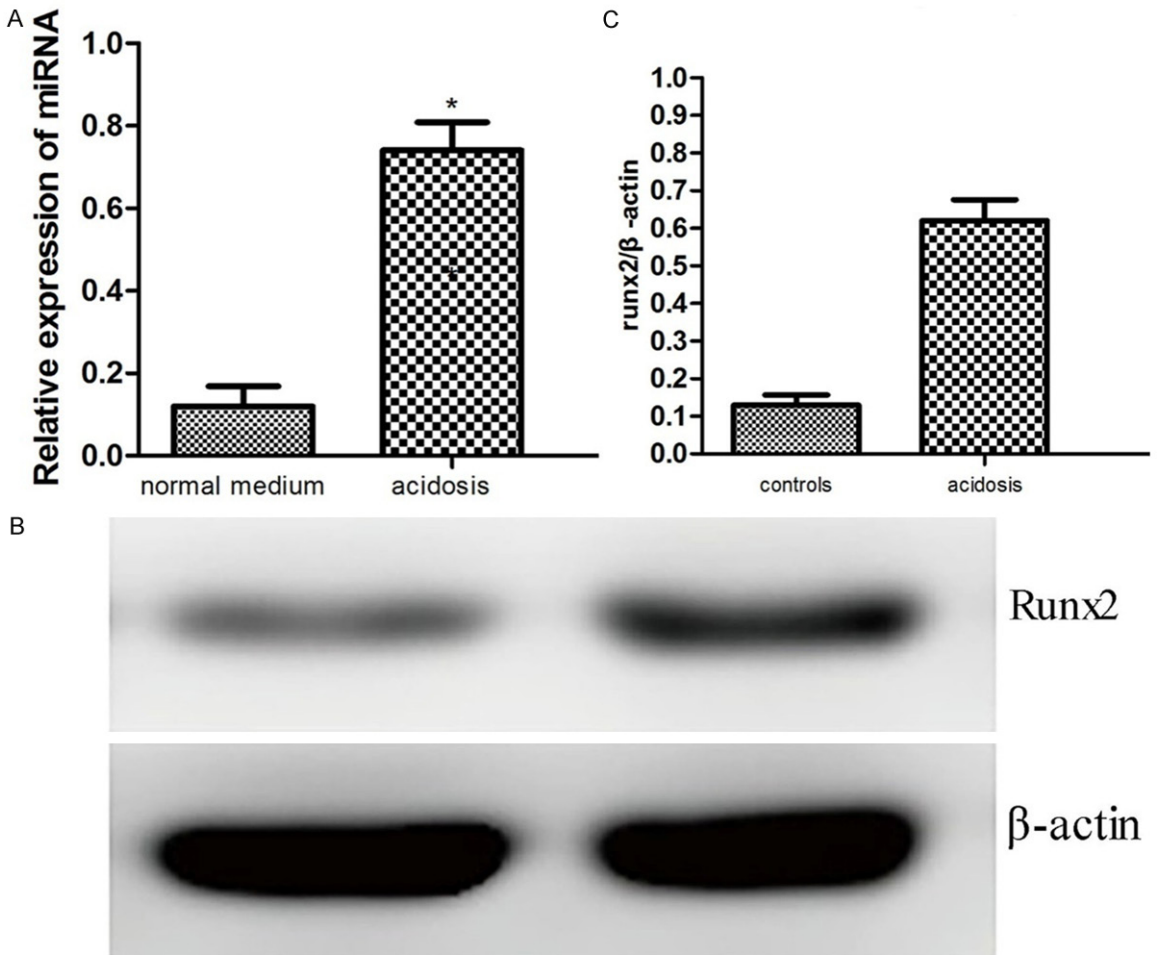

Figure 4. The effect of acidosis on the expression of runx2 gene. A: Real-time PCR was used to detect the runx2 mRNA expression level. B: Western blotting was used to detect the runx2 protein expression level in each group. C: The effect of acidosis on the expression of runx2 protein.

the fewer MSCs ($CD45^{neg}$ $CD34^{neg}$ $CD105^{pos}$) [15]. During expansion, hBMMSCs adhered to the culture plate, whereas hematopoietic cells detached and were eventually eliminated. The toxicity of acid on hBMMSCs was tested for up to 24 h in culture. Comparing with negative controls, acidosis did not result in any significant toxicity to the cells (**Figure 3A**); however, cell proliferation significantly declined (**Figure 3B**).

Effect of acidosis on related gene expression

The effect of respiratory acidosis on the expression of osteoblast transcription factor-runx2, which is necessary for osteoblast commitment [16], was subsequently examined. Cells were harvested at 5-day interval for further examination by RT-qPCR and Western blot analyses. The increase of runx2 mRNA and protein expression levels in acidosis was higher than those levels observed in negative controls (**Figure 4A-C**). Consequently, we examined the expression of type I collagen, the most abundant bone matrix protein produced by osteoblasts as well as a target gene for runx2 [14]. Corresponding to runx2, the levels type I collagen mRNA in acidosis appeared to be greater than levels observed in negative controls (**Figure 5A**). The quantification method has been shown to be sensitive and specific for collagen deposited on cultured osteoblasts [11]. Similar to the mRNA, the increase in collagen deposit after normalization by protein concentration was observed in cells cultivated in acidic environment (**Figure 5B**).

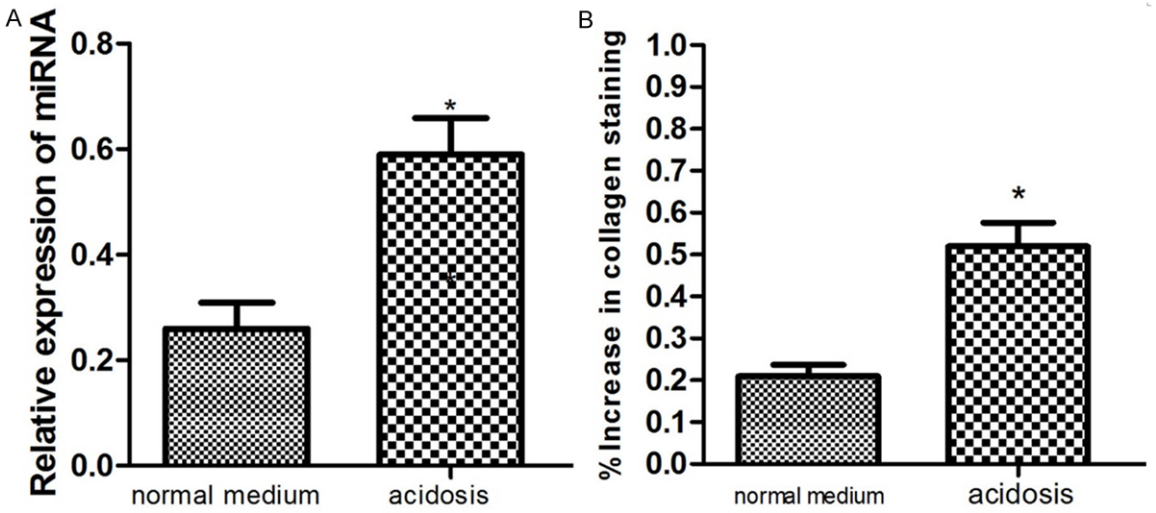

Figure 5. The effect of acidosis on the expression of type I collagen gene. A: Real-time PCR was used to detect the type I collagen mRNA expression level. B: The effect of acidosis on the expression of type I collagen.

Discussion

In this study, we demonstrated that simulated respiratory acidosis could inhibit the proliferation of hBMMSCs in vitro and play impaired roles on bone matrix mineralization due to reduction in alkaline phosphatase enzyme activity. And acidosis might downregulate the expressions of osteoblastic genes. However, there was no significant difference in cytotoxicity between acidic hBMMSCs and the negative controls.

In current study of respiratory acidosis, the expression of related osteoblastic genes and proteins was found to be altered during different stages of osteoblast differentiation. We initially examined the expression of a known essential transcription factor--runx2. Previous studies demonstrated that homozygous mutation of runx2 completely suppressed bone formation with arrest of osteoblast differentiation [16, 17]. Our report demonstrated that the expression of runx2 was increased under the effect of acidosis. Moreover, similar to that of runx2, the measurement of the most abundant bone matrix protein--type I collagen appeared to be an increased expression. Expression of type I collagen is improved during this early stage of osteoblast differentiation accompanying that of runx2 [8]. Therefore, the increased expression of runx2 and collagen could promote osteoblast differentiation in the early period. It could be explained that the increase in runx2's transcript as well as the parallel expressions of runx2 and collagen was due to type I collagen being one the target genes of runx2 [18]. According to the study of Tullberg-Reinert et al. [19], although uprelagutation of runx2 in mice heightened osteoblast differentiation during the early period, these animals were osteopenic as a result of less mature phenotype in their osteoblasts suggesting that overexpression of runx2 may play adverse roles in the later stage of osteoblast differentiation.

Metabolic acidosis actuates a net calcium efflux from bone and influences the function of osteoblasts and osteoclasts in vivo. Frick et al. reported that chronic metabolic acidosis could downregulate bone matrix protein levels including matrix Gla protein and osteopontin [20]. And it could vary osteoblast differentiation from hBMMSCs [5]. These studies in vitro presented that chronic metabolic acidosis directly inhibited osteoblast function and altered osteoblast differentiation from hBMMSCs through diverse effects on osteoblastic genes and proteins in vitro [5]. Furthermore, Frick et al. showed the inhibitory influence of acute metabolic acidosis on the induction of osteoblastic egr-1 and type I collagen in mouse calvarial cells [21]. Acidosis is also reported to potentiate osteoclast survival to activate bone resorption viaupregulation of osteopontin, promoting cell survival through integrin binding, augmentation of adhesion and spreading via activation of pyk-2, Cbl-b and src activation [22]. These findings suggest-

ed that the changes of external pH had a marked effect on the expression of certain genes which is important for bone formation or osteoblast differentiation.

The acid-base balance is a basic factor for the body. Acidosis is deeply involved in a variety of diseases and pathological conditions, promoting bone resorption and impeding bone formation. It therefore could be a candidate for intervention in the treatment of diseases. Although our data demonstrated that hBMMSCs exposed to acidic stress can increase the elevation of runx2 and type I collagen induced by low pH and thus affect differentiation for hBMMSCs, further experiments involving a more precise understanding of bone physiology and pathology of bone-related diseases are needed.

In this current study, our results revealed that the adverse effect of acidosis on bone formation was largely due to the impairment of bone matrix mineralization. Acidosis had diverse impact on osteoblastic genes and altered the differentiation of osteoblast, as well as attenuated the function of osteoblast. Our findings might serve as a suitable in vitro model to study underlying mechanisms involved low bone mass diseases.

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Disclosure of conflict of interest

None.

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References

[1] Kato K and Morita I. Promotion of osteoclast differentiation and activation in spite of impeded osteoblast-lineage differentiation under acidosis: effects of acidosis on bone metabolism. Biosci Trends 2013; 7: 33-41.

[2] Domrongkitchaiporn S, Pongsakul C, Stitchantrakul W, Sirikulchayanonta V, Ongphiphadhanakul B, Radinahamed P, Karnsombut P, Kunkitti N, Ruang-raksa C and Rajatanavin R. Bone mineral density and histology in distal renal tubular acidosis. Kidney Int 2001; 59: 1086-1093.

[3] Domrongkitchaiporn S, Pongskul C, Sirikulchayanonta V, Stitchantrakul W, Leeprasert V, Ongphiphadhanakul B, Radinahamed P and Rajatanavin R. Bone histology and bone mineral density after correction of acidosis in distal renal tubular acidosis. Kidney Int 2002; 62: 2160-2166.

[4] Disthabanchong S, Domrongkitchaiporn S, Sirikulchayanonta V, Stitchantrakul W, Karnsombut P and Rajatanavin R. Alteration of noncollagenous bone matrix proteins in distal renal tubular acidosis. Bone 2004; 35: 604-613.

[5] Disthabanchong S, Radinahamed P, Stitchantrakul W, Hongeng S and Rajatanavin R. Chronic metabolic acidosis alters osteoblast differentiation from human mesenchymal stem cells. Kidney Int 2007; 71: 201-209.

[6] Yan M, Wang Y, Yang M, Liu Y, Qu B, Ye Z, Liang W, Sun X and Luo Z. The effects and mechanisms of clinorotation on proliferation and differentiation in bone marrow mesenchymal stem cells. Biochem Biophys Res Commun 2015; 460: 327-332.

[7] Osyczka AM and Leboy PS. Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling. Endocrinology 2005; 146: 3428-3437.

[8] Aubin JE. Regulation of osteoblast formation and function. Rev Endocr Metab Disord 2001; 2: 81-94.

[9] Wang N, Wei H, Yin D, Lu Y, Zhang Y, Jiang D, Jiang Y and Zhang S. Cyclin D1b overexpression inhibits cell proliferation and induces cell apoptosis in cervical cancer cells in vitro and in vivo. Int J Clin Exp Pathol 2014; 7: 4016-4023.

[10] Fillmore N, Huqi A, Jaswal JS, Mori J, Paulin R, Haromy A, Onay-Besikci A, Ionescu L, Thebaud B, Michelakis E and Lopaschuk GD. Effect of fatty acids on human bone marrow mesenchymal stem cell energy metabolism and survival. PLoS One 2015; 10: e0120257.

[11] Tullberg-Reinert H and Jundt G. In situ measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: effects of transforming growth factor beta2 and ascorbic acid 2-phosphate. Histochem Cell Biol 1999; 112: 271-276.

[12] Kalaiarasan P, Kumar B, Chopra R, Gupta V, Subbarao N and Bamezai RN. In silico screening, genotyping, molecular dynamics simulation and activity studies of SNPs in pyruvate kinase M2. PLoS One 2015; 10: e0120469.

[13] Bushinsky DA. Stimulated osteoclastic and suppressed osteoblastic activity in metabolic

but not respiratory acidosis. Am J Physiol 1995; 268: C80-88.
[14] Stein GS and Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 1993; 14: 424-442.
[15] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
[16] Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S and Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997; 89: 755-764.
[17] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR and de Crombrugghe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002; 108: 17-29.
[18] Ducy P, Zhang R, Geoffroy V, Ridall AL and Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997; 89: 747-754.
[19] Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A and Komori T. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol 2001; 155: 157-166.
[20] Frick KK and Bushinsky DA. Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. Am J Physiol 1998; 275: F840-847.
[21] Frick KK, Jiang L and Bushinsky DA. Acute metabolic acidosis inhibits the induction of osteoblastic egr-1 and type 1 collagen. Am J Physiol 1997; 272: C1450-1456.
[22] Ahn H, Kim JM, Lee K, Kim H and Jeong D. Extracellular acidosis accelerates bone resorption by enhancing osteoclast survival, adhesion, and migration. Biochem Biophys Res Commun 2012; 418: 144-148.