http://www.cjmb.org

Open Access Original Article

Crescent Journal of Medical and Biological Sciences
Vol. 6, No. 1, January 2019, 100–104
eISSN 2148-9696

The Effects of Warfarin on Bone Metabolism

Farzin Rezazad[1*], Aliakbar Abolfathi[1*]

Abstract

Objectives: Osteocalcin which is s non-collagenous protein plays an important role in bone metabolism and its carboxylation depends on vitamin K. In addition, warfarin inhibits vitamin K epoxide reductase complex and has an anticoagulant effect and therefore interrupts the activity of osteocalcin. Accordingly, this study aimed to estimate the effects of warfarin on bone metabolism. In other words, it was attempted to evaluate the changes in serum levels of bone metabolism in warfarin-taking patients and find the relationship between the inhibition of vitamin K metabolism by warfarin and their synergistic effects on bone metabolism.

Materials and Methods: A total of 72 individuals were selected including healthy controls (n = 36) and patients (n = 36) who referred to clinical laboratories of Tabriz after consuming warfarin. Nearly 5 ml blood samples were collected and non-carboxylated osteocalcin experiments, parathyroid hormone (PTH), vitamin D3, calcium, phosphorus, magnesium, and alkaline phosphatase were performed for all the samples of each group.

Results: In this study, non-carboxylated osteocalcin, PTH, and international normalized ratio (INR) increased significantly in the case group while increasing of Mg^{2+} was non-significant in this group. However, vitamin D3, Ca2+, alkaline phosphatase (ALP), and P demonstrated a significant decrease in the case group compared to the control group.

Conclusions: In general, the consumption of warfarin leads to bone and vessel wall damages, causing cardiovascular diseases.

Keywords: Warfarin, Bone metabolism, Osteocalcin

Introduction

Bone is a metabolically active organ which has a permanent renewal in the life cycle. This constant renewal is necessary for maintaining the structural integrity and supplying the metabolic functions of the bone as a reservoir for calcium, phosphate, and magnesium (1). In addition, bone is structured by inorganic minerals such as calcium and phosphorus and the organic matrix. Approximately 90%-95% of the bone matrix is of type I collagen (1). The osteocalcin is considered a significant non-collagenous protein which plays an essential role in bone metabolism. Further, osteocalcin, which is known as the gamma-carboxy glutamic acid of the bone is a marker of bone formation whose carboxylation is dependent on vitamin K (2-4). Osteocalcin is a marker for a bone formation which is produced by the osteoblasts and plays an important role in the accumulation of bone minerals such as calcium which are involved in bone mineralization and calcium ion homeostasis (5). Furthermore, alkaline phosphatase is another marker for monitoring the activity of the bone metabolism which is a tetrameric glycoprotein and its serum level reflects the activities of bone metabolism (6,7). Moreover, warfarin is one of the most effective oral anticoagulant drugs prescribed to stabilize the international normalized ratio (INR). Totally, 99% of this drug binds to the albumin after oral absorption while only 1% becomes inactive by CYP2C9 liver enzyme and excretes in the bile. Warfarin exposes its anticoagulation effect by competitive inhabitation of vitamin K epoxide reductase enzyme complex which is responsible for recycling the restored vitamin K (K1H2) needed for gamma-carboxylation (8-12). The K1H2 is a required co-factor for the gamma-glutamyl carboxylase enzyme and is converted into inactive metabolite and epoxide vitamin K1 for conversion of glutamic to gamma-carboxy glutamic. Then, once again the inactive form of vitamin K is restored in the vitamin K cycle by VKORC1 liver enzyme to the active co-factor for the enzyme carboxylase. Warfarin inhibits this enzyme and reduces the restored vitamin K1 and activated coagulation factors delaying the blood clotting. It is suggested that vitamin K2 may play an important role in maintaining the health levels of the bone mineral density. The strong relationship between a lack of vitamin K2 and bone health disorders is proven by both clinical and laboratory studies. The osteocalcin is not converted from the inactive mode to activated carboxyl mode and thus calcium cannot be placed in the bone without the presence of vitamin K2

Received 25 December 2016, **Accepted** 10 April 2017, **Available online** 2 May 2017

[1]Department of Biology, Islamic Azad University, Ahar Branch, Ahar, Iran.

***Corresponding Authors:** Farzin Rezazad, Tel: +989128950747, Email: farzinre@ymail.com; Aliakbar Abolfathi, Tel: +989143115885, Email: Abolfathi.Aliakbar@gmail.com

(13-15). As previously explained, calcium is another major non-organic mineral (1) and the balance between vitamin D3 and parathyroid hormone (PTH) has a fundamental role in regulating the serum calcium homeostasis and ultimately bone deformities. Disruption of this balance is normally followed by a lack of vitamin D3, increased secretion of PTH, and in the long-term, a decrease in bone mass and eventually osteoporosis which is defined as the loss of bone mass per unit volume. In a situation of serum calcium deficiency, PTH increases the calcium absorption from the renal tubules by activating the osteoclasts and releasing the calcium from the bone and finally increases the conversion of vitamin D into its active form, namely, 1,25-dihydroxycholecalciferol in the kidney, facilitating the absorption of calcium from the gut. Accordingly, the most important biological function of vitamin D occurs in the skeletal system by increasing the intestinal absorption of the calcium. Vitamin D deficiency leads to a decreased intestinal absorption of calcium from 30%-15% which causes an increase in PTH and thus osteoporosis. In this study, the relationship between changes in bone markers among people who took warfarin and were subjected to changes in the metabolism of vitamin K are discussed. Finally, the risk of osteoporosis and cardiovascular disease among these patients was investigated (16-22).

Materials and Methods

Samples

The statistical population of this study included 36 patients (cases) with a history of at least 6 months of warfarin consumption who were referred to a medical laboratory in Tabriz, Iran, as well as 36 healthy individuals who took no warfarin (as the control group). They completed the questionnaires after giving written consent.

The Serum Factor Measurement

Blood samples (5 mL) were collected from each patient and the control group members, and after serum separation (centrifugation), the serum samples were stored at -70°C for further tests. PTH was measured by the ECL sandwich method (Electro-Chemi Luminescence) using the kits by the Swiss Roche and Cobas E411. Overall, measuring methods were as follows: Serum samples with biotinide and Ruthenium PTH monoclonal antibodies were added to the plates by the sandwich method. Then, streptavidin was added after the incubation and the result was read based on ECL phenomenon. The 25-OH vitamin D was measured by competitive ELISA using Immundiagnostik kit and Stat Fax ELISA reader. Additionally, data related to the patients and the control group' serums, standards, and the separator solution of vitamin D3 from its carrier protein (VDBP vitamin D3 from its carrier protein) were added to each well of the plate 1. Then, 20 Landa was removed from the microplate 1 and added to the corresponding wells in the plate, and the plate was then mixed for 20 seconds. In addition, the vitamin D3 antibody was added after incubation at room temperature and then the mixture of all the plates was washed. Further, the conjugate solution was added to all the wells and the wells were incubated. After washing, a substrate solution was added to all the wells and incubated, and then Stop solution was added to all the wells. These results were obtained by ELISA reader in the 450-630 nm wavelength. Ca was measured by auto-analyzer using the photometric method and Arsenazo III. In this experiment, calcium constructed a blue color complex in a neutral medium with the Arsenazo III. The intensity of the produced color was proportional to the calcium amount of the sample. Furthermore, the absorbance was read at 630 nm. The alkaline phosphatase was measured at 405 nm by the DGKC method (the Standard of Germany Society of Biochemistry) using the Hitachi auto-analyzer. Moreover, phosphor and magnesium were estimated employing the photometric method UV-test and Xylidyl blue by the Hitachi auto-analyzer at 340 and 546 wavelengths, respectively. Finally, undercarboxylated osteocalcin (ucOC) was measured by the sandwich ELISA method utilizing the crystal kites of Bioassay Technology Laboratory and Stat Fax ELISA reader. Additionally, to perform the experiment, the test samples were added to the wells and then ucOC antibody and streptavidin-HRP solutions were added and incubated at 37°C after mixing. The chromogenic solutions A and B were added after washing and incubated after mixing. Stop solution was then added to all the wells so that the color was changed from blue to yellow in the wells. Afterward, the results were obtained by ELISA reader at a wavelength of 450 nm. The prothrombin (PT) was estimated by coagulation method (clot) using the Stago Compact Max. Eventually, plasma and calcium thromboplastin solutions were mixed and the clotting time was measured by the device accordingly.

Statistical Analysis

The mean of each variable was calculated in each group, and the data were analyzed using the SPSS software, version 21 and independent sample *t* test method. All data are presented as the mean ± SD and the significance level was considered $P < 0.05$.

Results

Demographic Data

The mean of some clinical parameters was measured while no significant difference was observed between the two groups (Table 1).

The Serum Factors

Table 2 represents the levels of serum factors in both groups. Based on the data, the levels of all factors demonstrate a significant difference except for Mg^{++}.

The Correlation Between PTH and Other Factors

As shown in Table 3, there is a correlation between PTH

Table 1. Demographic Data of the Patients and Healthy Individuals

Parameters	Control	Case	*P* Value
Gender			
Male	16	16	0.12
Female	20	20	
Weight	69.88 ± 9.23	73.20 ± 8.18	0.53
Age	43 ± 12.69	42.08 ± 12.18	0.60

Table 2. Mean of Serum Parameters in Both Groups

Parameters	Control	Case	*P* Value
PTH (pg/mL)	39.08 ± 1.52	70.38 ± 5.91	0.02
OSN (ng/mL)	306.85 ± 68.09	600.05 ± 136.37	0.03
D3 (nmol/L)	44.31 ± 19.33	24.43 ± 6.11	0.01
ALP (U/L)	232.55 ± 70.41	184.60 ± 44.22	0.01
Ca^{2+} (mg/dL)	9.35 ± 0.280	9.03 ± 0.291	0.00
Mg^{2+} (mg/dL)	1.97 ± 0. 10	2.00 ± 0.20	0.46
P (mg/dL)	3.68 ± 0.40	3.35 ± 0.46	0.03
INR (min)	1.00 ± 0.0	2.42 ± 0.99	0.00
PT (min)	12.0 ± 0.0	17.66 ± 3.72	0.00

Abbreviations: PTH, parathyroid hormone; OSN, osteonectin; ALP, alkaline phosphatase; INR, international normalized ratio; PT, prothrombin time.

Table 3. Pearson Correlation of PTH With Other Biomarkers of the Bone Metabolism

Parameters	Mean ± SD	Correlation with PTH
PTH	70.38±5.91	1
OSN	600.05±136.37	0.78
D3	24.43±6.11	0.016
ALP	184.60±44.22	0.967
Ca	9.03±0.291	0.011
Mg	2.00±0.20	0.364
P	3.35±0.46	0.134
PT	17.66±3.72	0.031

Abbreviations: PTH, parathyroid hormone; OSN, osteonectin; ALP, alkaline phosphatase; PT, prothrombin time.

and other factors such as PT, Ca^{++}, and vitamin D.

Discussion

Warfarin is an anticoagulant which reduces blood clotting and is used to stabilize the INR which inhibits the synthesis of vitamin K-dependent clotting factors. Overall, 99% of the drug binds to albumin after the oral absorption and only 1% becomes inactive by the CYP2C9 liver enzyme and is excreted by the bile (8). Warfarin applies its anticoagulant effect by competitive inhibition of the enzyme complex (vitamin K epoxide reductase). The vitamin K epoxide reductase is responsible for recycling the restored vitamin K required for gamma-carboxylation (8-12). In this study, calcium significantly decreased in the case group compared to the control group (Table 2). Lack of vitamin K due to the use of warfarin and thus the increased INR results in decreasing the serum calcium. This is consistent with the results of Helin et al (23) and Sokol'nikov et al (24) (Tables 2 and 3). In some cases, warfarin causes a 50% increase in urinary excretion rather than affecting the absorption of calcium through the intestines. Additionally, warfarin leads to the calcification of blood vessels which reduces blood calcium accordingly (23). Osteocalcin is regarded as a non-collagenous protein in bone formation which is dependent on vitamin K (5). This protein is activated in the carboxylated glutamic residue after the translation process while this conversion requires vitamin K which is inhibited by warfarin. Therefore, it is anticipated that the ratio of ucOC to carboxylated osteocalcin increases over the period of warfarin consumption since carboxylated osteocalcin is a form of osteocalcin which has a high affinity for hydroxyapatite (25). Based on the findings, the osteocalcin significantly increased in the case group compared to the control group (Table 2), which is not in line with the results of Menon et al (25). As a result, there is a reverse relationship between the undercarboxylated osteocalcin and serum calcium. This implies that the amount of osteocalcin significantly increased in the control group while calcium significantly reduced in this group compared to the case group. PTH is considered another important factor in regulating blood calcium and bone metabolism (26). It facilitates the absorption of calcium from the gut in calcium deficiency situation, by activating the osteoclasts and absorbing the calcium from the bones. In addition, PTH increases the calcium absorption from the renal tubules and finally converts vitamin D to its active form, that is, 1, 25-dihydroxyvitamin cholecalciferol in the kidneys. Therefore, this hormone plays an indirect role in calcium metabolism in the intestinal environment (27). Based on the findings of this study, PTH levels increased significantly (Table 2). The other variables which were measured included serum levels of phosphorus and magnesium. Serum level of phosphate significantly decreased in the case group compared to the control group while that of the magnesium increased insignificantly in the case group compared to the control group. One reason for such a reduction in phosphorus is the lower level of PTH and calcium (Table 2). Magnesium absorption was subjected to the parameters which were discussed in the calcium case. However, the interference of vitamin D in absorbing the magnesium has not yet established. Similar to calcium, PTH increases reabsorbing the magnesium which is necessary for the natural release of PTH (27). Further, alkaline phosphatase is a metalloenzyme which contains zinc and plays a significant role in bone mineralization and substrate hydrolysis of the phosphate in bone mineralization. The level of alkaline phosphatase significantly reduced in the current study which is not consistent with the results of Stenova et al (29). The mechanism for this effect is not clear. However, it is thought that warfarin affects the expression levels of many proteins involved in bone metabolism including alkaline phosphatase (28). The level of vitamin D3 was another

variable which was evaluated in the current study (Table 2). The vitamin D3 level significantly reduced in the case group compared to the control group and this reduction is consistent with an increase in PTH since vitamin D3 inhibits PTH secretion. The result contradicts the results obtained by Stenova et al (29). Furthermore, INR is an index of the prothrombin time in which the effect of warfarin is related to the serum calcium concentration. The prothrombin or INR in case group increased significantly compared to the control group in the present study. Based on the results of this study, warfarin may increase the INR which is inconsistent with the findings of Helin et al (23). Moreover, a correlation was observed between the osteocalcin and INR while PTH had no correlation ($P > 0.05$) with other parameters of the study (Table 3). In general, the study sought to investigate the correlation between PTH and other variables. It was found that there is a correlation between this hormone and alkaline phosphatase. Additionally, a correlation was observed among vitamin D3, PTH, calcium, and PT. Conversely, alkaline phosphatase represented no correlation with any of the above parameters while calcium is only correlated with PTH. Finally, phosphorus and magnesium demonstrated no correlation with any of the parameters under investigation.

Conclusions

Despite the fact that bone metabolism is associated with different parameters, it seems that the three parameters osteocalcin, PTH, and vitamin D3 are more involved in this respect. Vitamin D3 changes were observed in patients by a reduction in taking warfarin. Therefore, using warfarin may have an impact on the absorption and metabolism of this vitamin. Based on the results of the current study, it can be concluded that the consumption of warfarin as an antagonist of vitamin K affects many body parameters associated with bone metabolism, especially calcium which in this case, it can cause cardiovascular diseases and bone diseases including osteoporosis.

Conflict of Interests

Authors have no conflict of interests.

Financial Support

None.

References

1. Sheth RD. Bone health in pediatric epilepsy. Epilepsy Behav. 2004;5 Suppl 2:S30-35. doi:10.1016/j.yebeh.2003.11.025
2. Price PA, Parthemore JG, Deftos LJ. New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. J Clin Invest. 1980;66(5):878-883. doi:10.1172/jci109954
3. Price PA, Baukol SA. 1,25-Dihydroxyvitamin D3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J Biol Chem. 1980;255(24):11660-11663.
4. Price PA, Nishimoto SK. Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. Proc Natl Acad Sci U S A. 1980;77(4):2234-2238.
5. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. 2007;130(3):456-469. doi:10.1016/j.cell.2007.05.047
6. Delmas PD, Christiansen C, Mann KG, Price PA. Bone Gla protein (osteocalcin) assay standardization report. J Bone Miner Res. 1990;5(1):5-11. doi:10.1002/jbmr.5650050104
7. Rudberg A, Magnusson P, Larsson L, Joborn H. Serum isoforms of bone alkaline phosphatase increase during physical exercise in women. Calcif Tissue Int. 2000;66(5):342-347.
8. Daly AK, King BP. Pharmacogenetics of oral anticoagulants. Pharmacogenetics. 2003;13(5):247-252. doi:10.1097/01.fpc.0000054071.64000.bd
9. Hirsh J, Dalen JE, Deykin D, Poller L, Bussey H. Oral anticoagulants. Mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 1995;108(4 Suppl):231s-246s.
10. Redman AR. Implications of cytochrome P450 2C9 polymorphism on warfarin metabolism and dosing. Pharmacotherapy. 2001;21(2):235-242.
11. Dumas TE, Hawke RL, Lee CR. Warfarin dosing and the promise of pharmacogenomics. Curr Clin Pharmacol. 2007;2(1):11-21.
12. Li T, Lange LA, Li X, et al. Polymorphisms in the VKORC1 gene are strongly associated with warfarin dosage requirements in patients receiving anticoagulation. J Med Genet. 2006;43(9):740-744. doi:10.1136/jmg.2005.040410
13. Booth SL, Broe KE, Peterson JW, et al. Associations between vitamin K biochemical measures and bone mineral density in men and women. J Clin Endocrinol Metab. 2004;89(10):4904-4909. doi:10.1210/jc.2003-031673
14. Knapen MH, Nieuwenhuijzen Kruseman AC, Wouters RS, Vermeer C. Correlation of serum osteocalcin fractions with bone mineral density in women during the first 10 years after menopause. Calcif Tissue Int. 1998;63(5):375-379.
15. Schurgers LJ, Knapen MHJ, Vermeer C. Vitamin K2 improves bone strength in postmenopausal women. Int Congr Ser. 2007;1297:179-187. doi:10.1016/j.ics.2006.08.006 Knapen MH, Schurgers LJ, Vermeer C. Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. Osteoporos Int. 2007;18(7):963-972. doi:10.1007/s00198-007-0337-9
16. Dawson-Hughes B, Harris S, Dallal GE. Serum ionized calcium, as well as phosphorus and parathyroid hormone, is associated with the plasma 1,25-dihydroxyvitamin D3 concentration in normal postmenopausal women. J Bone Miner Res. 1991;6(5):461-468. doi:10.1002/jbmr.5650060506
17. Weinstein RS, Underwood JL, Hutson MS, DeLuca HF. Bone histomorphometry in vitamin D-deficient rats infused with calcium and phosphorus. Am J Physiol. 1984;246(6 Pt 1):E499-505. doi:10.1152/ajpendo.1984.246.6.E499
18. Konis J. Osteoporosis, Black Well Science; 1994:56-62.
19. LeBoff MS, Kohlmeier L, Hurwitz S, Franklin J, Wright J, Glowacki J. Occult vitamin D deficiency in postmenopausal US women with acute hip fracture. JAMA. 1999;281(16):1505-1511.
20. Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud

SB, DeLuca HF. Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients: effect of age and dietary calcium. J Clin Invest. 1979;64(3):729-736. doi:10.1172/jci109516
21. Riggs BL, Nelson KI. Effect of long term treatment with calcitriol on calcium absorption and mineral metabolism in postmenopausal osteoporosis. J Clin Endocrinol Metab. 1985;61(3):457-461. doi:10.1210/jcem-61-3-457
22. Schacht E. Rationale for treatment of involutional osteoporosis in women and for prevention and treatment of corticosteroid-induced osteoporosis with alfacalcidol. Calcif Tissue Int. 1999;65(4):317-327.
23. Helin TA, Wickholm N, Kautiainen H, Vapaatalo H. Possible correlation between INR and serum calcium. Pharmacol Pharm. 2014;5(13):1180-1184. doi:10.4236/pp.2014.513129
24. Sokol'nikov AA, Kodentsova VM, Sergeev IN, Strunin SE, Sklimov OA. [Calcium metabolism in the case of vitamin D and K deficiencies]. Vopr Pitan. 1989(1):56-60.
25. Menon RK, Gill DS, Thomas M, Kernoff PB, Dandona P. Impaired carboxylation of osteocalcin in warfarin-treated patients. J Clin Endocrinol Metab. 1987;64(1):59-61. doi:10.1210/jcem-64-1-59
26. Uhlig K, Berns JS, Kestenbaum B, et al. KDOQI US commentary on the 2009 KDIGO Clinical Practice Guideline for the Diagnosis, Evaluation, and Treatment of CKD-Mineral and Bone Disorder (CKD-MBD). Am J Kidney Dis. 2010;55(5):773-799. doi:10.1053/j.ajkd.2010.02.340
27. Sakai R, Eto Y. Involvement of activin in the regulation of bone metabolism. Mol Cell Endocrinol. 2001;180(1-2):183-188.
28. Blair HC, Athanasou NA. Recent advances in osteoclast biology and pathological bone resorption. Histol Histopathol. 2004;19(1):189-199. doi:10.14670/hh-19.189
29. Stenova E, Steno B, Killinger Z, Baqi L, Payer J. Effect of long-term oral anticoagulant therapy on bone mineral density and bone turnover markers: a prospective 12 month study. Bratisl Lek Listy 2011;11(2):71-76.