ABSTRACT: Vitamin K (K) inadequacy may cause bone loss. Thus, K deficiency induced by anticoagulants (e.g., warfarin) may be an osteoporosis risk factor. The skeletal impact of long-term warfarin anticoagulation was evaluated in male monkeys. No effect on BMD or bone markers of skeletal turnover was observed. This study suggests that warfarin-induced K deficiency does not have skeletal effects.

Introduction: The skeletal role of vitamin K (K) remains unclear. It is reasonable that a potential role of vitamin K in bone health could be elucidated by study of patients receiving oral anticoagulants that act to produce vitamin K deficiency. However, some, but not all, reports find K deficiency induced by warfarin (W) anticoagulation to be associated with low bone mass. Additionally, epidemiologic studies have found W use to be associated with either increased or no change in fracture risk. Such divergent results may imply that human studies are compromised by the physical illnesses for which W was prescribed.

Materials and Methods: To remove this potential confounder, we prospectively assessed skeletal status during long-term W anticoagulation of healthy nonhuman primates. Twenty adult (age, 7.4–17.9 yr, mean, 11.7 yr) male rhesus monkeys (Macaca mulatta) were randomized to daily W treatment or control groups. Bone mass of the total body, lumbar spine, and distal and central radius was determined by DXA at baseline and after 3, 6, 9, 12, 18, 24, and 30 mo of W treatment. Serum chemistries, urinary calcium excretion, bone-specific alkaline phosphatase, and total and percent unbound osteocalcin were measured at the same time-points. Prothrombin time and international normalized ratio (INR) were monitored monthly. Serum 25-hydroxyvitamin D was measured at the time of study conclusion.

Results: W treatment produced skeletal K deficiency documented by elevation of circulating undercarboxylated osteocalcin (8.3% W versus 0.4% control, p < 0.0001) but did not alter serum markers of skeletal turnover, urinary calcium excretion, or BMD.

Conclusions: In male rhesus monkeys, long-term W anticoagulation does not alter serum markers of bone turnover or BMD. Long-term W therapy does not have adverse skeletal consequences in primates with high intakes of calcium and vitamin D.


Key words: osteoporosis, vitamin K, warfarin, nonhuman primate

INTRODUCTION

Vitamin K (K) is necessary for the γ-carboxylation of proteins including osteocalcin and confers calcium binding activity. Importantly, vitamin K–dependent proteins, such as osteocalcin and matrix Gla protein (MGP), account for 15–20% of the bone matrix noncollagenous proteins. Osteocalcin is an osteoblast product that appears in bone with the onset of hydroxyapatite deposition, its synthesis is increased by 1,25-dihydroxyvitamin D, and its bone concentration is directly proportional to skeletal calcium content. Similarly, MGP is present in high levels in bone and cartilage. Moreover, it has been documented using an osteocalcin knockout mouse model that vitamin K–dependent proteins have regulatory effects on bone mass. As such, it is not surprising that K insufficiency has been implicated in bone loss and fracture. Specifically, both low dietary K intake and elevated undercarboxylated osteocalcin concentration, a sensitive indicator of K inadequacy, are associated with increased fracture risk. However, not all studies find that low dietary vitamin K intake is associated with increased fracture risk. Moreover, if K deficiency causes bone loss, it is reasonable that patients receiving oral K antagonists as anticoagulants (e.g., warfarin [W]) would be at increased risk for osteoporosis and fracture. However, similar to the status with dietary K intake, some human studies report lower BMD in patients receiving W whereas others do not. Divergent results are also reported for fractures with some but not all studies finding W adminis-
tration to be associated with increased fracture risk. Thus, the role of vitamin K, and specifically of oral anticoagulants, in osteoporosis pathogenesis remains to be clarified.\(^{(20)}\)

Given the large number of anticoagulated patients, it might be assumed that human clinical studies could delineate a potential role of K antagonists in bone health. However, all human studies are confounded by the disease that necessitated anticoagulant therapy. In many cases, these indications consist of cardiac and/or vascular disease that could be anticipated to reduce physical activity. In such individuals, bone loss might result from reduced physical activity and not W. Because prospective clinical trials of W-induced K deficiency will not be conducted in healthy humans because of the potential for severe bleeding complications, this study used an animal model (adult male rhesus monkeys) that closely approximates human skeletal physiology to prospectively evaluate the effect of therapeutic W anticoagulation on markers of skeletal turnover, urinary calcium excretion, and BMD.

**MATERIALS AND METHODS**

**Animals**

Twenty adult male rhesus monkeys were selected from the colony at the Wisconsin National Primate Research Center. All animals were laboratory raised and had known birth dates. At time of study initiation, their mean age was 11.7 yr (range, 7.4–17.9 yr), and their mean weight was 9.9 kg (range, 6.7–14.6 kg). All animals were individually housed to enhance reliability of daily W administration. Twelve-hour light/dark cycles and constant temperature (18–26°C) and humidity (30–70%) were maintained. Free access to Purina monkey chow 5038 containing 0.9% calcium, 6.6 IU vitamin D, and 6 µg of vitamin K (menadione)/g was allowed. On this diet, an average adult male rhesus monkey consuming ~250 g of chow per day ingests 2250 mg calcium, 1650 IU vitamin D, and 1500 µg vitamin K. The animals were randomly assigned to W or control groups. Warfarin (Coumadin; DuPont Pharma, Wilmington, DE, USA) was administered daily in a small food treat (e.g., in gelatin, peanuts, bananas). Monthly protime measurement was used to adjust the warfarin dose with the goal of achieving therapeutic anticoagulation (international normalized ratio [INR] 2.5–3.5). BMD was measured, and blood/urine specimens were obtained at baseline and months 3, 6, 9, 12, 18, 24, and 30. This study was approved by the University of Wisconsin animal research committee and was conducted in compliance with all state and federal guidelines.

**Bone densitometry**

Ketamine/xylazine or inhaled isoflurane anesthesia was used for all DXA scans. A GE Healthcare Lunar DPX (Madison, WI, USA) densitometer with pediatric (v 1.5e) and small animal (v 1.0d) software was used to obtain BMD measurements of the total body, lumbar spine, and radius. Standardized positioning was used as previously described.\(^{(21)}\) Distal and central radius regions of interest chosen to mimic human clinical ulradistal and one-third sites were used. In our facility, BMD precision (%CV) was 1.4%, 1.1%, 1.8%, and 3.3% at the total body, L₁–L₄ spine, one-third radius, and ulradistal radius, respectively. External phantoms were measured at least weekly for the duration of this study and on all scanning days. No densitometer shift or drift was noted during this study.

**Biochemical measurements**

At all sampling time-points (baseline and months 3, 6, 9, 12, 18, 24, and 30), blood was obtained without use of anesthesia by femoral vein phlebotomy between 7:00 a.m. and 9:00 a.m. after an overnight fast. These samples were allowed to clot for 30–45 min at room temperature and centrifuged at 3300–3700 rpm for at least 10 min, and aliquots were frozen at −20°C until thawed for analysis. Urine specimens were obtained by straight catheterization using a sterile technique while the animals were anesthetized before performance of BMD measurement.

Prothrombin times were performed using Simplastin (Organon Teknika, Durham, NC, USA) and a fibrometer at least every 4 wk, and the INR was used to adjust daily warfarin dosing. The target INR (2.5–3.5) was chosen to mimic human therapeutic anticoagulation. Serum calcium, total alkaline phosphatase, albumin, creatinine, and urinary calcium/creatinine ratio was performed by Roche autoanalyzer at a local reference laboratory (General Medical Laboratories, Madison, WI, USA). Bone-specific alkaline phosphatase (BSALP) and osteocalcin (OC) were measured using commercially available kits (Alkphase-B; Mectra Biosystems and ELSA-osteo; CISbio International, respectively). Undercarboxylated OC was measured using a modified hydroxyapatite binding assay. All BSALP and OC assays were run using duplicate samples, and all time-points were included in the same kit to minimize assay variability. Inter- and intra-assay CVs for these assays are 5.1%/7.5% and 3.3%/7.7% for BSALP and OC, respectively. Undercarboxylated osteocalcin was measured using a modification of the hydroxyapatite binding assay as previously reported.\(^{(22)}\) Serum 25-hydroxyvitamin D [25(OH)D] was measured on samples from the time of study conclusion using reverse phase high-pressure liquid chromatography (HPLC) as previously reported.\(^{(23)}\) The intra-assay CV for this system at serum 25(OH)D concentrations of 45 and 82 ng/ml is 2.6% and 3.3% respectively.

**Statistical analysis**

Statview statistical software (Abacus Concepts, Cary, NC, USA) was used to perform repeated-measures ANOVA on all study endpoints. \(p \leq 0.05\) was considered significant.

**RESULTS**

**Animal status**

No animal mortality occurred in either the control or warfarin group during the course of this study. Hematuria was observed in four animals receiving warfarin during the course of this study, at which time an unscheduled protime shift was noted.
determination was performed. In each of these four animals, the INR was >3.5 at the time of clinical hematuria. The hematuria resolved in all cases with temporary discontinuation of W administration, and therapeutic anticoagulation was subsequently able to be re-established without recurrence of clinical bleeding. W was not able to be reliably dosed in one animal; as such, the n of the anticoagulated group was decreased to nine. A decline in body weight in both control and W groups occurred. A colony-wide alteration of feeding strategy was implemented at the Wisconsin National Primate Research Center ~9 mo into this study that led to an increase in body weight over time. W treatment had no effect on body weight (Fig. 1).

Anticoagulation

Therapeutic anticoagulation was achieved (Fig. 2) in the nine animals dosed with W. A mean W dose of 0.15 mg/kg/d was needed to attain an INR in the therapeutic range. If extrapolated to a 70-kg human, this W dose would be ~10.5 mg daily, substantially higher than routinely needed in humans. It is probable that this larger dose requirement reflects the high K content of laboratory monkey chow. Prothrombin time prolongation, and therefore INR elevation, reflects W-induced impairment of coagulation factor K–dependent γ-carboxylation. That W treatment in this study also impaired γ-carboxylation of the bone protein OC was documented by an elevation (p < 0.0001) of undercarboxylated OC at all time-points after treatment initiation (Fig. 3).

Serum/urine chemistry values

Serum creatinine, calcium, total alkaline phosphatase, albumin, aspartate aminotransferase (AST), and urinary calcium to creatinine ratio were unaffected by W administration (data not shown). Serum 25(OH)D was measured only at the time of study conclusion. The mean 25(OH)D did not differ between the warfarin and control groups (188.3 ± 12.5 and 189.3 ± 12.0 [SE]

FIG. 1. Body weight changed during the course of this study reflecting changes in animal feeding patterns at the colony. There was no effect of W anticoagulation on body weight. Data are mean ± SE.

FIG. 2. Effect of W on coagulation. As expected, W treatment increased the prothrombin time and thus the INR. Overall, the target of therapeutic anticoagulation (INR from 2.5 to 3.5) was attained for the study duration. Data are mean ± SE.

FIG. 3. Effect of W on serum undercarboxylated OC. That W administration led to impairment of γ-carboxylation of the bone protein OC is shown by an increase (p < 0.0001) in undercarboxylated OC (%ucOC). Data are mean ± SE.

ng/ml, respectively). Serum BSALP (Fig. 4A) and OC (Fig. 4B) concentrations were unaffected by warfarin administration.

Bone mass

No effect of W treatment on total body BMC or on BMD at the L1–L4 spine or one-third or ultradistal radius was observed (Figs. 5A–5D).

DISCUSSION

W has well-recognized effects on prenatal skeletal development (fetal W syndrome) characterized by nasal hypoplasia and epiphyseal stippling. However, skeletal effects of pharmacologic or dietary K deficiency in adults are far less clear. It has been suggested that K insufficiency may contribute to low bone mass and osteoporotic fracture. If this is the case, K deficiency produced by W would be a currently unrecognized osteoporosis risk factor. However, in this study, W administration was not associated with
bone loss, alteration in serum markers of bone turnover, or increased urinary calcium excretion.

That K inadequacy might contribute to osteoporosis development is suggested by the observation of low serum K concentrations in people with osteoporotic fracture.\(^\text{(25)}\) Additionally, elevated undercarboxylated OC concentration, a sensitive indicator of K insufficiency, is associated with low bone mass and predicts future fracture.\(^\text{(11,26)}\) Furthermore, low K intake is associated with increased osteoporotic fracture risk in epidemiologic studies.\(^\text{(9,10)}\) However, it is axiomatic that association does not prove causation; these observations could simply reflect general undernutrition, rather than a causal role of K insufficiency in bone loss. However, other work suggests that high K intake reduces markers of bone resorption,\(^\text{(27)}\) thus implying that low K status might lead to high bone turnover and bone loss. Consistent with this, some studies report that K supplementation reduces bone loss and fracture risk.\(^\text{(28,29)}\) Finally, strong evidence that K-dependent proteins play some role in regulation of skeletal mass is provided by elevated bone mass in OC knockout mice.\(^\text{(7)}\) Thus, it seems clear that K and/or the K-dependent proteins have the potential to play a role in bone physiology.

However, this study in healthy adult animals with skeletal physiology closely approximating humans finds no adverse effects of W on bone mass. W administration did not affect (A) total body BMC or BMD at the (B) lumbar spine, (C) one-third radius, or (D) ultradistal radius. Data are mean ± SE.

FIG. 4. Lack of effect of W on serum markers of bone turnover. W administration did not affect serum (A) BSALP or (B) OC. Data are mean ± SE.

FIG. 5. Lack of effect of W on bone mass. W administration did not affect (A) total body BMC or BMD at the (B) lumbar spine, (C) one-third radius, or (D) ultradistal radius. Data are mean ± SE.
skeletal effects accompanying long-term W administration. This suggests that the lower BMD and/or increased fracture risk shown in prior studies of W anticoagulation in patients are not the result of direct skeletal effects of K deficiency. Rather, as long-term oral anticoagulation is indicated generally for individuals with physical illnesses that are often cardiovascular in nature, it seems likely that such results reflect bone loss or increased fracture risk related to the diseases for which W was prescribed and are unrelated to K status.

It is worthy of emphasis that laboratory primate diets contain high amounts of calcium and vitamin D. In this study, routine monkey chow containing 0.9% calcium and 6.6 IU of vitamin D per gram was provided to all animals. As an average male rhesus monkey consumes ~250 g of chow per day and weighs ~10 kg, this diet provides 2250 mg of calcium and 1650 IU of vitamin D/kg daily. If directly translated on a per kilogram basis to a 70-kg human, such a diet would provide ~15,750 mg of calcium and 11,550 IU of vitamin D daily, clearly far exceeding current recommendations. As such, it is not surprising that the serum 25(OH)D concentration of these animals is much higher than currently considered optimal for humans.(30) It should be noted that vitamin D-binding kinetics differ between humans and rhesus monkeys, such that monkeys need a higher total 25(OH)D concentration.(31) However, even recognizing this between-species difference, the high dietary vitamin D intake produces 25(OH)D levels substantially greater than in humans. Because calcium and vitamin D supplementation preserve BMD and reduce fracture risk in humans,(32–34) it is possible that the high calcium and vitamin D intake provided by laboratory monkey chow contributed to bone mass preservation in these animals. An additional limitation of this study is the fact that only male monkeys were studied. To our knowledge, there has been no evaluation of vitamin K treatment on osteoporosis in men; however, vitamin K2 (menatetrenone) treatment has been reported to have beneficial skeletal effects in male rats.(35,36) Despite this, it is plausible that vitamin K deficiency may not have adverse skeletal consequence in males but could impact estrogen deficiency bone loss in women. In conclusion, long-term therapeutic anticoagulation with W does not alter serum chemistries, markers of skeletal turnover, urinary calcium excretion, or BMD in male pri- mates with a high dietary calcium and vitamin D intake. These results imply that long-term W anticoagulation does not contribute to osteoporosis in humans.

ACKNOWLEDGMENTS

This study was supported by NIH Grant AG 00801 and was made possible in part by Grant PS1 RR000167 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), to the Wisconsin National Primate Research Center, University of Wisconsin-Madison. This research was conducted at a facility constructed with support from Research Facilities Improvement Program Grants RR15459-01 and RR020141-01. This publication’s contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

REFERENCES


Address reprint requests to:
Neil Binkley, MD
2870 University Avenue, Suite 100
Madison, WI 53705, USA
E-mail: nbinkley@wisc.edu

Received in original form October 14, 2006; revised form December 23, 2006; accepted February 8, 2007.