文章编号:1004-7220(2015)01-0074-09

Combined 1, 25-dihydroxy vitamin D3 and mechanical strain treatment affect proliferation, differentiation, and expression of OPG and RANKL in osteoblastic MC3T3-E1 cells

LIU Lu[1], ZHANG Xin-chang[1*], ZHANG Xi-zheng[2], GUO Yong[2], LI Rui-xin[2](1. *Logistics Institute of Chinese People's Armed Police Forces*, *Tianjin* 300309, *China*; 2. *Institute of Medical Equipment of the Academy of Military Medical Sciences*, *Tianjin* 300161, *China*)

Abstract: **Objective** To investigate the effect of 1,25-$(OH)_2$-vitamin D3 (VD3) or mechanical strain alone and their combined treatment on proliferation and differentiation of pre-osteoblast MC3T3-E1 cells *in vitro*, as well as gene and protein expression of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) in those cells. **Methods** MC3T3-E1 cells were treated with 10 nmol/L VD3, intermitted mechanical strain or with a combination of these two factors. Cell proliferation was assessed with flow cytometry, and alkaline phosphatase (ALP) activity was measured using a fluorometric detection kit. The mRNA expression of ALP, runt-related transcriptional factor 2 (Runx2), OPG, and RANKL genes was determined by real-time PCR. The proteins expression of Runx2, OPG, and RANKL was determined by Western blotting. **Results** VD3 inhibited the proliferation of MC3T3-E1 cells, but the mechanical strain had no effect on cell proliferation. Mechanical strain, VD3, and the combined treatment enhanced the ALP activity of MC3T3-E1 cells as well as the protein expression of Runx2. The effect of combined treatment was less pronounced than the effect of VD3 or mechanical strain alone. Mechanical strain promoted the gene and protein expression of osteoprotegerin (OPG) and increased the ratio of OPG/RANKL. However, the combination of VD3 and mechanical strain led to a decrease in ratio of OPG/RANKL. **Conclusions** Mechanical strain might be effective in inducing osteogenic differentiation and increasing bone formation. A joint stimulation with VD3 and strain can decrease proliferation and osteogenic differentiation and increase RANKL expression, which might affect bone remodeling. This study supplies some new data, which might be important in theoretical and clinical research of osteoporosis (OP) and other related bone diseases.

Key words: 1, 25-$(OH)_2$-vitamin D3; Mechanical strain; Cell differentiation; Cell proliferation; Osteogenesis

中图分类号: R 318.01 **文献标志码**: A

DOI: 10.3871/j.1004-7220.2015.01.074.

收稿日期:2014-01-05; **修回日期**:2014-02-26

基金项目:国家自然科学基金资助项目(11302262, 31370942), 武警后勤学院创新团队基金(WHTD201307-1)。

通信作者:张西正, 教授, 博士研究生导师, E-mail: zxz84656717@163.com。

* 共同第一作者

活性维生素 D3 联合力学拉伸对成骨细胞 MC3T3-E1 增殖、分化及 OPG 和 RANKL 表达的影响

刘　璐[1]，张新昌[1*]，张西正[2]，郭　勇[2]，李瑞欣[2]
（1. 武警后勤学院，天津 300309；2. 军事医学科学院 卫生装备研究所，天津 300161）

摘要：目的　体外模拟成骨细胞在体内的生存环境，考察活性维生素 D3（VD3）、力学拉伸以及两者联合对成骨细胞 MC3T3-E1 增殖、分化及破骨细胞抑制因子（OPG）和破骨细胞分化因子（RANKL）表达的影响。**方法**　将 10 nmol/L VD3、间断性力学拉伸以及两者联合作用于成骨细胞。流式细胞术检测细胞增殖。荧光探针试剂盒检测碱性磷酸酶（ALP）活性。实时定量 PCR 检测核心转录因子 Runx2、OPG、RANKL mRNA 水平，Western blotting 检测其蛋白表达。**结果**　VD3 抑制成骨细胞增殖，力学拉伸不能改变这种抑制效应。力学拉伸和 VD3 单独作用成骨细胞均能增加 ALP 活性及提高 ALP、Runx2 mRNA 水平，但当联合刺激后这些指标均降低，且成强度依赖性。力学拉伸增加 OPG/RANKL 比值，增强成骨作用，联合 VD3 后，OPG/RANKL 比值下降。**结论**　力学拉伸能有效诱导成骨分化，增加骨形成。VD3 与力学拉伸联合抑制成骨细胞增殖和分化，并通过增加 RANKL 表达而影响骨重建。研究结果为骨质疏松及相关骨疾病的理论和治疗提供有意义的探索。

关键词：活性维生素 D3；力学拉伸；细胞分化；细胞增殖；成骨

Bone remodeling comprises the coordinated processes of bone resorption and formation where old bone is replaced by new bone[1]. Inappropriate balance between osteoblasts (OBs) and osteoclasts (OCs) is one of the determinants of the development of osteoporosis (OP), a reduction in skeletal mass due to an imbalance between bone resorption and bone formation[2]. 1,25-$(OH)_2$-vitamin D3 (VD3) regulates bone remodeling and balances interactions between OBs and OCs. It functions through vitamin D receptor (VDR), inducing the expression of various calcium-binding and transport proteins in the intestine to stimulate active calcium uptake, thus preserving normocalcemia and, indirectly, maintaining bone mineralization[3]. VD3 also acts directly on OBs and OCs, by inhibiting the proliferation of these two cell populations and modulating differentiation. It affects osteocalcin[4], alkaline phosphatase (ALP), and collagen 1 (COL 1) levels[5] and takes part in the regulation of extracellular matrix mineralization. Osteoclastic differentiation and maturation are important stages in the process of bone resorption, directly associated with interactions of RANKL, produced by OBs, and the receptor activator of NF-κB (RANK), produced by osteoclast precursor cells. OBs secrete the osteoprotegerin (OPG) to block this interaction. Tsukii *et al*[6] proposed that VD3 might enhance osteoblastic expression of RANKL.

Mechanical loading also stimulates bone formation and remodeling. Many experiments have shown that mechanical forces are crucial in the regulation of osteoblastic proliferation, differentiation, and apoptosis[7-8]. In our previous work, we found that mechanical strain at the physiological level (1 000, 2 500 με) directly up-regulated the expression of osteogenic marker mRNA and was involved in the regulation of OPG and RANKL expression[9]. However, few studies have focused on the regulation mechanism of mechanical strain combined with VD3. Body environment is a complex system. Cell functions are regulated not only by biochemical factors, like cytokines, but also by physical factors, such as tensile stress and shear stress. These stresses affect cell differentiation and maturation and biological functions of OBs and OCs. Therefore, the aim of this study is to investigate the proliferation and differentiation of OBs and the secretion of OPG and RANKL induced by mechanical strain combined with VD3.

1　Materials and Methods

1.1　Materials

VD3 was purchased from Sigma. Anti-OPG, anti-RANKL and anti-RUNX2 antibody were purchased from Boster Biology (Wuhan, China). ALP fluorometric detection kit was obtained from Nanjing Jiancheng Biotechnology

Co. Ltd (Nanjing, China). The pre-osteoblast cell line MC3T3-E1 was provided from Institute of Basic Medicine of Peking Union Medical College.

1.2 Cell culture and application of mechanical strain

The pre-osteoblast cell line MC3T3-E1 was cultured in culture flasks with α-MEM (Invitrogen, USA) supplemented with 10% FBS and antibiotic-antimycotic solution at 37 ℃ under a humidified atmosphere of 95% air/5% CO_2. When reached 80% confluence, cells were seeded at the density of $10^4/cm^2$ in the cell culture dishes. After 24 h cultured, cells were treated with 10 nmol/LVD3 for 72 h. 2 hours after VD3 was added, cells were subjected to intermitted mechanical strain of 1 000 and 2 500 με at 0.5 Hz for 1 h once a day or without mechanical strain. Uniaxial and homogeneous mechanical strain was generated by a specially designed 4-point bending device described previously[10]. Control groups were simultaneously subjected to mechanical strain of 1 000 με and 2 500 με or without mechanical strain. The cells were cultured for 72 h and processed for flow cytometry, ALP assay, real-time RT-PCR and Western blotting analysis.

1.3 Flow cytometry

Proportions of cells undergoing proliferation were determined by flow cytometry. Briefly, cells was harvest, washed 3 times with PBS, and collected by centrifugation before fixed by 75% cool ethanol and kept for 4 ℃. Before detection, samples were washed 2 times with PBS so as to remove ethanol, then, were stained with 10 pg/mL propidium iodide (PI) for 30 min. DNA content was detected and cell cycle was analyzed by EPICS flow cytometer (Coulter, USA). FCM-DNA quantitative analysis divided diploid DNA content distribution into three parts, namely, the G_0/G_1, S, and G_2/M phases. Scattered cells above the ploidy-establishing peak and in the S and G_2/M range represented the proliferation index (PI) and were counted as the percentage of the total number of OBs. PI of each samples were calculated as follows to represent the proliferation of cell populations.

$$\mathrm{PI} = \frac{S + G_2/M}{G_0/G_1 + S + G_2/M} \times 100\%$$

1.4 ALP activity assay

At the end of 72 h, cells were lysed in 200 μL/dish of lysis buffer (10 mmol/L Tris pH 8.0, 1 mmol/L $MgCl_2$, 0.5% Triton X-100), sonicated, and centrifuged to remove the cell debris. ALP activity in the cellular fraction was measured by a fluorometric detection kit. ALP activity of each sample was normalized by protein concentration.

1.5 RNA isolation and real-time RT-PCR

Cells were collected and total RNA was extracted with Trizol (Invitrogen, USA), and integrity of the extracted RNA was verified by denaturing agarose gel electrophoresis. The concentration of total RNA was determined by the Quant-iT RNA assay kit (Invitrogen, USA). Reverse transcription was performed with 1 μg of RNA in a total volume of 20 μL per reaction using Rever Tra Plus (TOYOBO, China). Quantitative RT-PCR was performed to determine mRNA levels of ALP, Runx2, OPG and RANKL using a pair of primers specific to these genes (Tab.1) using 7700 real-time PCR System (ABI, USA) with Brilliant SYBR Green master mix. The amplification reaction included 3 steps: (1) incubation at 94 ℃ for 3 min; (2) incubation at 94 ℃ for 15 s; and (3) annealing and extension at each annealing temperature for 30 s. The step (2) and (3) were repeated for 35 cycles. The fold change wascalculated using the control sample Ct values at each specified time point as a calibrator by means of $2^{-\triangle\triangle CT}$ method. Three independent experiments were carried out to determine relative mRNA levels.

Tab.1 Oligonucleotides used in real-time RT-PCR

Gene	Primer sequence (5'-3')	Products size/(bit · s^{-1})	Annealing temperature/℃
ALP (NM 007431.2)	F:CGGGACTGGTACTCGGATAA R:ATTCCACGTCGGTTCTGTTC	157	58
Runx2 (NM 001145920.1)	F: AGTAGCCAGGTTCAACGAT R:GGAGGATTTGTGAAGACTGTT	90	58
OPG (NM 008764.3)	F:AGTCTGAGGAAGACCATGAG R:AAACAGCCCAGTGACCATTC	205	56
RANKL (NM 011613.3)	F:CCAAGATGGCTTCTATTACC R:TCCCTCCTTTCATCAGGTTAT	152	53

1.6 Western blotting

Cells were solubilized in a modified RIPA buffer. Approximately 30 μg proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine Runx2, OPG and RANKL. Subsequently, the proteins were transferred to nitrocellulose membranes. The blots were blocked by incubation in 5% milk with TBS-T for 1 h and probed overnight at 4 ℃ with rabbit anti-Runx2, rabbit anti-OPG and rabbit anti-RANKL, respectively. After washing, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase. The immunoreactive bands were visualized using enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, USA). Optical density of the protein bands was determined with Gel Doc 2000 (Bio-Rad, USA). The expression of GAPDH was used as a loading control and data were normalized against those of corresponding GAPDH. Results were expressed as relative to control.

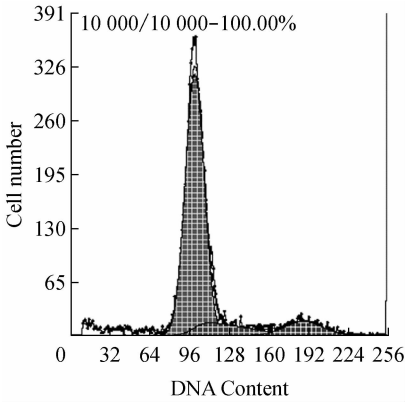
1.7 Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were presented as mean ± SD and analyzed with one-way ANOVA to determine significance between groups. Statistical analysis was performed using SPSS 13.0 software. $P < 0.05$ was considered statistically significant.

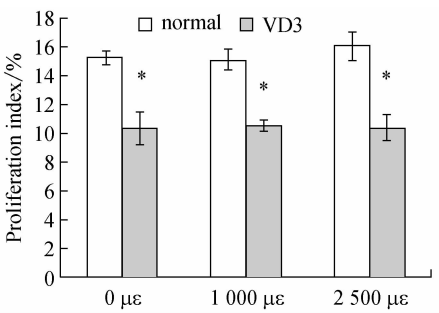
2 Results

2.1 Mechanical strain slightly increased cell proliferation, but decreased it when combined with VD3

M7C3T3-E1 cells were subjected to intermittent mechanical strain, VD3 treatment or combination of VD3 and strain for 72 h. Flow cytometry was used to examine cell cycle [Fig. 1(a)] and the effects of mechanical strain, with or without VD3, on cellular proliferation were evaluated. As shown in Fig. 1(b), mechanical strain alone slightly increased PI values. VD3 (10 nmol/L) alone depressed the proliferation of MC3T3-E1 cells. Combination of these two factors inhibited OB cell proliferation, regardless of the size of mechanical strain applied.



(a) Cell cycles of the control group



(b) PI values

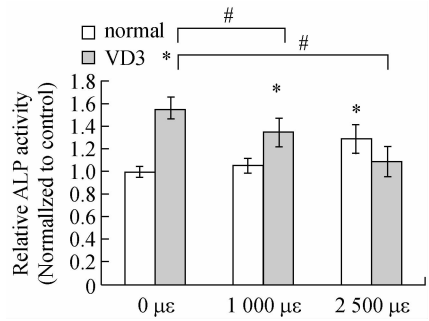
Fig. 1 Effect of mechanical strain on cell proliferation with or without VD3 (* $P < 0.05$ versus control)

2.2 Mechanical strain increased ALP activity and mRNA expression, but decreased them when combined with VD3

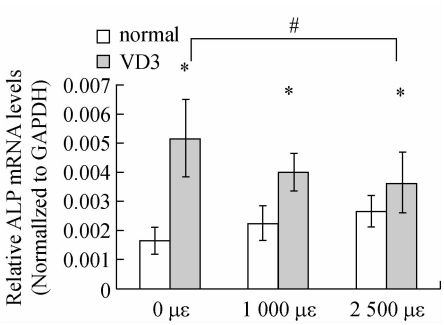
ALP is an early differentiation marker for OBs. Fig. 2(a) showed the effect of strain, VD3, and the combination of VD3 and strain on ALP activity. Mechanical strain alone at 2 500 με promoted ALP activity [Fig. 2(a), $P < 0.05$]. VD3 alone enhanced ALP activity significantly, to 1.5 times of the control value (normal cells with 0 με strain, no VD3). When VD3 was combined with strain, ALP activity was decreased, and the extent of inhibition was inversely related to strain intensity. ALP mRNA expression showed the same trend as ALP activity [Fig. 2(b)]. VD3 alone increased ALP mRNA expression in comparison with control, but decreased it when combined with mechanical strain.

2.3 Mechanical strain increased Runx2 protein expression, but decreased it when combined with VD3

Runx2 is an essential mediator of osteoblast phenotype



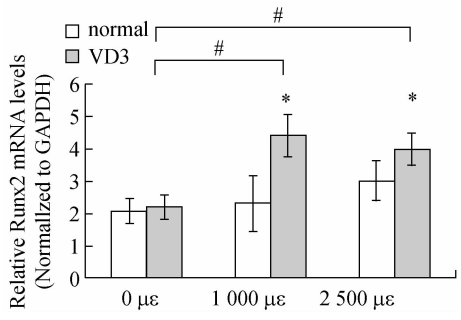
(a) ALP activity



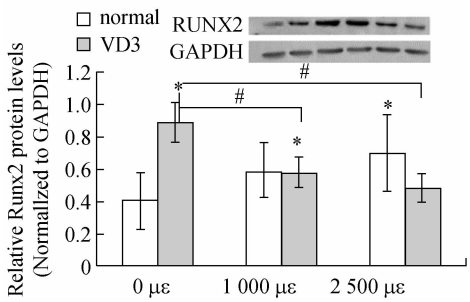
(b) ALP mRNA expression

Fig. 2 The effect of mechanical strain, with or without VD3, on ALP activity and mRNA expression

($^{*}P<0.05$ versus control, $^{\#}P<0.05$ represents a significant difference between groups)



(a) Gene expression



(b) Protein expression

Fig. 3 The effect of VD3, mechanical strain, and combined treatment on gene expression and protein expression of Runx2

($^{*}P<0.05$ versus control, $^{\#}P<0.05$ represents a significant difference between groups)

and plays a pivotal role in the process of osteoblastic differentiation[11]. Fig. 3(a) shows the effects of VD3 and mechanical strain on Runx2 expression. Mechanical strain slightly decreased Runx2 mRNA expression. When the strain was combined with VD3, Runx2 expression was up-regulated in a strain magnitude-dependent manner [Fig. 3(a), $P<0.05$]. In contrast, mechanical strain increased Runx2 protein expression, with a significant increase at 2 500 με [Fig. 3(b), $P<0.05$]. VD3 alone significantly promoted Runx2 protein expression ($P<0.05$), but showed a strain magnitude-dependent decrease when combined with strain. The regulation pattern of Runx2 protein expression resembled the pattern of ALP activity.

2.4 Mechanical strain increased OPG/RANKL ratio at both mRNA level and protein level, but decreased it when combined with VD3

Mechanical strain significantly increased OPG mRNA expression [Fig. 4(a), $P<0.05$]; the expression was enhanced 4-fold under 2 500 με strain in comparison with the control value (normal cells with 0 με strain, no VD3). However, VD3 had little effect on OPG expression at mRNA level. When VD3 was combined with strain, a slight increase was observed at mRNA level. In contrast, mechanical strain had no effect on RANKL mRNA expression [Fig. 4(b)]. VD3 significantly increased RANKL expression, and the effect strengthened when VD3 was combined with strain ($P<0.05$). The maximum RANKL expression was achieved under 2 500 με strain with VD3, which was 22 times higher than that in the control group. This demonstrated that a high-magnitude strain could promote RANKL expression induced by VD3. OPG/RANKL ratio is shown in Fig. 4(c). Mechanical strain increased the ratio due to the high expression of OPG; however, the combined VD3 and strain treatment decreased the ratio due to the high expression of RANKL. These results suggest that VD3 combined with mechanical strain might effectively modulate bone remodeling in favor of osteoclastogenesis and promote bone resorption.

OPG and RANKL protein expression profiles were consistent with the patterns of mRNA expression. Mechanical strain increased OPG expression in a magnitude-depended manner [Fig. 4(d)] but had no effect on RANKL expression [Fig. 4(e), $P < 0.05$], thus raising OPG/RANKL ratio [Fig. 4(c)]. VD3 significantly inhibited OPG expression but had no effect on RANKL expression. When combined with mechanical strain, VD3 significantly inhibited OPG expression [Fig. 4(f), $P < 0.05$] and significantly promoted RANKL expression ($P < 0.05$). As a result, OPG/RANKL ratio decreased under combined treatment conditions.

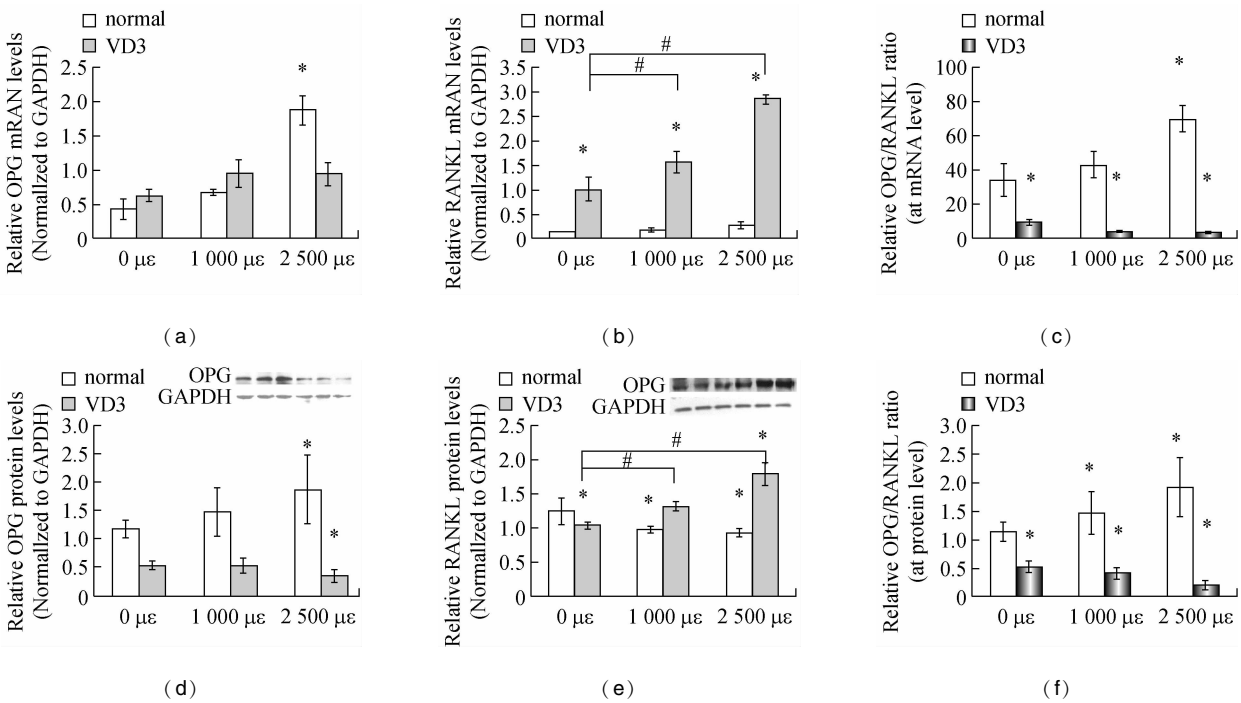


Fig. 4 The effect of VD3, mechanical strain, and combined treatment on gene and protein expression of OPG, RANKL, and the OPG/RANKL ratio (a), (b) Gene expression, (d), (e) Protein expression, (c), (f) OPG/RANKL ratio of mRNA and protein expression ($^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ represents a significant difference between groups)

3 Discussion

OBs are the most important cells involved in bone formation, the process including proliferation, ECM maturation, mineralization, and apoptosis. All these stages require precise control to achieve normal bone formation[12]. A variety of cytokines, hormones, and physical factors affect OBs and regulate bone cell function. VD3 plays an important role in the regulation of bone formation and development[13]. In this study, VD3 (hormonal factor) was combined with mechanical strain (physical factor) to investigate the regulation of bone remodeling, and the results showed that both VD3 and mechanical strain were involved in bone remodeling by inhibiting osteoblastic proliferation and differentiation, and increasing RANKL expression.

VD3 is one of the most important hormones involved in the maintenance of calcium and phosphorus balance[14]. Some studies have shown that vitamin D deficiency can seriously affect the osteoblastic differentiation and proliferation, resulting in infant skeletal dysplasia[15]. ALP activity and gene expression might be early markers of osteoblastic differentiation. Our results confirmed that the osteogenic differentiation could be substantially promoted by VD3 and mechanical strain, which was consistent with several existing reports[16-17]. However, when VD3 was combined with mechanical strain, ALP activity and its mRNA expression were significantly down-regulated. We also examined Runx2 expression to confirm the inhibition caused by the

combined treatment with mechanical strain and VD3. Runx2 is the most critical transcription factor that regulates osteoblastic differentiation and bone formation *in vitro* and *in vivo*[18]. It binds to the osteoblast-specific cis-acting element 2 (OSE2), which was found in the promoter regions of all the major osteoblast-specific genes (encoding osteocalcin, type I collagen, bone sialoprotein, osteopontin, ALP, and collagenase-3) and controls their expression[19]. Our study confirmed that mechanical strain and VD3 increased Runx2 protein expression, and the combined VD3 and strain treatment decreased its expression. These results were consistent with the pattern of ALP activity and ALP mRNA expression. It is well known that the changes in VDR expression affect the biological function of VD3. Cross-talk of Runx2 and VD3 at bone-specific promoters may represent an important component of the mechanisms mediating tissue-specific expression of osteoblast phenotypic genes[20]. Recent *in vitro* studies have shown that the deletion of VDR gene enhances osteoblastic differentiation, which results in an increase in ALP activity, mineralized matrix formation, and bone sialoprotein expression, indicating that VDR plays a negative role in osteoblastic differentiation *in vitro* and suggesting that other factors might participate in the modulation of osteoblastic differentiation *in vivo*[21]. However, the profile of Runx2 mRNA expression obtained in this study was not consistent with its protein expression pattern. It is possible that the level of this gene product is controlled, via negative feedback, by its own abundance. There are at least three self-binding sites in the 5′-regulatory sequence of this gene; it is possible that overexpression of Runx2 protein inhibits its gene expression[22].

RANKL controls the differentiation, maturation, and activation of OCs. OPG acts as a non-signaling decoy receptor, binds RANKL, and prevents activation of RANK, resulting in decreased osteoclast recruitment. Osteotropic factors, such as parathyroid hormone, VD3, or prostaglandin, increase the ratio between RANKL and OPG in favor of RANKL and can support osteoclastogenesis, whereas other factors such as estrogens can inhibit osteoclast recruitment by changing the OPG /RANKL ratio in favor of OPG[23]. Our study showed that VD3 changed the ratio between OPG and RANKL in favor of RANKL. Moreover, we found that mechanical strain changed the ratio in favor of OPG. When the two regulators co-operated, RANKL expression (both at the mRNA and protein level) was upregulated while OPG expression decreased; thus the ratio declined sharply. This result suggested that VD3 combined with mechanical strain could increase osteoclastogenesis and bone remodeling. Some studies have found that RANKL gene, which has a functional vitamin D response element VDRE in its promoter region, is one of the target genes of VD3/VDR[24]. The significant RANKL upregulation during VD3 treatment could be attributed to the VD3/VDR-mediated promotion of RANKL mRNA transcription, which might be augmented by the VD3- induced increase of VDR expression[25]. Horwood *et al*[26] has proposed that VD3 might enhance the RANKL/OPG expression ratio in OBs, and promote bone resorption, via three different routes: VDR pathway, protein kinase A pathway, or gp130 pathway. The changes in OPG and RANKL expression in response to joint stimulation with VD3 and strain were more complex than the responses to each of those factors administered separately. The exact mechanisms underlying the effect of drugs and mechanical strain still need to be determined.

In clinical practice, the usual treatment for OP is taking calcium and vitamin D and promoting physical exercise. Harter *et al*[27] has found that human osteoblast-like cells respond to mechanical strain in the presence of VD3 with increased bone-matrix protein production. However, our results do not support these findings. Mechanical loads have an important effect on the bone, but differences in direction, frequency, magnitude, and duration of mechanical stimulation may produce different effects. To make meaningful comparisons of the results, the sensitive mechanical parameters that trigger osteoblastic response *in vitro* results should be comparable to the *in vivo* mechanical strain conditions. Fritton *et al*[28] have comprehensively reviewed peak bone strain and strain rates measured during various activities in numerous species, including humans. For most animals, peak functional strains range from less than 1 000 με during walking to between 2 000 ~ 3 200 με for more vigorous activities, and can almost reach 5 000 με in galloping racehorses. These values reflect the deformation that

the bone normally experiences. They have been helpful in defining the parameters within which *in vitro* experiments should be designed to characterize the cellular mechanisms that initiate an appropriate loading-related adaptive response. Yan *et al*[29] has found that strains above the physiological level (5 000 με) are disadvantageous in bone formation.

Hormones and mechanical strain are two important regulators in bone remodeling. The effects of these two kinds of stimuli might be positive or negative, and no one factor can be universal. VD3 can cause different adjustments in bone tissue[30]. Excessive intake of VD3 can inhibit bone formation, so can mechanical loading. However, when combined, these two factors can trigger a different set of changes depending on the intensity of mechanical strain and the levels of the hormone. Rehabilitation exercises exert a low intensity mechanical stimulation that might be helpful in senile OP. When such stimulation is administered together with an appropriate dose of the hormone, the combined effects might restore homeostasis in the bone microenvironment. Further research in this field should lead to improved understanding of these complex processes.

References:

[1] Parfitt M. Bone resorption is not driven by formation in adult skeleton [J]. J Bone Miner Res, 2010, 25(3): 681.

[2] Iacovino JR. Mortality outcomes after osteoporotic fractures in men and women [J]. J Insur Med, 2001, 33(4): 316-320.

[3] Darias MJ, Mazurais D, Koumoundouros G, *et al*. Overview of vitamin D and C requirements in fish and their influence on the skeletal system [J]. Aquaculture, 2011, 315(1-2): 49-60.

[4] Norman AW, Nemere I, Zhou LX, *et al*. $1,25(OH)_2$-vitamin D3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways [J]. J Steroid Biochem Mol Biol, 1992, 41(3-8): 231-240.

[5] Maehata Y, Takamizawa S, Ozawa S, *et al*. Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: Roles of osterix, an osteoblast-related transcription factor [J]. Matrix Biol, 2006, 25(1): 47-58.

[6] Tsukii K, Shima N, Mochizuki S, *et al*. Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha, 25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone [J]. Biochem Bioph Res Co, 1998, 246(2): 337-341.

[7] Katsumi A, Orr AW, Tzima E, *et al*. Integrins in mechanotransduction [J]. J Biol Chem, 2004, 279(13): 12001-12004.

[8] Akisaka T, Yoshida H, Inoue S, *et al*. Organization of cytoskeletal F-actin, G-actin, and gelsolin in the adhesion structures in cultured osteoclast [J]. J Bone Miner Res, 2001, 16(7): 1248-1255.

[9] Wang L, Zhang X, Guo Y, *et al*. Involvement of BMPs/Smad signaling pathway in mechanical respinse in osteoblasts [J]. Cell Physiol Biochem, 2010, 26(6): 1093-1102.

[10] Tang LL, Wang YL, Pan J, *et al*. The effect of step-wise increased stretching on rat calvarial osteoblast collagen production [J]. J Biomech, 2004, 37(1): 157-161.

[11] Katerina KP, Demetrios NK, Kostas AP, *et al*. Mechanotransduction in osteoblast [J]. Trends Mol Med, 2009, 15(5): 208-216.

[12] Siggelkow H, Rebenstorff K, Kurre W, *et al*. Development of the osteoblast phenotype in primary human osteoblasts in culture: Comparison with rat calvarial cells in osteoblast differentiation [J]. J Cell Biochem, 1999, 75(1): 22-35.

[13] Bortman P, Folgueira MA, Katayama ML, *et al*. Antiproliferative effects of 1, 25-dihydroxyvitamin D3 on breast cells [J]. Braz J Med Biol Res, 2002, 35(1): 1-9.

[14] Dusso AS, Brown AJ, Slatopolsky E. Vitamin D [J]. Am J Physiol Renal Physiol, 2005, 289(1): F8-28.

[15] Santo L, Marisa G, Giorgio G, *et al*. The human cytomegalovirus [J]. Pharm acol Ther, 2003, 98(3): 269-297.

[16] Kawase T, Oguro A. Granulocyte colony-stimulating factor synergistically augments 1, 25-dihydroxyvitamin D3-induced monocytic differentiation in murine bone marrow cell cultures [J]. Horm Metab Res, 2004, 36(7): 445-452.

[17] Wang L, Zhang X, Guo Y, *et al*. Involvement of BMPs/Smad signaling pathway in mechanical respinse in osteoblasts [J]. Cell Physiol Biochem, 2010, 26(6): 1093-1102.

[18] Wu M, Hesse E, Morvan F, *et al*. Zfp521 antagonizes Runx2, delays osteoblast differentiation in vitro, and promotes bone formation in vivo [J]. Bone, 2009, 44(4): 528-536.

[19] Ducy P, Zhang R, Geoffroy V, *et al*. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation [J]. Cell, 1997, 89(5): 747-754.

[20] Maehata Y, Takamizawa S, Ozawa S, *et al*. Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: roles of osterix, an osteoblast-related transcription factor [J]. Matrix Biol, 2006, 25(1): 47-58.

[21] Sooy K, Sabbagh Y, Demay MB. Osteoblasts lacking the vitamin D receptor display enhanced osteogenic potential in vitro [J]. J Cell Biochem, 2005, 94(1): 81-87.

[22] Drissi H, Luc Q, Shakoor IR, *et al*. Transcriptional autoregulation of the bone related CBFα1 / RUNX2 gene [J]. J Cell Physiol, 2000, 184(3): 341-350.

[23] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation [J]. Nature 2003, 423(6937): 337-342.

[24] Kitazawa S, Kajimoto K, Kondo T, *et al*. Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter [J]. J Cell Biochem 2003, 89(4): 771-777.

[25] Kitazawa R, Kitazawa S. Vitamin D3 augments osteoclastogenesis via vitamin D-responsive element of mouse RANKL gene promoter [J]. Biochem Biophys Res Commun, 2002, 290(2): 650-655.

[26] Horwood NJ, Elliott J, Martin TJ, *et al*. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells [J]. Endocrinology, 1998, 139(11): 4743-4746.

[27] Harter LV, Hruska KA, Duncan RL. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation [J]. Endocrinology, 1995, 136(2): 528-535.

[28] Fritton SP, Rubin CT. In vivo measurement of bone deformations using strain gauges [M]// Cowin SC. Bone mechanics handbook. USA: CRC Press, 2001: 8-10-34.

[29] Yan YX, Zhang XZ, Song M, *et al*. Effects of substrate-streching strain on mouse MC3T3-E1 cells in Biological responses and its mechanism [J]. J Med Biomech, 2009, 24(s1): 93-94.

[30] Liu WF, Li YY. Analysis on alendronate sodium combined with VD3 therapy of senile osteoporosis [J]. New Med, 2012, 43(4): 261-263.

·致读者·

关于图表的要求

表和图的设计应科学、简洁、合理,有自照性,均分别按其正文出现先后次序连续编号,并冠以图(表)序号和题目。说明性的资料应置于图(表)下方注释中,并在注释中标明图标中使用的全部非公知公用的缩写及表中的统计学处理。均采用三线表,表内数据同一指标有效位数一致,均数及标准差小数点后保留位数一致。图题、表题及图注、表注均应中、英文对照书写。

黑白图片必须清晰度及对比度良好,层次分明,彩色照片要求色彩鲜明,图像清晰。图片或照片大小要基本一致。图不宜过大,最大宽度半栏图不超过7.5 cm,通栏图不超过16.5 cm,高与宽比例以5 ∶ 7为宜。图注应放在图题之上及固定脚注之前,照片中需说明的部位请以箭头或字母标注,在图注中说明。图片及照片不得折损。若刊用人像,应征得本人书面同意,或遮盖其能辨认出系何人部分(眼睛)。大体标本照片在图内最好有尺度标记。病理照片要求注明染色方法和放大倍数。

试验数据的曲线图必须根据测试数据绘制,线条必须光滑清晰,有横纵坐标的统计图必须有确切的标目名称和规范的计量单位;实验装置等示意图一定要精心绘制,对图内的结构要有必要的注释,使读者一目了然;引用他人的图(表)一定要用文献角码表明出处。

本刊编辑部