High dietary intake of retinol leads to bone marrow hypoxia and diaphyseal endosteal mineralization in rats

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A B S T R A C T

Vitamin A (retinol) is the only molecule known to induce spontaneous fractures in laboratory animals and we have identified retinol as a risk factor for fracture in humans. Since subsequent observational studies in humans and old animal data both show that high retinol intake appears to only have small effects on bone mineral density (BMD) we undertook a mechanistic study of how excess retinol reduces bone diameter while leaving BMD essentially unaffected. We fed growing rats high doses of retinol for only 1 week. Bone analysis involved antibody-based methods, histology, pQCT, biomechanics and bone compartment-specific PCR together with Fourier Transform Infrared Spectroscopy of bone mineral. Excess dietary retinol induced weakening of bones with little apparent effect on BMD. Periosteal osteoclasts increased but unexpectedly endosteal osteoclasts disappeared and there was a reduction of osteoclastic serum markers. There was also a lack of capillary erythrocytes, endothelial cells and serum retinol transport protein in the endosteal/marrow compartment. A further indication of reduced endosteal/marrow blood flow was the increased expression of hypoxia-associated genes. Also, in contrast to the inhibitory effects in vitro, the marrow of retinol-treated rats showed increased expression of osteogenic genes. Finally, we show that hypervitaminotic bones have a higher degree of mineralization, which is in line with biomechanical data of preserved stiffness in spite of thinner bones. Together these novel findings suggest that a rapid primary effect of excess retinol on bone tissue is the impairment of endosteal/marrow blood flow leading to hypoxia and pathological endosteal mineralization.

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Introduction

Vitamin A is the only molecule known to induce spontaneous fractures in laboratory animals and can only be derived from the diet as no animal species has the capability for de novo synthesis [1,2]. The important regulatory role of vitamin A was recently highlighted when the nuclear receptors of its active metabolite, retinoic acid (RA), were shown to have 5310 target genes, in breast cancer cells [3]. Vitamin A deficiency is a public health problem in over 120 developing countries and significantly increases the risk of severe illness and death from common childhood infections [4]. In the industrialized part of the world, we have identified high serum retinol levels as a risk factor for fracture [5,6]. However subsequent observational studies relying on BMD measurements were unable to associate increased vitamin A intake with reduced BMD. For example, of the nine published observational studies, investigating the association between circulating levels of vitamin A and adverse skeletal effects, three relied on fracture as an endpoint and in two of these a positive association with fracture risk was demonstrated. More importantly, none of the studies relying on BMD as endpoint where able to demonstrate that serum retinol affected BMD negatively, on the contrary, three of the studies noticed a positive correlation [7]. This may indicate that excess retinol induces fragile bones without a large effect on BMD. In line with this, several hypervitaminosis A studies on laboratory animals were unable to demonstrate significant differences in ash weight between the thinner hypervitaminotic bones and control bones [8–10]. Hypervitaminosis A
has been studied since 1925 and consistently show induced loss of appetite together with reduced weight gain, bone thinning, spontaneous fractures of long bones together with general (including bone) tissue hemorrhage and increased periosteal resorption [11]. Recently, Kneissl and colleagues showed that excess retinoid treatment induced bone thinning in both young and aged rodents and also suggested a bone compartment-specific action of retinoids by means of measuring decreased cortical BMD but increased trabecular BMD in animals receiving RA [12]. Furthermore, our recent finding in an experimental study of subclinical hypervitaminosis A showed that although bending strength decreased, there was no reduction of BMD (cortical or trabecular) [13] and as the ash weight was unchanged and since the hypervitaminotic bone volume was smaller than in control bones, the actual volumetric BMD was increased [14]. As osteoporosis is defined as a reduction of BMD of 2.5 standard deviations or more, compared to healthy young bone, it is important to further investigate the mechanism behind this phenomenon.

Therefore the aim of this study was to identify the mechanism behind how excess retinol induces fragile bones with minimal effects on BMD. We have used the rat as a model, since this species has been consistently used in hypervitaminosis A investigations. Importantly, as bone thinning and lack of reduced ash weight were noticed in studies using both high and subclinical doses of vitamin A, we have in the present study mimicked earlier hypervitaminosis A protocols using high doses of retinol to induce distinct changes in a short time. Also important, it has previously been shown that high retinol doses, comparable to what is used in this report, given to rats for 2 weeks result in plasma concentrations of about 3–4 μM, which is similar to what is seen in hypervitaminosis A toxicity in humans [56,57]. We have applied modern methods, not used before in hypervitaminosis A studies, such as specific antibody-based immunohistochemistry and ELISA analysis, differential bone compartment-specific real-time PCR to assess changes in gene expression together with Fourier Transform Infrared (FTIR) Spectroscopy, to analyze the bone mineral content and crystallinity [15].

Materials and methods

Animals and experimental design

Twenty-four male Sprague-Dawley rats, 5 weeks of age, were obtained from Møllegaards Breeding Centre, Ltd. (Skensved, Denmark). They were acclimatized for 1 week and kept in groups of three animals and had free access to water and commercial pellet diet (Lactamin R36, Stockholm, Sweden). The rats were divided into two groups, each with 12 animals. They were fed a standard diet containing 12 IU vitamin A/g pellet ("cont"), or a standard diet supplemented with 1700 IU ("Ex. vitA") vitamin A/g pellet. The vitamin A was added to the pellets in the form of retinyl palmitate and retinyl acetate. Food intake was continuously measured by weighing (grams of pellet per cage of three rats). Body weight was measured at the beginning of the study (initial weight), at day three and before the rats were killed (final weight). After 8 days, the rats were killed by exsanguinations from the abdominal aorta under Equivetä anesthesia (chloral hydrate 182 mg/kg, pentobarbital 41.7 g/kg). Blood was left at room temperature for at least 30 min before centrifuging at 200 x g for 10 min to separate sera, aliquots were stored at −70 °C pending biochemical analyses. Animal experiments were conducted in accordance with Swedish regulations.

Peripheral quantitative computed tomography (pQCT)

The left femora were scanned using pQCT (Stratec XCT Research SA+) with version 5.50 R software (Norland-Stratec Medizintechnik GmbH, Birkenfeld, Germany) using a voxel size of 0.07 mm. The precision and accuracy of the method have been verified previously [13,16]. The femur diaphysis was scanned at the midshaft and the threshold value was set to 710 mg cm−3, values above were defined as cortical bone. Trabecular variables were determined by metaphyseal scans at a point located at 14% of the total bone length from the proximal tip. Values ranging from 280 to 400 mg cm−3 were considered to be trabecular bone. The thresholds were set according to the manufacturer's recommendations.

Determination of serum markers

Commercially available kits were utilized for measurement of serum markers as follows: osteocalcin, Rat-MID Osteocalcin (Nordic Bioscience Diagnostics, Herlev, Denmark); TRACP 5b, RatTRAP (Immunodiagnostic Systems, Boldon, UK); N-terminal propeptide of type I collagen, PINP EIA (Immunodiagnostic Systems, Boldon, UK); C-terminal telopeptides of type I collagen (CTX-1), RatLaps (Nordic Bioscience Diagnostics, Herlev, Denmark); free soluble RANKL (Biomedica Medizinprodukte, Vienna, Austria), soluble Icam1 (R&D Systems) according to the manufacturer's instructions and rat ICTP as previously published [17]. Data represents 11 control and 12 excess retinol rats and are presented as mean±SD.

Immunohistochemistry

The bone (humeri) preparation and immunohistochemistry have previously been described in detail [18]. The bones from all animals were sectioned in the same orientation in order to make comparable sections. Immunostaining for cathepsin K was achieved by the use of polyclonal antimouse cathepsin K antisemur at a dilution of 1:300 [19] and for preparation of a polyclonal antibody against osteopontin a synthetic peptide corresponding to amino acid 46–58 (PDPSQKQNLLAPQ) to the rat osteopontin protein sequence was conjugated to keyhole limpet hemocyanin (Innovagen AB, Lund, Sweden). A rabbit was immunized subcutaneously with 200 μg of the peptide conjugate mixed with Freund's complete adjuvant at week 0 and followed by booster injections of 100 μg peptide conjugate mixed with Freund's incomplete adjuvant at week 2, 5 and 11. Serum was collected 2 weeks after the last booster injection. The specificity of the antibody was verified by Western blot analysis and used in immunohistochemistry at a dilution of 1:800. For protein localization, sections were incubated with goat anti-rat Icatr (Abcam/CD54 (1:200, R&D Systems), rabbit antimouse Pecam1/CD31 (1:400, SantaCruz-1506), rabbit antirat Dmp1 (1:400, Takara Bio), rabbit antihuman VWF (1:800, DAKO), goat antimouse Mmp2 (1:400, R&D Systems), goat antihuman osteocalcin (1:800, SantaCruz-18319), and mouse antihuman Rbp4 (1:48, 1:800) [20]. Visualization of the antibodies where achieved by incubation with secondary biotinylated antibody at a dilution of 1:200 in 10% serum and PBS followed by an avidin–biotin–peroxidase complex incubation using the Vectastain ABC-kit (Vector Laboratories) and the substrate diaminobenzidine tetrahydrochloride (DAB, DAKO).

Cell culture

Mouse stromal bone marrow derived cell line, ST2, was cultured essentially as described previously [21]. Briefly, cells were maintained in α-minimal essential medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. To induce osteoblastogenesis, confluent cells were switched to media containing 25 μg/ml ascorbic acid and 10 mM ß-glycerophosphate, treated with ± 400 nM retinoic acid (Sigma-Aldrich). RA was dissolved in 95% ethanol in a dark room under the flow of nitrogen. The 0.5 μg/ml (1.86 mM) stock solution was stored in −70 °C and shielded from light until use. Experiments were stopped and total RNA was extracted using TRI Reagent® (Sigma-Aldrich).
Diaphyseal bone tissue RNA isolation

Humeri were isolated, and all connective tissue, including periosteum, were completely removed as were also both epiphyses (including growth plates). For samples with cortical bone including marrow (CortB+M) the samples (n = 3 for cont and n = 4 for Ex. vita) were snap-frozen in liquid nitrogen and quickly crushed into a fine powder using a mortar and pestle followed by total RNA extraction with TRI Reagent® (Sigma-Aldrich). For cortical bone without marrow (CortB) (n = 4 for cont and n = 3 for Ex. vita), diaphyseal bones were cut in pieces, vortexed in ice-cold PBS three times at 10 s to remove marrow cells followed by snap-freezing in liquid nitrogen, crushing into a fine powder and RNA extraction. Isolated RNA was quantitated using spectrophotometry by measuring the absorbance at 260 nm, and the 260/280 nm ratio was calculated, and RNA was kept only when this ratio was 1.9–2.0 to ensure the absence of protein contamination and limited RNA degradation. The integrity of sample RNAs was confirmed by capillary electrophoresis separating 18S and 28S ribosomal RNA on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Quantitative RT-PCR

Four hundred nanograms of total RNA were transcribed to cDNA using the TaqMan system (Applied Biosystems). Quantitative PCR was performed using inventoried TaqMan® Gene Expression Assays for Cyp26a1 (Rn00590308), Stra6 (Rn01418200), Hif1α (Rn00577560), Twist1 (Rn00585470), Mmp2 (Rn01538174), tissue non-specific alkaline phosphatase (Alp or Alp) (Rn00564931), osteocalcin (Rn00566386), cathepsin K (Rn00580723), Runx2 (Rn01512298), OPG (Rn00563499) and RANKL (Rn00589289) according to the manufacturer’s protocol, on a TaqMan 7000 apparatus. Cycling protocol: 50 °C for 2 min followed by 95 °C for 10 min and then 40 cycles of 95 °C 15 s followed by 60 °C for 1 min. Expression levels were divided by β-actin (Rn00667869) levels for standardization. Each sample was run as single points at each experiment and experiments were performed at least twice.

Mechanical three-point bending analysis of femur diaphyses

Three-point bending test was performed on right femora using an electromechanical material testing machine (Avalon technology Inc., Rochester, MN, USA) with a span length of 15 mm and a loading speed of 0.48 mm/s, as described previously [13]. Bone strength was measured at 50% of the total length of the bone. The load and displacement were sampled with a frequency of 50 Hz and stored digitally. Displacement at failure (the total amount of movement of the load in mm from the moment of contact with the bone surface until the bone fractured) and the load applied (Newtons; N) at the moment when the bone fractured were recorded. The data accumulated during each test were used to construct a load–displacement curve. The area under the curve defines the amount of energy absorbed until failure (breakage) measured in Nmm. The maximum slope of the load–displacement curve is defined as the bone’s stiffness (N/mm).

Bone chemical composition

Bone chemical composition was analyzed by Fourier transform infrared spectrometry (FTIR). The entire left femora, after the pQCT analysis, were cleaned with a scalpel, rinsed with Milli-Q water and powdering using a cryogenic mill (CertiPrep 6750 Freezer/Mill, SPEX). For the FTIR analyses, 5 mg of bone powder was mixed with 90 mg of FTIR-grade KBr and pressed under vacuum. Infrared spectral data were collected on a Fourier transform infrared spectrometer (Magna IR200, Nicolet) from 4000 to 400 cm⁻¹ at 2 cm⁻¹ resolution over 128 scans. The amounts of phosphate, carbonate, and organic matrix in bone were determined from the peak area of absorption bands associated with phosphate, carbonate, and amide groups in the infrared spectra. Overlapping peaks under the above mentioned bands were resolved and their integrated areas measured using a curve fitting software (Peakfit v.4.11) and applying a second derivative methodology. Peak areas of isolated bands were normalized to the area of 3800–2800 cm⁻¹ band region associated with OH groups and were represented by a capital “A” (e.g. A1660). On the other hand, the individual sub-peak area under a band were normalized to the associated band and were designated using “a” (e.g. a956, a994, a1020). The procedure is described in detail elsewhere [22]. This methodology allows for a detailed and quantitative analysis of different molecular constituents of bone mineral. In particular, different compositional variables were determined to quantify the potential effects of hypervitaminosis on bone mineralization. To describe bone degree of mineralization and carbonate content in the mineral from FTIR analyses: degree of mineralization of bone (mineral) was defined as the ratio between the peak area of phosphate and amide I bands: (mineral = (A1200_900)/(A1405)). Carbonate in bone mineral (minCO₃) was defined as the ratio between the peak area for 1405 cm⁻¹ (carbonate type B substitution) to phosphate band area: (minCO₃ = A1405/(A900_1200)).

Statistical analyses

STATVIEW 5.0 software (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. The data were evaluated by t-test. In every case, p < 0.05 was considered statistically significant.

Results

One week of excess retinol induces brittle bones

Growing (6-week old) male rats were fed either standard chow or a diet fortified with a high dose of vitamin A for 7 days. All animals survived and appeared healthy during the experimental period of 8 days (which includes fasting for the last 24 h) although the classical gross phenotypical characteristics of excess retinol consumption such as reduced food intake (−13%) and weight gain (−36%) were noticed (Table 1). The length of femur was slightly affected by the large excess of retinol (−2.4%) (Table 1). pQCT measurements showed that metaphyseal trabecular and total bone mineral contents were drastically reduced (34% and 32%, respectively), whereas the respective densities were less reduced (9% and 5%) (Table 2). At mid-diaphysis, total cross-sectional areas were reduced by 15% and cortical mineral content was lowered by 15%, while the cortical density was only 3.2% lower than control bones. Importantly, a three-point bending test showed that bones from retinol-treated rats were weaker as the energy absorption and load at failure was reduced by 46% and 19%, respectively (Table 2).

<table>
<thead>
<tr>
<th>Phenotypic characteristics from 1 week of excess ingestion of vitamin A.</th>
<th>Control</th>
<th>Excess vitamin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 12)</td>
<td>(n = 12)</td>
<td>p-value</td>
</tr>
<tr>
<td>End weight (g)</td>
<td>124 ± 2.1</td>
<td>105 ± 1.6</td>
</tr>
<tr>
<td>Weight increase (g)</td>
<td>50.7 ± 1.06</td>
<td>32.4 ± 0.60</td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>25.5 ± 0.58</td>
<td>24.8 ± 0.72</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>102 ± 4.16</td>
<td>89.1 ± 26.1</td>
</tr>
</tbody>
</table>

Values are mean ± SE. n=number of individuals.
Serum markers for osteoclast activity (cathepsin K degradation of fibrillar type I collagen) show a reduction by 36% (CTX-1) and osteoclast number was reduced 31% (TRACP 5b) (Fig. 1A). Thus, a significant reduction of microvessel associated erythrocytes in pathognomonic areas of hypervitaminotic animals compared to similar areas in controls (Fig. 3C). These corresponding areas were highly cellular (Fig. 3A) but completely negative for the osteoclastic proteins, cathepsin K (Fig. 2A), TRACP and Mmp9 (not shown). Upon close inspection, control sections showed numerous erythrocytes densely packed in vessel-like structures. In contrast, sections from hypervitaminotic animals showed either no or scattered erythrocytes outside vessel structures, indicative of hemorrhage and/or a non-functional blood supply (Figs. 3A and B). Counting of erythrocytes around the endocortical surface and were not readily observed on the periosteal surface except in the highly active metaphyseal area close to the growth plate. In contrast, osteoclasts in retinol-treated animals were abundant at the periosteal surface of the mid-diaphysis together with an almost complete lack of endosteal osteoclasts (Fig. 2A). Counting osteoclasts along the periosteal and endosteal surfaces of the diaphysis confirmed this reversed pattern of osteoclast positioning (Fig. 2B). Notably, there were no apparent osteoclast differences in the trabecular compartment (Fig. 2C). Hough et al. previously described hypervitaminosis A rats with a pathognomonic appearance of endosteal bone, characterized by “highly vascularized bone lacunae” [23].

**Excess retinol reverses diaphyseal osteoclast positioning and endothelial staining**

In diaphyseal bones from control rats, osteoclasts were scattered along the endocortical surface and were not readily observed on the periosteal surface except in the highly active metaphyseal area close to the growth plate. In contrast, osteoclasts in retinol-treated animals were abundant at the periosteal surface of the mid-diaphysis together with an almost complete lack of endosteal osteoclasts (Fig. 2A). Counting osteoclasts along the periosteal and endosteal surfaces of the diaphysis confirmed this reversed pattern of osteoclast positioning (Fig. 2B). Notably, there were no apparent osteoclast differences in the trabecular compartment (Fig. 2C).

**Table 2**

Results of pQCT analysis and three-point bending test (Biomech.) of rat femurs.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=12)</th>
<th>Excess vitamin A (n=12)</th>
<th>p-value</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total BMC (mg/mm)</td>
<td>5.74±0.18</td>
<td>3.90±0.01</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Total BMD (mg/cm³)</td>
<td>349±2.6</td>
<td>331±4.5</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Trab BMC (mg/mm)</td>
<td>1.90±0.067</td>
<td>1.25±0.043</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Trab BMD (mg/cm³)</td>
<td>192±3.88</td>
<td>174±3.77</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Total CSA (mm²)</td>
<td>16.4±0.44</td>
<td>11.8±0.28</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Trab CSA (mm²)</td>
<td>9.87±0.20</td>
<td>7.18±0.22</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>PERIC (mm)</td>
<td>14.4±0.19</td>
<td>12.2±0.15</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Diaphyseal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total BMC (mg/mm)</td>
<td>3.78±0.06</td>
<td>3.13±0.06</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Total BMD (mg/cm³)</td>
<td>507.4±4.75</td>
<td>497.4±11.79</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Total CSA (mm²)</td>
<td>7.45±0.13</td>
<td>6.28±0.15</td>
<td>&lt;0.001</td>
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<tr>
<td>Cort BMC (mg/mm)</td>
<td>2.76±0.06</td>
<td>2.21±0.06</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>Cort BMD (mg/cm³)</td>
<td>1053±5.82</td>
<td>1019±8.13</td>
<td>&lt;0.01</td>
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<tr>
<td>Cort CSA (mm²)</td>
<td>2.62±0.05</td>
<td>2.16±0.05</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Cort THKC (mm)</td>
<td>0.30±0.004</td>
<td>0.27±0.07</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>PERIC (mm)</td>
<td>9.67±0.087</td>
<td>8.88±0.105</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>ENDOC (mm)</td>
<td>7.78±0.083</td>
<td>7.18±0.13</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Polar SSI (mm³)</td>
<td>4.01±0.141</td>
<td>2.71±0.096</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Marrow cavity (mm³)</td>
<td>4.83±0.10</td>
<td>4.12±0.15</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Biomech.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Displacement (mm)</td>
<td>2.08±0.46</td>
<td>1.65±0.29</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>Load (N)</td>
<td>33.5±3.3</td>
<td>27.1±4.54</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Energy (N×mm)</td>
<td>47.8±9.7</td>
<td>28.7±5.40</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>16.8±4.3</td>
<td>17.1±5.26</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SE. n=number of individuals; BMC=bone mineral content; BMD=bone mineral density; trab=trabecular; CSA=cross-sectional area; PERIC=periosteal circumference; cort=cortical; THKC=thickness; ENDOC=endosteal circumference.

**Fig. 1.** Serum bone cell markers, measured with immuno-assays. (A) Osteoclast markers and, (B) osteoblast markers. White bars represent control (cont, n=11) rats and black bars show results from rats on excess vitamin A (Ex. vitA, n=12). Not significant (ns), *p<0.05, **p<0.01 and ***p<0.001.
endothelial Icam1 and showed very few intact blood vessel structures (Fig. 4A). Conversely the periosteal blood vessels appeared both larger and stronger in hypervitaminotic bones (Fig. 5A). To test if the local differential delivery of vitamin A in hypervitaminotic animals. Gene expression analysis suggests that the marrow cells in hypervitaminotic animals are osteogenic. Immunostaining of the numerous cells in the marrow/endosteal pathognomonic compartment confirm the mRNA expression data and show that these cells are positive for osteoblastic proteins, including osteocalcin, osteopontin and Mmp2 (Fig. 6A). A further indication of increased endosteal/marrow osteoblast activity (as mineral producing cells) in retinol-treated rat bones is from the biomechanical data as a 3-point bending test show preserved stiffness although the bones are thinner (Table 2). This indicates that hypervitaminotic bones are harder (contain more mineral) which is in line with the significantly decreased displacement, also noticed for the hypervitaminotic bones (Table 2). This, together with the measured reduced force needed for breaking show that excess retinol induce brittle bones (Table 2). To directly test if we could measure changed mineral composition in hypervitaminotic bone tissue we analyzed its mineral content, composition and crystallinity using FTIR Spectroscopy. This revealed that retinol bones indeed have a higher degree of mineralization compared to control bones. Although not statistically significant the amount of mature highly crystalline phosphate in hypervitaminotic bones were reduced (−5.1%), but contained more poorly crystalline phosphate mineral (+2.7%) compared to normal bone (Fig. 6B). Finally, as mineral trafficking in bone is believed to be controlled by osteocytes which are embedded inside mineralized bone and are organized in an osteocyte lacunar canalicular system (OLCS), we analyzed those structures. The OLCS can be visualized by Dmp1 staining and it has been shown that when the speed of bone deposition is high, the OLCS are irregular [25]. Significantly, Dmp1 staining in the pathognomonic area of hypervitaminotic bones was diffuse and irregular, indicative of fast growing immature/non-remodeled endosteal bone (Fig. 6C). Importantly, at both the periosteal site and mid-cortical the pattern of the OLCS in hypervitaminotic bones was preserved indicating that these sites are not affected although the staining intensity at the periosteal site was stronger in hypervitaminotic bones (Fig. 6C). These results suggest a
local endosteal/marrow increase in mineralization in hypervitaminosis A animals.

Discussion

We have previously shown experimentally that a subclinical dose of vitamin A for three months causes weakening of bones in rats, without reduction or slight increase of BMD, depending on the method of calculation [14]. Here we show that three times this subclinical dose of vitamin A in food induces a measurable reduction in both diaphyseal cortical area and bone strength after only 8 days in younger male rats. Serum markers for osteoclast number, (TRACP 5b), differentiation, (RANKL) and activity, (CTX-1) were also found to be decreased in vitamin A supplemented animals. In previous rat studies,
hypervitaminosis A has been associated with increased markers for bone resorption, as determined by urinary secretion of hydroxyproline [23] or serum tartrate resistant acid phosphatase (TRACP) activity [12]. The discrepancy with our results could be explained by several experimental differences such as time of sampling (day 8 here, day 5 for Tracp activity and day 20 for hydroxyproline secretion) or the fact that earlier results rely on less specific biochemical analyses and thus does not specifically reflect osteoclast number/function [23] or the route of administration i.e. oral and subcutaneous [12]. Subcutaneously injected synthetic retinoids have been used together with thyroparathyroidectomy in rats as a model to study increased bone resorption to evaluate osteoclast inhibitors in vivo [26]. Today the standard osteoporosis animal model is ovariectomy (OVX), which mimics postmenopausal osteoporosis. OVX affects mostly trabecular bone and has less effect on cortical bone as reflected in reduced resistance to compression of vertebrae but preserved bending strength of long bones. It is important to stress the differences between vitamin A-induced fragile bones and the OVX model. In addition, although the present study on hypervitaminosis A and bone weakening involves young male rats, similar results are noticed with both young and aged female rats [12,13]. The dissimilarity observed in long-bone bending strength between OVXed and hypervitaminosis A animals could at least in part be explained by the observation that vitamin A affects bone perimeter considerably more than BMD, compared to the opposite in ovariectomized animals. Furthermore, OVX induces a persistent increase in serum markers of both osteoclast and osteoblast activities [27,28], which is contrary to our findings of excess dietary vitamin A. Also, OVX treatment induces, whereas excess vitamin A reduces weight gain. It is well known that growth and food intake influences the growth hormone/insulin-like growth factor 1 (IGF-1)–axis which is a major regulator of postnatal growth, including bones. In our study hypervitaminotic rats gained less weight which could be a consequence of reduced serum levels of IGF-1 [29]. Also a recent study investigating the impact of short term calorie restriction on bone parameters showed that calorie restriction reduced serum IGF-1 and PINP without affecting CTX-1 levels [31]. They also showed that calorie restriction decreases

**Fig. 4.** Endothelial marker analysis of bone and serum. (A) Staining blood vessel cells (endothelial) with lcam1 in controls show thin circular staining close to endosteat bone (left part of picture, b=bone, asterix=central large vessel). In bones from rats on a high vitamin A diet there is a distinct reduction in staining throughout the marrow except for the large central vessel. Lower panel show a close-up view of the endosteal/marrow area. Bar = 100 μm in upper panel and 25 μm in lower panel. (B) In controls, periosteal vessels are hardly visible using different endothelial immunological markers (left panel, arrows) whereas in hypervitaminotic animals endothelial staining of the periosteum shows presence of engorged vessels (arrowheads). Bar = 25 μm. (C) lcam1 staining of trabecular compartment. (D) Serum levels of soluble lcam1 measured by ELISA (cont, n = 11 and Ex. vitA, n = 12).
expression of marrow osteoblastic genes and decreases bone stiffness which is in sharp contrast to our findings indicating that the effects we see in our study are mainly vitamin A specific. Furthermore, many hypervitaminosis A studies have investigated other serum factors influencing bone remodeling such as: Ca, phosphate, vitamin D and PTH but there are generally very little changes in these parameters [9,10,14,23,32].

We did not notice any changes in the staining of markers for osteoclast or endothelial cells in metaphyseal trabecular bone compartment which probably reflects that this area is better perfused than the diaphysis. Instead the indications of reduced diaphysal endosteal/marrow blood flow in hypervitaminotic animals are: 1) lack of visible capillary erythrocytes, 2) absence of endothelial staining, 3) loss of luminal blood vessel labeling for the retinol-binding protein, 4) lack of induced expression of RA targets, and 5) increased expression of hypoxia induced genes. A further indication of disturbed blood distribution in hypervitaminotic animals was the increased serum levels of a marker for endothelial injury, sIcam1 [24].

Fig. 5. Bone compartment–specific vitamin A delivery and gene expression. (A) Immunohistochemical staining of bone sections for the serum retinol-binding protein, Rbp4, shows clear intraluminal vessel staining in endosteal and periosteal compartment in control rats (top panel, arrows). In contrast, although animals on excess vitamin A diet show intraluminal vessel staining at the periosteal site (right lower panel, arrows) there is complete lack of staining at the endosteal site (left lower panel, arrowheads). (B) TaqMan analysis of RA direct target genes in CortB and CortB+M (n = 3 or 4 per group). (C) Hypoxia induced gene expression in CortB and CortB+M (n = 3 or 4 per group). (D) CortB+M expression of osteoblastic genes (n = 3 or 4 per group). (E) In vitro analysis of how RA affects osteoblastic and hypoxia gene expression in a murine bone marrow stromal cell line (n = 3 per group).
periosteal osteoclasts considering the potent capacity of RA to inhibit apoptosis [52]. It is also interesting to note the appearance of prolonged endosteal hypoxia could in turn induce osteoclasts at the same time as endosteal ones disappear. An associated the cortical bone may explain how periosteal osteoclasts suddenly antiangiogenic effects, a reduced number of blood vessels penetrating are unlikely to be a major mechanism. Instead, since vitamin A has known osteoclast formation in vitro [33]. However this discrepancy could involve special local osteoclast precursors which readily transform into mature osteoclasts upon stimulation [34]. In line with this, organ culture experiments show that RA induces bone degradation of both calvarial and long bones [35,36]. Although the endosteum in hypervitaminosis A animals has been described with a pathognomonic appearance resembling highly vascularized bone lacunae we were unable to label these structures with endothelial markers and a close histological examination revealed a lack of erythrocytes in these areas, indicating that these structures do not contain functional blood vessels. In fact, hypervitaminotic bones showed a clear gradual reduction of labeling for endothelial markers beginning from the large central marrow vessel. A disturbed endothelial function is consistent with regular findings of hemorrhage in hypervitaminosis A and Ingber and Folkman observed over two decades ago that retinoids are potent angiogenic factors [42]. Similar observations have also been observed in the clinic, in RA treated patients with acute promyelocytic leukemia (APL). Twenty-five percent of APL patients treated with RA develop an adverse complication called retinoic acid syndrome (RAS) which is manifested by fever, respiratory distress, pulmonary edema, episodic hypotension, and occasionally bone pain [37], bone marrow failure [38] and bone marrow necrosis and fibrosis [39]. Importantly, post-mortem studies have suggested that the series of events leading to RAS involves damaged microvasculature and moreover bone marrow biopsies from APL patients before and after treatment with RA, show that RA suppresses angiogenesis [40,41]. These clinical observations are in line with our findings of disturbed endosteal/marrow blood flow. Also consistent with this, marrow from hypervitaminotic animals was found to have increased expression levels of hypoxia induced genes. It is well acknowledged that epithelial (or endothelial) cells transform to a mesenchymal type (EMT, a key profibrotic stimulus) during hypoxia. EMT involves an increased expression of alpha smooth muscle actin (αSMA) and Twist1 expression [43]. In accordance with this we find in hypervitaminotic bone marrow an exclusive increase of Twist1 expression and an αSMA induction capacity of RA has been shown in previous angiogenesis experiments [44]. To get a feeling for the complexity of hypoxia at the gene expression level it was shown in transcriptome experiments with colon carcinoma cells that 4005 genes were altered by a 24 h hypoxic exposure [45]. In vivo experiments with genetically manipulated mice harboring osteoblastic overexpression of Hif1α develop extremely dense bones [46]. It was concluded that increased bone formation was tightly connected to increased blood flow. However, it has also been shown that reduced or lack of blood flow in bones increases BMD or pathological calcification of the marrow, respectively [47,48]. Here we found increased labeling and expression of osteoblastic osteoclasts in the marrow of hypervitaminotic animals apparently lacking functional blood vessels, which resembles descriptions of pathological calcification occurring in ischemic bone marrow [49]. The mechanism behind this may involve precipitation of hydroxyapatite emanating from ischemic tissue, as described recently when the appearance of abnormal calcification after liver transplants were investigated [50]. Here a direct measurement of the bone mineral content using FTIR showed clearly that hypervitaminotic bones contain more phosphate mineral relative to organic matrix content compared to control bones. Although apparently contradictory as this increase is not reflected as an increase in cortical BMD from the pQCT data, this probably reflects the limitations of the pQCT method to accurately measure the density of thin cortical bone. It has been shown that pQCT underestimates BMD values if cortical bone thickness decreases, as is the case with our hypervitaminosis A bones [51]. The FTIR analysis further indicated that the mineral was of immature nature, suggesting that it was not normal bone. Abnormal endosteal bone was also suggested by the diffuse and irregular appearance of the osteocyte lacunar canalicular system in the pathognomonic areas of hypervitaminotic bones. Additional evidence

![Fig. 6. Immunohistological appearance of osteogenic cells, bone mineral analysis and osteocyte network analysis. (A) Osteoblastic staining of control and pathognomonic endosteal area in hypervitaminosis A diaphyseal long bones (bar=25 μm). (B) FTIR analysis of the bone mineral shows that hypervitaminotic bones have a higher degree of mineralization and that the mineral shows a tendency towards being less crystalline (n=12 per group). (C) Staining of the osteocyte network with Dmp1 shows in controls strong staining (arrowheads) close to the marrow and weak staining (arrows) close to the periosteum. In the hypervitaminosis A bone (lower panel) there is strong staining close to the periosteum and staining of the endosteal pathognomonic area is weak and diffuse.](image)

The endosteum is a niche with abundant osteoclasts in young bone, and with age there is an increase of periosteal osteoclasts. In line with earlier observations we find increased numbers of periosteal osteoclasts in hypervitaminotic rat bones. However, the novel observation in this study is that hypervitaminotic animals display a remarkable lack of endosteal osteoclasts. Since the normal life span of osteoclasts in vivo are between 2–4 weeks, inhibition of formation is unlikely to be a major mechanism. Instead, since vitamin A has known antiangiogenic effects, a reduced number of blood vessels penetrating the cortical bone may explain why periosteal osteoclasts suddenly appear at the same time as endosteal ones disappear. An associated prolonged endosteal hypoxia could in turn induce osteoclast apoptosis [52]. It is also interesting to note the appearance of periosteal osteoclasts considering the potent capacity of RA to inhibit episodic hypotension, and occasionally bone pain [37], bone marrow failure [38] and bone marrow necrosis and fibrosis [39]. Importantly, post-mortem studies have suggested that the series of events leading to RAS involves damaged microvasculature and moreover bone marrow biopsies from APL patients before and after treatment with RA, show that RA suppresses angiogenesis [40,41]. These clinical observations are in line with our findings of disturbed endosteal/marrow blood flow. Also consistent with this, marrow from hypervitaminotic animals was found to have increased expression levels of hypoxia induced genes. It is well acknowledged that epithelial (or endothelial) cells transform to a mesenchymal type (EMT, a key profibrotic stimulus) during hypoxia. EMT involves an increased expression of alpha smooth muscle actin (αSMA) and Twist1 expression [43]. In accordance with this we find in hypervitaminotic bone marrow an exclusive increase of Twist1 expression and an αSMA induction capacity of RA has been shown in previous angiogenesis experiments [44]. To get a feeling for the complexity of hypoxia at the gene expression level it was shown in transcriptome experiments with colon carcinoma cells that 4005 genes were altered by a 24 h hypoxic exposure [45]. In vivo experiments with genetically manipulated mice harboring osteoblastic overexpression of Hif1α develop extremely dense bones [46]. It was concluded that increased bone formation was tightly connected to increased blood flow. However, it has also been shown that reduced or lack of blood flow in bones increases BMD or pathological calcification of the marrow, respectively [47,48]. Here we found increased labeling and expression of osteoblastic osteoclasts in the marrow of hypervitaminotic animals apparently lacking functional blood vessels, which resembles descriptions of pathological calcification occurring in ischemic bone marrow [49]. The mechanism behind this may involve precipitation of hydroxyapatite emanating from ischemic tissue, as described recently when the appearance of abnormal calcification after liver transplants were investigated [50]. Here a direct measurement of the bone mineral content using FTIR showed clearly that hypervitaminotic bones contain more phosphate mineral relative to organic matrix content compared to control bones. Although apparently contradictory as this increase is not reflected as an increase in cortical BMD from the pQCT data, this probably reflects the limitations of the pQCT method to accurately measure the density of thin cortical bone. It has been shown that pQCT underestimates BMD values if cortical bone thickness decreases, as is the case with our hypervitaminosis A bones [51]. The FTIR analysis further indicated that the mineral was of immature nature, suggesting that it was not normal bone. Abnormal endosteal bone was also suggested by the diffuse and irregular appearance of the osteocyte lacunar canalicular system in the pathognomonic areas of hypervitaminotic bones. Additional evidence
of increased mineralization of hypervitaminotic bones was provided by the biomechanical properties which changed after 1 week of excess vitamin A. Although hypervitaminotic bones were thinner, stiffness was unchanged and the displacement before failure was significantly less. Both these parameters indicate that hypervitaminotic bones were harder and contained more mineral compared to control bones. Furthermore, bending strength is not only dependent on cortical area but also of bone width as is shown by the formula that the radius of long-bone diaphysis will decrease its resistance to torsion or bending loads by a factor raised to the fourth power. Thus, a decrease in failure load cannot readily be explained by a similar decrease in cortical area without taking the bone width into account.

Also, in this study hypervitaminotic rats showed decreased osteoblastic markers in serum, in spite of increased osteoblastic expression and mineralization in their bones. We believe this is a consequence of reduced blood flow around the endostal site, the very site where the increased mineralization takes place, thus resulting in local inability of proper delivery of osteoblastic markers from these sites by secretion to the bloodstream. Collectively, our data indicate that excessive ingestion of vitamin A induces both bone thinning and increased endosteal mineralization, leading to brittle bones.

In conclusion, we have identified that a relatively rapid primary effect of excess dietary retinol appears to be a reduction in the number of bone vessels penetrating the cortical bone. This in turn leads to hypoxia in the endosteal/marrow compartment, reversal of osteoclastic positioning around diaphyseal bone and ultimately to pathological endosteal mineralization and brittle bones. Revealing the key initiating mechanism of how excess retinol impacts bone tissue is important for designing new observational studies and for understanding/interpretation of acquired data. It is important to recognize novel types of brittle bone conditions which differ from the “standard” postmenopausal osteoporosis condition and is not detected by BMD measurements. Notably, in spite of the rapid bone thinning, serum markers for bone resorption were reduced in hypervitaminotic rats showing decreased hypervitaminotic condition and is not detected by BMD measurements. Notably, in spite of the rapid bone thinning, serum markers for bone resorption were reduced in hypervitaminotic rats showing decreased hypervitaminotic condition and is not detected by BMD measurements. Notably, in spite of the rapid bone thinning, serum markers for bone resorption were reduced in hypervitaminotic rats showing decreased hypervitaminotic condition and is not detected by BMD measurements. Notably, in spite of the rapid bone thinning, serum markers for bone resorption were reduced in hypervitaminotic rats showing decreased hypervitaminotic condition and is not detected by BMD measurements.

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