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Effect of Excessive Vitamin A on Alkaline Phosphatase Activity and Concentrations of Calcium-binding Protein and Bone Gla-protein in Culture Medium and CaBP mRNA Expression in Osteoblasts of Broiler Chickens*

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ABSTRACT : This study was conducted to determine the effects of excess vitamin A on alkaline phosphatase (ALP) activity, contents of calcium-binding protein (CaBP), bone gla-protein (BGP) in culture medium and CaBP mRNA expression in chicken osteoblasts *in vitro*. Osteoblastic cells in the tibia from 1-day-old Arbor Acre broiler chickens were isolated using enzyme digestion. The subconfluenced cells were divided into eight treatments with six replicates in each treatment and cultured in a medium containing either vehicle or different levels of vitamin A (0, 0.2, 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml), and the control received an equivalent volume of ethanol. The incubation lasted 48 h. The results showed that vitamin A down-regulated ALP activity in the culture medium as well as CaBP mRNA expression of osteoblasts in a linear dose-dependent manner ($p = 0.124$ and $p<0.10$, respectively), and suppressed the contents of BGP and CaBP in the culture medium in a quadratic dose-dependent manner ($p<0.05$ and $p<0.10$, respectively) with increasing addition of vitamin A. The addition of 0-0.2 μg/ml vitamin A to the culture medium increased ALP activity, BGP and CaBP contents as well as CaBP mRNA expression compared with other groups, but positive effects of vitamin A tended to be suppressed when vitamin A was increased to 1.0 μg/ml, and adverse effects occurred when vitamin A was increased to 10.0-20.0 μg/ml. These results implied that there was a threshold level of vitamin A inclusion beyond which inhibitory effects occurred, and the mechanism by which overdose of vitamin A reduced bone growth in chickens was probably reduced osteoblastic cell activity, and inhibited expression of CaBP mRNA and CaBP secretion. (**Key Words :** Vitamin A, Broiler Chicken, Calcium-binding Protein, mRNA Expression, Osteoblast, Bone Gla-protein)

INTRODUCTION

It is well known that vitamin A plays very important roles in normal skeletal development and growth of animals and humans. However, overdose of vitamin A is hazardous for the body. Accumulated evidence suggested that excess vitamin A was associated with reduced bone mineral density and increased risk of hip fracture in humans and rats (Hough et al., 1988; Melhus et al., 1998; Whiting and Lemke, 1999). Studies in broiler chickens demonstrated that a diet containing excessive vitamin A (15,000-45,000 IU/kg) could result in reductions in growth, contents of calcium and ash in bone and bone mineral density (BMD) (Yan et al., 2007; Chen et al., 2008). There is, however, very little data that examines the mechanisms，especially molecular mechanisms, by which high levels of vitamin A affect bone development of broiler chickens. It was reported that calcium-binding protein (CaBP) was responsible for binding free Ca^{2+} (Linse et al., 1997) and acting as a cytosolic Ca^{2+} buffer in many tissues, resulting in modulation of Ca^{2+} adsorption (Thomasset et al., 1990). Our previous study measured the *in vivo* effect of excessive dietary vitamin A on CaBP mRNA expression in the tibia of broiler chickens and indicated that vitamin A decreased the expression of CaBP mRNA in tibia in a linear dose-dependent manner (Feng et al., 2007). It was also reported that CaBP was present in cartilage and bone, and was distributed in osteoblasts in bone (Balmain, 1991). This implied that reduction of BMD in broiler chickens by excessive vitamin A was probably associated with

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decreased expression of CaBP mRNA in osteoblasts. However, *in vitro* the action of vitamin A on osteoblasts of broiler chickens is less clear. Rohde and DeLuca (2003) indicated that vitamin A acted directly on bone cells and up-regulated the expression of several proteins in osteoblasts and osteoclasts, including matrix proteins and lysosomal enzymes that might play a direct role in bone resorption. Alkaline phosphatase (ALP) is produced by bone-forming cells called osteoblasts, and is one of the most frequently used biochemical markers of osteoblast activity (Magnusson et al., 1999), and bone gla-protein (BGP) is also produced by mature osteoblasts and primarily deposited in the extracellular matrix of skeletal tissue (Wada and Kamiya, 2006), and both ALP and BGP are related to activity of osteoblastic cells. In this study, we examined the effects of vitamin A on ALP activity and contents of BGP and CaBP in culture medium, as well as CaBP mRNA expression in broiler osteoblastic cells *in vitro*, to explore the probable mechanism by which excess vitamin A affects bone growth.

MATERIALS AND METHODS

Cell isolation and culture

Osteoblastic cells in tibia from 1-day-old Arbor Acre broiler chickens were isolated according to a modified procedure outlined elsewhere (Wang et al., 2001). Briefly, the bone specimens were minced into small pieces about $1mm^3$ in size after soft connective tissues and peri-osteal and cortical bone were carefully removed. The bone fragments were thoroughly and repeatedly rinsed and vortexed three times in sterile phosphate buffered solution (PBS) with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) to remove adherent marrow cells and to expose the trabecular surfaces of the bone specimen. The bone fragments were digested with 5 ml TE (0.25% Trypsin (Gibco)+0.02% EDTA (Sigma)) at 37°C for 20 min in a flask. The small bone chips were then placed into another flask containing 5 ml 0.1% II-type collagenase, and digested for 60 min at 37°C and rocked every 5 min. Digestion was concluded with 6ml medium composed of DMEM/F-12 (Gibco), 10% fetal bovine serum (FBS)(TBD, Inc. Tianjin, China) and 100 U/ml penicillin and 100 μg/ml streptomycin. The flask was thoroughly rocked and stood for 5 min. The digested liquid containing cells was centrifuged at 1,500 r/min for 5 min in a 1.5 ml Eppendorf centrifuge tube, the supernatant liquid was removed, the precipitate was rinsed with PBS and rocked and centrifuged at 1,500 r/min for 5 min. The above process was repeated three times. Cells obtained were plated in a flask containing DMEM/F-12 medium supplemented with 20% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and inoculated at 37°C in a humidified (95%) atmosphere with 5% CO_2. After 24 h the medium was replaced to remove non-adhering cells and debris. Then the adherent cells were cultured until confluence, and the medium was replaced every other day.

Experimental design

The experiment was divided into 8 treatments with 6 replicates in each treatment. Vitamin A acetate (2.8×10^6 IU/g, Sigma) was dissolved in absolute alcohol at a stock concentration of 0.2 μg/μl and maintained at -20°C. The confluenced cells were trypsinized, counted, pooled, re-suspended in DMEM/F12 medium containing 20% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin, and plated at a density of 2×10^5 cells/well in 24-well plates. The medium was changed every 2 days. The cells were cultured for 72 h, and the cells with sub-confluency were changed to a medium containing either vehicle or different levels of vitamin A. The stock solutions of vitamin A acetate in absolute ethanol were added to the culture medium at the final concentrations (0, 0.2, 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml), and the control cultures did not receive vitamin A (0 μg/ml) but an equivalent volume of ethanol. The incubation lasted 48 h. The osteoblastic cells obtained were morphologically observed with an inverted microscope. The culture medium was collected for the assay of ALP activity, BGP and CaBP contents, and the cell layers were rinsed 3 times with PBS for assay of CaBP mRNA expression.

Measurement of ALP activity and CaBP and BGP in culture medium

ALP activity in the cell medium was analysed using ALP kits (Biosino bio-technology and science INC) according to the manufacturer's instructions. The instrument used was an Alcyan 300 automatic biochemistry analyzer (USA). CaBP concentration was determined using a CaBP kit (Sigma) and BGP content was analysed using a BGP kit (Beijing Huaying bio-technology Inc) by radioimimunoassay (RIA) according to the manufacturer's instructions.

Extraction of total RNA of osteoblasts and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from the above cell layers rinsed 3 times with PBS using RNAfast200 (FASTAGEN, Inc. Shanghai, China) according to manufacturer's protocol. Then 500 ng of total RNA was reverse-transcribed into First-Strand complementary DNA (cDNA) using an ExScript™ RT reagent Kit (TaKaRa, Inc. Dalian, China). The RT reaction mixture (10 μl) contained 2 μl 5×ExScript™ RTase Buffer, 0.5 μl dNTP Mixture (10 mmol/L), 0.5 μl Random 6 mers (100 μmol/L), 0.25 μl

ExScript™ RTase (200 U/μl), 0.25 μl RNase Inhibitor (40 U/μl) and 6.5 μl Total RNA. The RT products (cDNA) were stored at -20°C pending quantitative PCR assay.

Real-time RT-PCR assay for CaBP mRNA expression

Relative levels of CaBP mRNA were quantified using SYBR® PrimeScriptTM RT-PCR Kit (TaKaRa, Inc. Dalian, China) following the manufacturer's instructions and a light cycler real-time DNA amplification system (MJ research Opticon-2, USA) according to optimized PCR protocols. Reactions were also performed with negative controls (water replacing cDNA). The primers used were as follows: β-Actin (337 bp, GenBank accession no. NM_205518) 5'- CCC AAG GCC AAC CGT GAG AAG AGT - 3' (forward) and 5'- CGA AGT CCA GGG CCA CGT AGC AGA G -3' (reverse); CaBP-D_{28K} (298 bp, GenBank accession no. M14230) 5'- TTA AAT CTG CGT TGC TTC CAT ACA - 3' (forward) 5'- GGC CCA TCC TGC ACT CCA TAA C -3' (reverse). The PCR reaction was performed in 2 μl cDNA template, 10 μl SYBR Premix Ex Taq™ (2×), 7 μl dH_2O and 1 μl each (10 μM) of forward and reverse specific primers in a final volume of 20 μl. The same dilution was used for both CaBP and β-Actin. The PCR reaction was done at 95°C for 1 min, 95°C for denaturation (5 sec), 57°C for annealing (30 sec), and 72°C for extension (6 sec) for 40 cycles. Detection of the fluorescent product was carried out at the end of melting curve program (70 to 95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement). The specific CaBP and β-Actin gene amplification of the PCR products was confirmed by 1.0% agarose gel electrophoresis stained with ethidium bromide, and bands were visualized by exposure to ultraviolet light. Sequences were confirmed by Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). The primer CaBP and β-actin amplified a product of 297 bp and 337 bp, respectively. CaBP gene amplified a product of 265 bp, and showed 95% (253/265) identity to CaBP-D_{28K}. Thereafter, PCR products were analyzed by generating a melting curve. The melting curve of a product is sequence-specific and can be used to distinguish non-specific from specific PCR products. The amplification efficiency curves were obtained by amplification of a dilution series of CaBP mRNA and β-actin mRNA. The melting curve after amplification showed a single melting peak, indicating a specific product. Relative expression levels of CaBP mRNA were calculated by $2^{-\Delta CT}$ method, where ΔCt = Ct CaBP-Ct β-actin (Schmittgen et al., 2000). Tube normalization was used to remove background fluorescence.

Statistical analysis

All data were subjected to the General Linear Model procedure of SAS software (SAS Version 8, SAS Institute, Cary, NC) and used to detect the statistical significance of the treatment groups. Regression analysis was conducted to evaluate linear and quadratic effects of vitamin A on the various response criteria. A level of $p<0.05$ was used as the criterion for statistical significance, while a level of 0.10 was taken to indicate a statistical trend.

RESULTS

Cell morphology

The osteoblastic cells were cultured in the presence of 0, 0.2, 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml of vitamin A for 48 h. The cells showed a spindle-shaped fibroblastic morphology or relatively polygonal morphology (Figure 1A-H). The results also showed that the cell number tended to be suppressed when the addition of vitamin A was increased to 2.0 μg/ml (Figure 1E); especially, the addition of 10.0-20.0 μg/ml was more effective (Figure 1G-H).

Effect of vitamin A on ALP activity in culture medium

Table 1 shows the dose-response effects of vitamin A on ALP activity in culture medium. The results indicated that ALP activity in culture medium treated with vitamin A tended to decrease linearly or quadratically with increasing addition of vitamin A from 0 to 20.0 μg/ml ($p = 0.124$, $p = 0.143$); the control and the 0.2 μg/ml vitamin A addition had higher ALP activity than other treatments, and treatments with vitamin A addition from 10 to 20 μg/ml to the culture medium had lower ALP activity.

Effect of vitamin A on BGP and CaBP concentrations in culture medium

As shown in Table 1, BGP content in the culture medium was depressed linearly or quadratically with increasing addition of vitamin A ($p<0.05$). CaBP content in the culture medium also tended to decrease quadratically with increasing addition of vitamin A ($p<0.10$). The culture medium with supplementation from 0 to 2.0 μg/ml vitamin A had higher BGP and CaBP contents than other treatments, and those with addition of 5.0 to 20.0 μg/ml vitamin A had lower BGP and CaBP contents.

Effect of vitamin A on CaBP mRNA expression of osteoblasts

As shown in Table 1, excessive vitamin A in the culture medium down-regulated CaBP mRNA expression of osteoblasts. Regression analysis revealed that the expression of CaBP mRNA tended to reduce ($p<0.10$) linearly with increasing addition of vitamin A. The control and treatment with addition of 0.2 μg/ml vitamin A to the culture medium had higher CaBP mRNA expression than

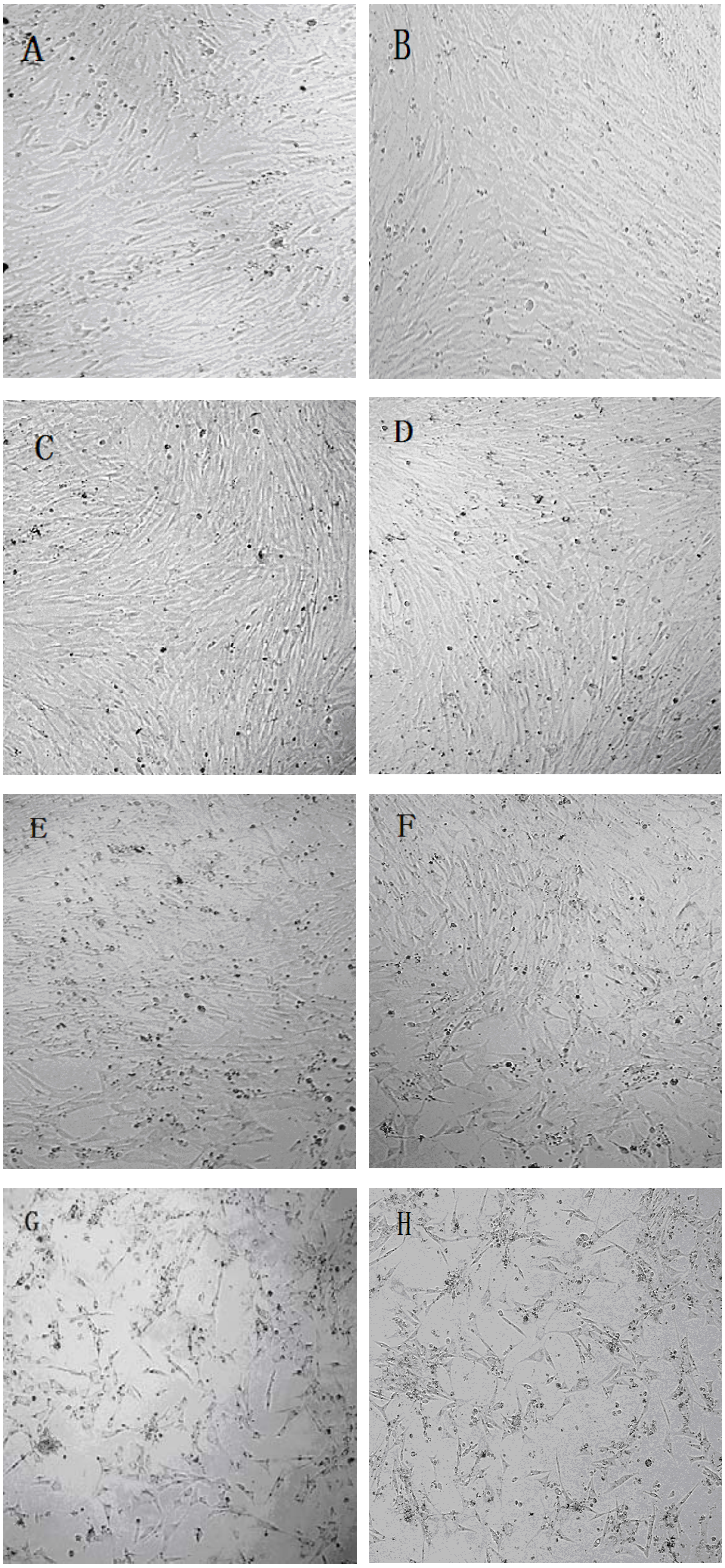

Figure 1. The cells were cultured in the presence of 0, 0.2, 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml of vitamin A for 48 h (×100), and showed a spindle-shaped fibroblastic or relatively polygonal morphology (A-H). The cell number tended to be suppressed when the addition of vitamin A was increased to 2.0 μg/ml(E), and especially the addition of vitamin A from 10 to 20.0 μg/ml was more effective (G-H).

Table 1. Effects of vitamin A levels on ALP activity and concentrations of BGP and CaBP in culture medium and CaBP mRNA expression of osteoblasts *in vitro*

Vitamin A in culture medium (μg/ml)	ALP activity (U/L)	BGP concentrations (μg/L)	CaBP concentrations (ng/ml)	CaBPmRNA expression ($2^{-\Delta Ct}$)
0	23.87	5.17	44.524	0.0070
0.2	23.33	5.40	52.625	0.0097
0.6	19.53	5.64	53.046	0.0028
1	20.62	5.51	48.165	0.0031
2	20.07	4.83	48.702	0.0029
5	19.53	4.72	42.297	0.0038
10	18.99	4.51	39.331	0.0005
20	18.99	4.62	44.901	0.0003
SEM	1.005	0.264	9.884	0.0034
Linear	0.124	0.047	0.194	0.072
Quadratic	0.143	0.027	0.079	0.136

ALP = Aalkaline phosphatase; CaBP = Calcium-binding protein; BGP = Bone gla-protein.

other treatments, and those with the addition of 10 to 20 μg/ml vitamin A had lower CaBP mRNA expression.

DISCUSSION

Alkaline phosphatase is one of the most frequently used biochemical markers of osteoblast activity and differentiation (Risteli and Risteli, 1993; Magnusson et al., 1999). The higher ALP activity is necessary for initiating mineralization, because ALP can decompose phosphoric acid of organic matter, increase inorganic phosphoric acid concentration and thereby enhance mineralization (Owen et al., 1990). Osteoblasts secrete ALP and calcium salt crystals into the extracellular matrix and up-regulate the concentration of phosphoric acid and then support mineralization. There is much evidence that vitamin A may alter ALP activity, and thereby relates to activity and differentiation of osteoblasts and bone growth. For example, retinol and carotenoids at physiological concentrations induced a significant amount of ALP activity, and thereby had a stimulatory effect on activity and differentiation of osteoblasts in the newborn mouse *in vitro* (Park et al., 1996). All- trans-retinoic acid (ATRA, 10^{-5}, 10^{-6}, and 10^{-7} M) could up-regulate proliferation of osteoblastic cells in the newborn rat after 72 h culture, and increase ALP activity *in vitro* (Zhi et al., 2001). By contrast, Wolke et al. (1969) indicated that excess vitamin A caused bone injury because large amounts of vitamin A inhibited osteoblastic cell activity. Anderson (2002) reported that retinoic acid (RA) suppressed osteoblastic cell activity and stimulated osteoclast cell formation. The probable reasons were that RA could be capable of inducing bone resorption in the rat (Fell et al., 1952; Raisz, 1965; Rohde et al., 2003). Few data have reported the effects of vitamin A on ALP activity of osteoblast cells in broiler chickens. The results of the present study showed that ALP activity in culture medium of osteoblast cells in broiler chickens was affected by vitamin A supplementation, and vitamin A down-regulated ALP activity in culture medium in a linear dose-dependent manner ($p = 0.124$) with increasing addition of vitamin A. The addition of 0-0.2 μg/ml vitamin A to culture medium produced higher ALP activity compared with other groups, but positive effects of vitamin A tended to be suppressed when the addition of vitamin A was increased to 0.6 μg/ml; especially, the addition of 10.0-20.0 μg/ml was more effective in reducing ALP activity. It would therefore appear that a low dose of vitamin A had a stimulatory effect on osteoblasts, and that overdose of vitamin A had adverse effects on osteoblast cell activity of broiler chickens, inhibited the proliferation and differentiation of chicken osteoblasts *in vitro* and was known to be a negative regulator of osteoblast activity.

BGP is produced and secreted by osteoblasts, is related to osteoblastic cells and plays an important role in the regulation of skeletal metabolism (Wada and Kamiya, 2006). It sustains the normal rate of bone mineralization, and suppresses the formation of abnormal hydroxyapatite crystals. Furthermore, BGP inhibits mineralization rate of growing cartilage, and is a marker of differentiation and maturation of osteoblasts (Wang, 2001). As a result, BGP was considered to be the important item to study the effects of many active factors and cell growth on osteoblastic activty and to investigate the influenced mechanisms using cell culture *in vitro*. There is however, very little data that examines the relationship between vitamin A and BGP content in chicken osteoblastic culture *in vitro*. In the present study, a dose-dependent relationship ($p<0.05$) was shown between supplemental dosage of vitamin A and BGP content in a culture medium of osteoblasts from broiler chickens. The addition of 0.2-0.6 μg/ml vitamin A to culture

medium produced higher BGP content compared with other groups, but positive effects tended to be suppressed when vitamin A was increased to 1.0 μg/ml, and, especially, the addition of 5.0-20.0 μg/ml vitamin A was more effective in decreasing BGP content. These results implied that excess vitamin A down-regulated the synthesis and secretion of BGP in osteoblasts of broiler chickens, and inhibited differentiation and mineralization of osteoblast cells, and influenced osteogenesis.

CaBP is the principal vehicle for active transport in the absorption process of calcium (Naveh-Many et al., 1990), directly participating in the Ca^{2+} transport process. Some evidence has suggested that tissue concentrations of CaBP-D_{28k}, which were higher in such tissues as intestinal tract, kidney and placenta, are closely related to calcium transport (Balm and Cai, 1991). Vitamin A has been known for many years to be of special importance for normal bone growth, differentiation and function (Mellanby, 1947). Balm et al. (1995) indicated that CaBP existed in osteoblasts of growing and embryo rats, and that osteoblast cells and extracellular matrix *in vitro* also contained CaBP. However, few data have been reported on the relationship between vitamin A and CaBP synthesis and CaBP mRNA expression in osteoblast cells. The present study investigated the effects of different levels of vitamin A on the CaBP content in culture medium and the expression of CaBP mRNA of osteoblasts *in vitro*. The findings suggested that the concentration of extracellular matrix CaBP or CaBP mRNA expression of osteoblasts had a significant tendency to be decreased quadratically or linearly, respectively, along with incremental addition of vitamin A dose ($p<0.10$). The supplementation of 0.2-0.6 μg/ml or 0-0.2 μg/ml vitamin A to culture medium resulted in higher CaBP content or CaBP mRNA expression, and CaBP content as well as CaBP mRNA expression was lower than in other treatments when vitamin A addition to culture medium was increased to 10.0-20.0 μg/ml. These findings suggested that a low dose of vitamin A enhanced expression of CaBP mRNA in osteoblasts, and improved CaBP concentration in culture medium, and that excess vitamin A to a certain extent restrained the expression of CaBP mRNA in osteoblasts, and led to a depression of CaBP concentration in culture medium. The causes of this result are unclear. Retinoic acid (RA) receptors (RARs), retinoid X-receptors (RXRs) and cellular retinol-binding protein I (CRBP-I) play important roles in facilitating trafficking and metabolism of vitamin A. Harada et al. (1995) indicated that the actions of vitamin A in bone were exerted through RARs and RXRs by regulating target gene (RAR and RXR) expression for vitamin A, and through CRBP-I by regulating target gene CRBP-I expression and modulating the intracellular transport of vitamin A. These results also indicated that mRNA levels of these genes in vitamin A-deficient rats decreased to half of those in normal rats and that excess RA given to normal rats doubled the mRNA levels of these genes. These results verified that bone was a target for vitamin A in terms of target gene expressions. Rohde and DeLuca (2003) indicated that an antagonistic interaction between vitamin A and vitamin D has been established for many years and that large amounts of vitamin A were shown to affect the ability of vitamin D to normalize bone mineral. Therefore, the actions of excess vitamin A on the expression of CaBP mRNA in osteoblasts are probably related to its effect on the expression of target genes for vitamin A (RAR, RXR and CRBP-I) and its antagonistic action toward vitamin D is related to CaBP. So more research is required to confirm and interpret the phenomenon. In addition, the present results for cell morphology showed that addition of vitamin A from 10 to 20.0 μg/ml to the culture caused decreased cell number. These results suggested that vitamin A influenced the osteoblast cell growth in a dose-dependent manner and that overdose of vitamin A inhibited the cell growth and then down-regulated the expression of CaBP mRNA of osteoblast cells. However, cell number was not estimated by cell counting in the present study, therefore more studies are needed.

Our previous study explained the mechanism by which excessive vitamin A decreased bone growth and development using an *in vivo* method and indicated that excess vitamin A reduced bone density, depressed ALP activity and contents of BGP and CaBP in serum of broiler chickens, and down-regulated the expression of CaBP mRNA in the tibia and duodenum of broilers (Feng et al., 2007; Yan et al., 2007). Our present study explained, in part, the mechanism by which excessive vitamin A decreased bone growth and development in *in vitro* culture, and suggested that overdose of vitamin A inhibited osteoblast cell activity and growth and then depressed the expression of CaBP mRNA of osteoblast cells.

In addition, our present study indicated that the control treatment had higher ALP activity, BGP and CaBP contents and CaBP mRNA expression, although the control did not contain extra vitamin A in the culture medium. The causes of this result were partly that the culture medium for the control contained 20% FBS containing about 0.08 μg/ml vitamin A. Thereby, the control culture medium contained about 0.016 μg/ml vitamin A. These results further suggested that a lower dose of vitamin A enhanced the osteoblast cell activity. However, the supplemental levels of vitamin A in culture medium were limited, and further work is needed.

IMPLICATIONS

Our results suggested that ALP activity, BGP and CaBP

concentrations in culture medium, as well as CaBP mRNA expression of osteoblasts, were affected in a linear or quadratic dose-dependent manner with increasing addition of vitamin A, and addition of 0 to 0.2 μg/ml vitamin A showed the best enhanced effect, while supplementing with 5.0 to 10 μg/ml vitamin A showed adverse effects. This implied that there was a threshold level of vitamin A inclusion beyond which inhibitory effects occurred, and the mechanism by which overdose of vitamin A reduced bone growth in chickens was probably that a large dose of vitamin A reduced osteoblast cell activity and inhibited expression of CaBP mRNA and CaBP secretion.

REFERENCES

Anderson, J. J. 2002. Over supplementation of vitamin A and osteoporotic fracture in the elderly: To supplement or not to supplement with vitamin A. J. Bone Miner. Res. 17(8):1359-1362.

Balmain, N. 1991. Calbindin-D9k. A vitamin-D-dependent, calcium-binding protein in mineralized tissues. Clin. Orthop. Relat. Res. 265:265-276.

Balm, N. and B. F. Cai. 1991. Calcium-binding protein: Calbindin-D9K. J. Foreign Medical Sciences: Section of Orthopaedics. 12(4):223-226.

Chen, Y. J., S. M. Yan, H. Y. Li, P. Cao and Z. X. Xu. 2008. Effect of vitamin A and vitamin E on growth performance and immune functions in broilers. Chinese Journal of Inner Mongolia Agricultural University 29(1):14-19.

Feng, Y. M., S. M. Yan, H. Y. Li and L. H. Fang. 2007. Effect of dietary vitamin A on CaBP-m RNA expressions in tissues of broilers. Chinese J. Animal Husbandry and Veterinary Medicine. 39:7-9.

Fell, H. B. and E. Mellanby. 1952. The effect of hypervitaminosis A on embryonic limb-bones cultivated *in vitro*. J. Physiol. 116:320-349.

Hough, S., L. V. Avioli, H. Muir, D. Gelderblom, G. Jenkins, H. Kurasi, E. Slatopolsky, M. A. Bergfeld and S. L. Teitelbaum. 1988. Effects of hypervitaminosis A on the bone and mineral metabolism of the rat. Endocrinology 122:2933-2939.

Harada, H., R. Miki, S. Masushige and S. Kato. 1995. Gene expression of retinoic acid receptors, retinoid-X receptors, and cellular retinol-binding protein I in bone and its regulation by vitamin A. Endocrinology 136:5329-5335.

Linse, S., E. Thulin, L. K. Gifford, D. Radzewsky, J. Hagan, R. R. Wilk and K. S. Akerfeldt. 1997. Domain organization of calbindin D28k as determined from the association of six synthetic EF-hand fragments. Protein Sci. 6(11):2385-2396.

Melhus, H., K. Michaelsson, A. Kindmark, R. Bergstrom, L. Holmberg, H. Mallmin, A. Wolk and S. Ljunghall. 1998. Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fracture. J. Ann. Intern Med. 129:770-778.

Magnusson, P., L. Larsson, M. Magnusson, M. W. J. Davie and C. A. Sharp. 1999. Isoforms of bone alkaline phosphatase: Characterization and origin in human trabecular and cortical bone. J. Bone Miner. Res. 14:1926-1933.

Mellanby, E. 1947. Vitamin A and bone growth: the reversibility of vitamin A deficiency changes. J. Physiol. 105:382-399.

Naveh-Many, T., R. Marx, E. Keshet, J. W. Pike and J. Silver. 1990. Regulation of 1,25-dihydroxyvitamin D_3 receptor gene expression by 1,25-dihydroxyvitamin D_3 in the parathyroid *in vivo*. J. Clin. Invest. 86(6):1968-1975.

Owen, T. A., M. Aronow, V. Shalhoub, L. M. Barone, L. Wilming, M. S. Tassinari, M. B. Kennedy, S. Pockwinse, J. B. Lian, G. S. Stein, A. Michael and S. Victoria. 1990. Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationship in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracelluar matrix. J. Cell Physiol. 143:420-430.

Park, C. K., Y. Ishimi, M. Ohmura, M. Yamaguchi and S. Ikegami. 1996. Vitamin A and carotenoids stimulate differentiation of mouse osteoblastic cells. J. Nutr. Sci. Vitaminol. (Tokyo). Jun; 43(3):281-296.

Rohde, C. M. and H. DeLuca. 2003. Bone resorption activity of all-trans retinoic acid is independent of vitamin D in rats. J. Nutr. 133:777-783.

Raisz, L. G. 1965. Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. J. Clin. Invest. 44:103-116.

Risteli, L. and J. Risteli. 1993. Biochemical markers of bone metabolism. Ann. Med. 25:385-393.

Schmittgen, T. D. and B. A. Zakrajsek. 2000. Effect of experimental treatment on house-keeping gene expression: validation by real-time, quantitative RT-PCR. Biochem. Biophys. Methods 46(1-2):69-81.

Thomasset, M., J. M. Dupret, A. Brehier and C. Perret. 1990. Calbindin-D9K (CaBP9K) gene: a model for studying the genomic actions of cacitriol and calcium in mammals. Adv. Exp. Med. Biol. 269:35-36.

Whiting, S. J. and B. Lemke. 1999. Excess retinol intake may explain the high incidence of osteoporosis in northern Europe. Nutr. Rev. 57(6):192-195.

Wada, S. and S. Kamiya. 2006. Bone and bone related biochemical examinations. Bone and collagen related metabolites. Osteocalcin(OC). Clin. Calcium 16(6):1017-1021.

Wang, H. F. 2001. Spectrum of a graph of osteocyte and the technique of osteocyt cultivation *in vitro*. M. Shanghai: shanghai scientific and technical publishers. 61-63.

Wolke, R. E., H. D. Eaton, S. W. Nielsen and C. F. Helmboldt. 1969. Qualitative and quantitative osteoblastic activity in chronic porcine hypervitaminosis A. J. Pathol. 97:677-686.

Yan, S. M., Y. M. Feng, H. Q. Zhang and B. L. Shi. 2007. Effects of vitamin A and vitamin D on metabolism of calcium and phosphorous in broilers. Chin. J. Anim. Nutr. 19(3):218-224.

Zhi. H. Y., E. Li and J. Zhang. 2001. Effect of all trans retinoid acid on newborn rat calvarial osteoblastic cells *in vitro*. J. Acta Nutrimenta Sinica. 2:167-169.