Perinatal Exposure to Vitamin A Differentially Regulates Chondrocyte Growth and the Expression of Aggrecan and Matrix Metalloprotein Genes in the Femur of Neonatal Rats[1–3]

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Abstract

Vitamin A (VA) and its active form, retinoic acid (RA), are regulators of skeletal development. In the present study, we investigated if maternal VA intake during pregnancy and lactation, as well as direct oral supplementation of neonates with VA + RA (VARA) in early life, alters neonatal bone formation and chondrocyte gene expression. Offspring of dams fed 3 levels of VA (marginal, adequate, and supplemented) for 10 wk were studied at birth (P0) and postnatal day 7 (P7). One-half of the newborns received an oral supplement of VARA on P1, P4, and P7. Tissues were collected on P0 and 6 h after the last dose on P7. Pup plasma and liver retinol concentrations were increased by both maternal VA intake and VARA ($P <$ 0.01). Although maternal VA did not affect bone mineralization as assessed by von Kossa staining, newborn femur length was increased with maternal VA ($P < 0.05$). VARA supplementation of neonates increased the length of the hypertrophic zone only in VA-marginal pups, close to that in neonates from VA-adequate dams, suggesting VARA caused a catching up of growth that was limited by low maternal VA intake. Maternal diet did not alter type X nor type II collagen mRNA. However, VARA-treated pups from VA-supplemented dams had reduced mRNA for aggrecan, a major component of cartilage matrix, and increased mRNA for matrix metalloproteinase (MMP)13, which catalyzes the degradation of aggrecan and collagens. These results suggest that moderately high maternal VA intake combined with neonatal VARA supplementation can reduce the ratio of aggrecan:MMP, which may unfavorably alter early bone development. J. Nutr. 142: 649–654, 2012.

Introduction

Prenatal and postnatal skeletal growth is a complex and tightly controlled process (1,2). The growth plate is responsible for bone elongation and the formation of most elements. Cartilage growth occurs through the proliferation of chondrocytes and production of matrix, which primarily consists of type II collagen and a large proteoglycan, aggrecan. As chondrocytes differentiate, these cells enlarge into prehypertrophic and hypertrophic chondrocytes, extracellular matrix is replaced by type X collagen, and MMP13[4] is increased, which catalyzes the

degradation of collagen and aggrecan (3,4). The chondrocytes at the lower border of the HZ undergo apoptosis. The matrix eventually becomes mineralized and is replaced by bone and marrow. Aggrecan is one of the major extracellular matrix components of cartilage and it is essential for normal bone growth and function (1,2). The increased catabolism of aggrecan is a key pathological mechanism in arthritis (5,6).

Maternal VA status affects embryonic development, including the growth of cartilage and bone (7,8). Studies on VA deficiency in pregnant animals have revealed a large array of congenital malformation in the embryos, including defects in the development of the eye, heart, lung, kidney, central nervous system, genitourinary tract, and the skull, skeleton, and limbs (7,8). On the other hand, the administration of excessive VA during pregnancy also results in congenital anomalies, such as of the external surface of the head, cranial deformity, ectopic teeth, and heterotopic cartilage (9,10). All of these studies have established a critical role of VA in skeletal development. RA, a major bioactive metabolite of VA, has been detected at a higher concentration in hypertrophic chondrocytes (11), where it

stimulates chondrocyte terminal differentiation and alters cartilage matrix synthesis (12–15). RA can directly affect the expression of genes involved in extracellular matrix, such as type X collagen, MMP13, and aggrecan (12,13,15,16). RA regulates gene expression through its nuclear RARs (RA receptors), consisting of 3 subtypes: RARα, RARβ, and RARγ (17). RARγ is the most strongly expressed in the growth plate from proliferative to hypertrophic chondrocytes (18,19). RARγ functions in both the unliganded and liganded forms in the regulation of chondrocyte proliferation and differentiation (15,19).

Despite this knowledge that overt VA deficiency and toxicity affect chondrocyte and bone development, little is known about whether exposure to moderately elevated or marginally deficient VA states, during the perinatal period, is likely to alter bone parameters in newborns or neonates. Nor is it known how direct VA supplementation of neonates may affect bone development. Therefore, in the present study, we investigated if the level of maternal VA intake in the perinatal period as well as direct oral supplementation of neonates with affect aspects of neonatal bone formation in terms of bone growth and the expression of genes important for matrix formation. Previous studies conducted in our laboratory indicated that VARA increases retinol uptake and retinyl ester formation in neonatal lung to a level higher than that produced by VA or RA alone (20). Therefore, in the current study, we treated neonatal rats with orally administered VARA as a means to enhance the effects of VA supplementation. The diets used in this study were selected to represent the range of VA found in most human diets, whereas the amount of VA in the VARA supplement represented the dose of VA given to newborns and young children (15 mg retinol/child, which we estimated to be ~2.5 kg) in VA supplementation programs, as previously described (21); thus, VA in the dose was equal to 0.6 mg retinol/kg. The amount of RA in VARA, equal to ~0.5 mg/kg body weight, represented a clinical dose, as was previously given to neonatal rats and noted to promote lung maturation (22). Several outcomes related to retinol homeostasis, bone growth, and matrix-related gene expression were evaluated to determine under which conditions of maternal VA status and neonatal VARA supplementation these outcomes might be altered in the early neonatal period.

Materials and Methods

Animal procedures. Animal procedures were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Female Sprague-Dawley rats with 1-wk-old female pups were purchased from Charles River. Upon arrival, the dams were fed a controlled VA-deficient diet. To achieve differences in maternal VA status, the weanling female rats were fed 1 of 3 levels of VA in a standard AIN-93G purified diet for 10 wk before mating and through pregnancy and lactation. These diets are denoted VAM (VA marginal; 0.4 μg retinol/g diet), VAA (VA adequate; 7.67 μg retinol/g diet), and VAS (VA supplemented; 104 μg retinol/g diet) (*n* = 3–4 litters/diet). Plasma total retinol concentrations of mothers were measured after 10 wk of feeding, before mating. The offspring of these mothers were studied at birth (P0) and on P7 (*n* = 3–6 pups/litter). Additionally, one-half of the newborns were orally supplemented with VARA, or oil as placebo, on P1, P4, and P7. For the oral doses, VA (all-*trans*-retinyl palmitate) and RA were purchased from Sigma-Aldrich; VA and RA were admixed, dissolved in oil, and given in a concentration that was equivalent to 11 μg of retinol and 0.6 μg of RA/g pup (10:1 molar ratio).

Pups were killed with isoflurane, blood was collected into heparinized capillary tubes from the vena cava or heart, and tissues were collected.

HPLC. The total retinol concentration in plasma, pup's stomach milk (indicative of maternal milk retinol concentration), and total retinol storage in the liver and lung of the pups were determined by HPLC as previously described (23). Briefly, portions of the liver and lung were weighed and extracted overnight in 20 or more volumes of chloroform: methanol (2:1, v:v) per weight of tissue. The tissue extracts were filtered, washed, and dried under argon. Then the samples were subjected to hydrolysis by a saponification procedure, trimethylmethoxyphenyl-retinol was added as an internal standard, and the samples were dried again and reconstituted in methanol and subjected to HPLC analysis. Plasma was collected from fed rats. Milk curd, which represents recently ingested milk, was expressed from the stomach of the neonates. Plasma and milk samples underwent direct hydrolysis without prior extraction.

Anatomical measurement and histology staining. Femurs were dissected out and length was measured with digital calipers (1/100 mm). Femurs were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissue blocks were cut at 8-μm thickness on a microtome. Sections were transferred onto Probe-On Plus microscope slides (Fisher Scientific) and then the slides were dried on a 37°C plate overnight and stored at room temperature. For staining, sections were deparaffinized and rehydrated through xylenes and graded alcohol series and then stained with 1% Alcian blue (pH 2.5) for 20 min and counterstained with 0.1% nuclear fast red for 5 min. The HZ in the growth plate was measured on Alcian blue-stained images using a SPOT camera and software (Diagnostic Instruments) on samples that were coded by subject group before measurement. For von Kossa staining, sections were incubated in 1% silver nitrate for 20 min, unreacted silver was removed by 5% sodium thiosulfate for 5 min, and slides were counterstained with nuclear fast red.

qPCR. Femur and tibia were broken and the bone marrow was flushed out. Total RNA was extracted from bone using Trizol (Invitrogen) and quantified by spectrophotometry. mRNA transcript levels were determined by real-time q-PCR (24). Briefly, 1 μg of total RNA was subjected to RT and one-twentieth of the diluted reaction mixture was used for real-time qPCR analysis using Real Time PCR iQ SYBR Green Supermix (BioRad) in a final volume of 20 μL. *Gadph* mRNA was amplified at the same time as an internal control. Primers were *Col2a1*: 5′-GAGTCAAGGGTGAT-CGTGGT-3′ (forward), 5′-TCTGGTTGTTCAGCGACTTG-3′ (reverse); *Col10a1*: 5′-CAGGGTTACCAGGACCAAAA-3′ (forward), 5′-AGCCA-CACCTGGTCATTTTC-3′ (reverse); *Aggrecan*: 5′-CAACCTCCTGGGT-GTAAGGA-3′ (forward), 5′-GCTTTGCAGTGAGGATCACA-3′ (reverse); *Mmp13*: 5′-CCCTCGAACACTCAAATGGT-3′ (forward), 5′-GAGCTGC-TTGTCCAGGTTTC-3′ (reverse); and *Gapdh*: 5′-ATGGGAAGCTGGT-CATCAAC-3′ (forward), 5′-GGATGCAGGGATGATGTTCT-3′ (reverse). mRNA levels were measured by q-PCR and calculated based on a dilution series (standard curve) run at the same time as the samples. *Gadph* was used as internal control; all numbers were normalized to results for the P0 VAA group (=1.0).

Statistics. Results are shown as the mean ± SEM of at least *n* = 3 observations. Prism 5 software (GraphPad Software) was used for analysis. Either 1-way (maternal diet) or 2-way (maternal diet, direct oral treatment with VARA) ANOVA was conducted, followed by Bonferroni's post hoc multiple comparison test to determine the significance between multiple groups. If variances were unequal, log-10 transformation was performed. $P < 0.05$ was considered significant.

Results and Discussion

Neonatal VA status is regulated by both maternal VA status and neonatal treatment with VARA. After female rats received 1 of the 3 diets for 10 wk, the plasma total retinol in the VAS group (dams) was more than twice that in VAA dams, whereas VAM and VAA dams did not differ (**Fig. 1A**). Because milk is the only source of VA for the accumulation of retinol in the offspring after birth, and milk retinol concentration is related to the mother's dietary VA intake (25,26), we measured retinol
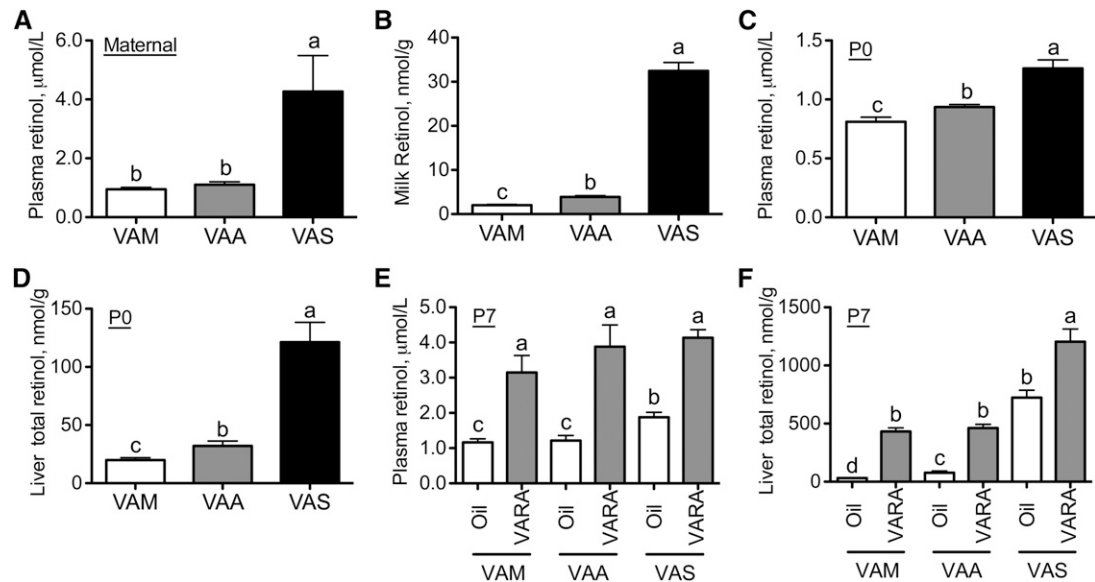
FIGURE 1 Total retinol concentrations in adult rat plasma (*A*) and milk (*B*) of rat dams fed a VAM, VAA, or VAS diet, and in the plasma (*C,E*) and liver (*D,F*) of their offspring at P0 (*C,D*) and P7 (*E,F*). Data are means ± SEM, $n \geq 5$/group, except for maternal plasma retinol, determined before mating, $n \geq 3$/diet group. Maternally derived milk retinol was determined on clotted stomach contents of pups that did not receive VARA, $n \geq 5$/group. (*A–D*) Means without a common letter differ, $P < 0.05$. P, postnatal day; VAA, vitamin A-adequate diet; VAM, vitamin A-marginal diet; VARA, vitamin A combined with 10% retinoic acid; VAS, vitamin A-supplemented diet.

in milk curd collected from the pups' stomachs as an indicator of maternal retinol transfer after birth. Total retinol concentrations were 8-fold higher in milk from VAS than from VAA dams ($P < 0.001$) and were reduced 50% in VAM dams (Fig. 1*B*). The plasma retinol concentrations of the newborns, which reflect differences in maternal dietary VA only, were slightly but significantly different among pups from dams fed the 3 different levels of dietary VA (Fig. 1*C*). Liver retinol accumulation in P0 pups showed the same differences, indicating that VA storage in newborn liver was directly related to maternal VA intake during gestation (Fig. 1*D*).

At P7, plasma retinol continued to be higher in control (oil-treated) pups from VAS dams, concordant with differences at P0 and indicating a continuing effect of maternal VA supplementation during lactation. Plasma retinol concentrations were similar in VAA and VAM dams (Fig. 1*E*). Direct oral feeding with VARA increased neonatal plasma retinol, regardless of maternal VA intake, with a similar increase in all maternal VA groups (Fig. 1*E*). Neonatal liver retinol differed significantly in the order VAM < VAA < < VAS at both P0 (Fig. 1*D*) and P7 (Fig. 1*F*).

These results provide support that our experimental design was appropriate for altering VA homeostasis in the offspring both pre- and postnatally. Maternal VA supplementation affected the offspring's VA status at P0 as well as P7 and direct oral supplementation with VARA further altered VA status in P7 neonates from all 3 maternal diet groups.

Maternal VA status and oral feeding alter bone formation. We next asked whether bone formation was also changed. First, bone length was determined. At P0, femur length was slightly but significantly greater in neonates of VAS dams compared with the control group ($P < 0.05$) (**Fig. 2***A*). At P7 (Fig. 2*B*), maternal VA intake affected the length of both the femur ($P < 0.01$) and tibia (not shown). Direct oral supplementation with VARA, however, did not affect femur length.

Growth plate activity is essential for skeletal growth. Therefore, the histology of the growth plate was examined.

Sections of femurs were stained with 1% Alcian blue (**Supplemental Fig. 1**) and then the HZ was quantified. The HZ was shorter in newborns from VAM dams compared to VAA dams ($P < 0.05$) (Fig. 2*C*). At P7, the formation of the HZ was also changed in response to maternal dietary VA ($P < 0.001$) (Fig. 2*D*), similar to total bone length (Fig. 2*A*). Only in neonates in the VAM group did oral feeding with VARA increase the length of the HZ (Fig. 2*D*) ($P < 0.05$), which was close to the HZ length in pups from VAA and VAS dams either with and without VARA, suggesting that VARA caused a catching up of growth in the neonates of VAM mothers. In addition, however, bone formation (mineralization) assessed by von Kossa staining appeared similar among all groups (**Supplemental Fig. 2**), which might have been due to the sensitivity of this method.

Maternal VA status and oral feeding alters gene expression in bone. Bone growth is also affected by extracellular matrix production and composition (2,27). We therefore examined whether genes involved in matrix synthesis and accumulation are altered by maternal VA status and direct oral feeding of neonates with VARA. At P0 and P7, the relative mRNA levels for neither type II collagen (**Fig. 3***A,B*) nor type X collagen (Fig. 3*C,D*) were affected by maternal VA. At P7, VARA treatment did not affect type II collagen, whereas there was a main effect of VARA for type X collagen mRNA (Fig. 3*D*).

Aggrecan and MMP13 are related to proteoglycan synthesis and degradation, respectively; aggrecan has been shown to be reduced and MMP13 increased by RA treatment in vitro (12,13,15,16). In our study, aggrecan mRNA did not differ with maternal VA intake at P0 or P7 (**Fig. 4***A*). However, in neonates in the VAS group at P7, treatment with VARA reduced aggrecan mRNA levels (Fig. 4*B*) ($P < 0.05$). MMP13 expression increased strongly from P0 to P7 but did not differ with maternal diet (Fig. 4*C*). However, concordant with but opposite to aggrecan mRNA, the level of MMP13 mRNA was higher in P7 VARA-treated neonates in the VAS group (Fig. 3*D*) ($P < 0.05$).

Either too much or too little VA is known to alter skeletal growth. Li et al. (28) reported that VA deficiency caused
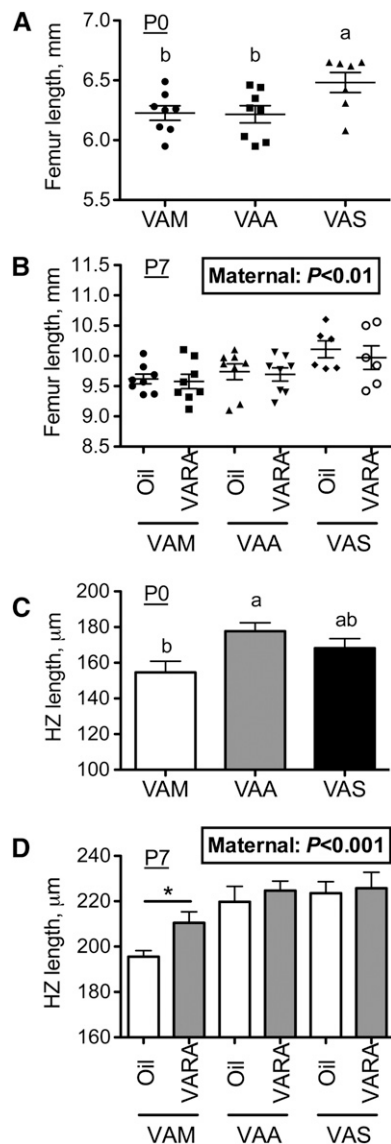
FIGURE 2 Length of femur (*A,B*) and HZ (*C,D*) in newborn and neonatal offspring, without and with oral VARA supplementation, of dams fed a VAM, VAA, or VAS diet. Data are means ± SEM, *n* = 7–9/ group (*A*), 6–8/group (*B*), or 6/group (*C,D*). (*A,C*) Means without a common letter differ, *P* < 0.05; in *D*, **P* < 0.05. Significant differences for maternal diet from 2-way ANOVA are shown in boxes in *B* and *D*. HZ, hypertrophic zone; P, postnatal day; VAA, vitamin A-adequate diet; VAM, vitamin A-marginal diet; VARA, vitamin A combined with 10% retinoic acid; VAS, vitamin A-supplemented diet.

that had been fed VAM, VAA, and VAS diets from weaning until young, middle, and old age (29). In these studies, continuous feeding of the VAM diet after weaning altered cortical bone dimension, and the VAS diet increased medullary area and decreased cortical thickness in young, but not in old, rats. Thus, the VAS diet, used in both studies, appears unlikely to result in an increased risk of fracture-related bone changes in old rats. However, in our aging study, the experimental diets were initiated at the time of weaning. The present study sheds light on the consequences of early life exposure to VA, over an earlier and much shorter period when cartilage and bone formation is rapid.

Aggrecan, a large proteoglycan, forms a major structural component of cartilage (1,2). It is responsible for hydrating cartilage, rendering the bone capable of accommodating physical loading, and thereby plays a major role in maintaining cartilage integrity and normal functions (30). A depletion of aggrecan leads to a decrease in compressibility and resilience during joint loading. Over time, this process can lead to irreversible cartilage damage. The increased catabolism of aggrecan is a key pathological mechanism in arthritis (5,6). MMP13, one of a family of metalloproteinases, catalyzes the degradation of collagen and aggrecan, and is induced in
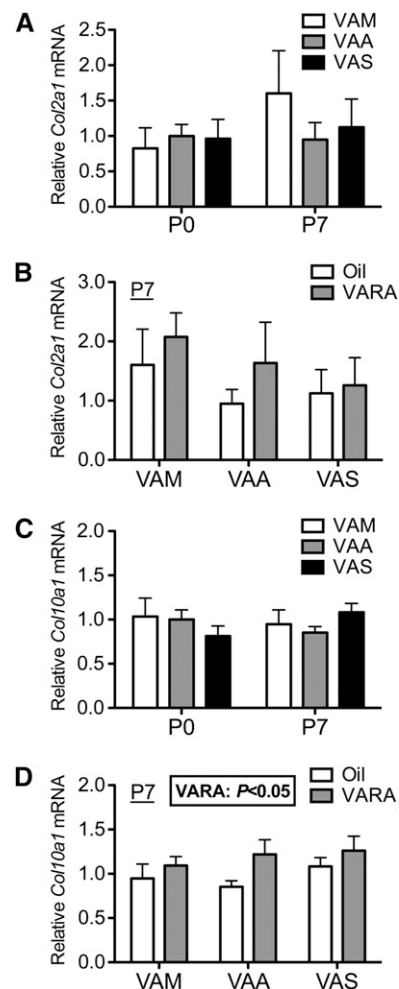
embryonic skeleton hypoplasia, including retardation of ossification and reduction in the size of the skeleton, and limb dysostosis. In our study, which we designed to examine a narrower physiological range of VA intake that is representative of most human diets, we observed a reduction in limb size (femur length) in neonates from VAM dams, whereas obvious severe dysostosis was not observed. This may be due to the fact that our VAM diet was not extremely low in VA, ~5 times more than that used by Li et al. (28). Moreover, the HZ of femur chondrocytes was significantly shorter in pups from VAM dams, and only in the VAM group did oral feeding with VARA significantly increase the length of the HZ. These results suggest that VARA caused a catching up of chondrocyte growth that was slowed due to insufficient retinoid provided by the mother's VAM diet. Recently, we examined bone qualities in adult rats

FIGURE 3 Collagen type II (*A,B*) and type X (*C,D*) mRNA expression in bone of newborn and neonatal rats. Data are means ± SEM, *n* = 6/ group. Significant differences from 2-way ANOVA are shown in the box in *D*. *Col2a1*, type II collagen; *Col10a1*, type X collagen. P, postnatal day; VAA, vitamin A-adequate diet; VAM, vitamin A-marginal diet; VARA, vitamin A combined with 10% retinoic acid; VAS, vitamin A-supplemented diet.
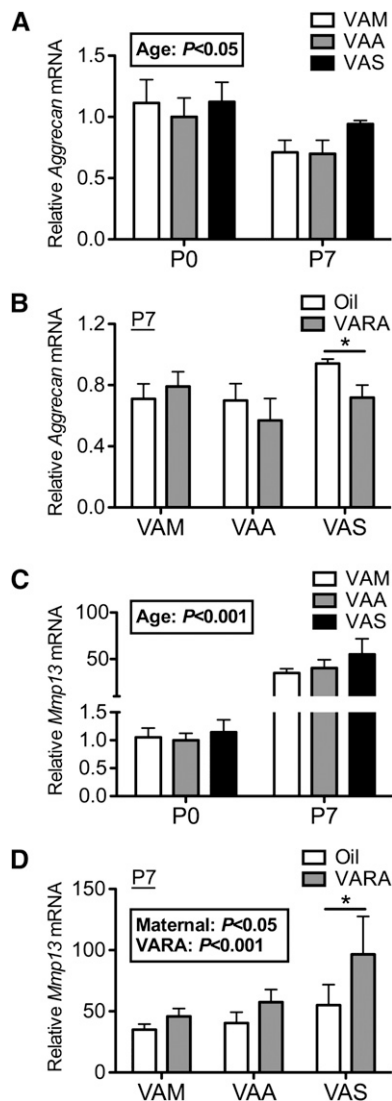
FIGURE 4 Aggrecan (*A,B*) and MM13 (*C,D*) mRNA expression in femur of newborn and neonatal rats. Data are means ± SEM, *n* = 6/group. Significant differences for maternal diet from 2-way ANOVA are shown in boxes in *A, C,* and *D.* *, *P* < 0.05. MMP, matrix metalloproteinase; P, postnatal day; VAM, VAA and VAS, vitamin A marginal, adequate and sufficient maternal diet, respectively.

terminally differentiated chondrocytes in the growth plate (3,4). Our results indicate that the alteration of aggrecan and MMP13 expression by short-term oral VARA supplementation depends on maternal VA status. Only in the pups from VAS dams did oral dosing with VARA significantly decrease aggrecan expression, along with a significant increase in MMP13. This result suggests that a combination of maternal VA supplementation and excess VA in the neonatal period could potentially be unfavorable for cartilage development. It can be speculated from our study that neonatal VA supplementation may be beneficial for bone only if the mother's VA status is low. Thus, further studies are needed to evaluate if there are long-term, persistent consequences of early-life exposure to marginal VA status, or VA supplementation, on bone growth and composition.

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