The Effect of the Combination of Vitamin K2 and Genistein, Coumestrol and Daidzein on the Osteoblast Differentiation and Bone Matrix Formation

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Abstract
Multiple studies have been reported the stimulatory effect of the combinations of nutrients factors on bone formation. One such factor is vitamin K2 which can be associated with bone protective activities. The effect of vitamin K2 alone and in combination with genistein, coumestrol and daidzein on osteoblast differentiation and mineralization were tested. Significantly, vitamin K2 increased bone mineralization in combination with genistein (10^{-5}M), coumestrol (10^{-7}M) and daidzein (10^{-5}M). However, there is no additive effect of this vitamin on alkaline phosphatase (ALP) levels in osteoblasts. By contrast, vitamin K2 enhanced the stimulatory effect of type I collagen and osteocalcin expression. Vitamin K2 alone increased RUNX and OSX expression while there is no synergistic effect with tested compound; this vitamin also did not modulate nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) ratio expression. These results suggested that vitamin K2 can be more effective factor in the presence of phytoestrogens on the improvement of bone formation after menopause.

Keywords: vitamin K2, phytoestrogens, osteoblast, bone resorption, bone matrix

Introduction
The skeleton remodels in response to changes in mechanical load, Ca^{2+} and damage [1,2]. Bone remodeling is performed by osteoblasts that secrete and mineralize new bone matrix and osteoclasts that resorb bone. Osteoblast and osteoclast are regulates during bone formation and any disruption causes bone resorption such as post-menopausal osteoporosis and osteomyelitis [3]. Previous study shown that phytoestrogens (PEs) suppressed osteoclast differentiation and bone resorption, induced osteoblast differentiation and bone matrix formation as well [4] and also increased bone matrix formation following phytoestrogen supplementation in women [5,6]. Vitamin K identified as a fat-soluble nutrient required for coagulation and also acts as a co-factor for carboxylase, an essential enzyme for γ-carboxylation of vitamin K-dependent osteocalcin and Gla proteins [7]. However, the main function of vitamin K is the maintenance of the blood clotting proteins levels that synthesized as inactive proteins in the liver. Importantly, vitamin K species (K1 and K2) is known to promote γ-carboxylation of glutamic acid residues to vit K-dependent osteocalcin bone matrix and also possess both anti-osteoclastic and pro-osteoblastic action [8,9]. Moreover, vitamin K2 has long been considered as potent protective factor against osteoporosis, hepatocarcinoma, and atherosclerosis [10,11]. Interestingly, several clinical trials detected the positive correlation between bone fracture and vitamin K deficiency [12-14]. Additionally, vitamin K1 acts as antioxidant agent, whereas vitamin K2 (MK-4) proposed as a protective agent for neural cells from apoptosis by decreasing oxidative stress [15]. Flavones found to contain soybeans, menaquinon-7 (K7) and cryptoxanthin, which have been revealed to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption in vitro. It was found that the supplementation with vitamin K2 prevented
bone loss in ovariectomised (OVX) rats, and its intake has a stimulatory effect on bone mass in human. Therefore, accumulated studies paid the attention toward the important role of vitamin K in bone metabolism. Natural vitamin K₂ (MK-7) with seven isoprene unites is abundant in fermented soybeans (natto) and has anabolic effect on bone calcification. In contrast, vitamin K₂ has been caused a significant increase in alkaline phosphatase (ALP) activity and calcium content in femoral-metaphyseal tissues in vitro. Furthermore, MK-7 (10⁻⁶ or 10⁻⁵M) caused a significant increasing in calcium content, ALP activity and DNA contents in cortical bone and trabecular bone in ovx rats. The oral administration of Vitamin K₂ to neuroectomised rats also was found to reduce trabecular bone loss, prevention of osteoblast dysfunction, and increasing bone formation rate [16]. In addition, this effect enhanced in combination with genistein (10⁻⁵, 10⁻⁶M), suggestion the mode of action of this vitamin is differs from genistein, and MK-7’s activity was abolished in the presence of cyclohexamide [17].

Thus, the combination of these two factors may be useful in the prevention of bone resorption and disorders. Recent study found that vitamin K increased the mineralisation in primary human cells, although this was associated with decreasing in type-I collagen expression [18]. Vitamin K₂ also influenced bone formation through inhibited apoptotic cell death and maintenance of osteoblast via a mechanism involves inhibition of Fas expression on osteoblast treated with or without TNF-α [19].

Several studies suggest that the dietary factors such as Zn and phytoestrogens have a positive effect on bone cell activity [4, 20–22]. However, these studies have not fully examined the effect of combinations of phytoestrogens (PEs) and Vit K₂ on osteoblast differentiation and activity. Therefore, the current study examined the effect of genistein, coumestrol and daidzein in the presence of vitamin K₂ on osteoblast differentiation, function and bone matrix formation in-vitro.

Materials and Methods

Cell culture

Saos-2 human osteoblast like cells were obtained from ECACC, Porton Down, UK and cultured in RPMI1640 media supplemented with 10% charcoal stripped fetal calf serum, 2mmol/l glutamine, 100IU/mL benzylpenicillin and 100 mg/mL streptomycin [23].

Effect of Vitamin K₂ on alakaline phosphatase (ALP) activity and mineralization

The effect of coumestrol, daidzein and genistein at (10⁻⁵ to 10⁻⁹ M) concentration, in the presence or absence of vitamin K₂ (10⁻⁵ M) on alkaline phosphatase activity was assessed as follows. Saos-2 cells were incubated incubated with treatments in presence of β-glycerophosphate (β-GP) (10 mM) and L-ascorbic acid (L-AA) (50 mg/l) for four days. ALP activity measured by staining cultures with p-nitrophenyl phosphate (1 mg/ml) at 37ºC for 30 min [24]. Absorbance was measured at 405 nm. Mineralization was assessed using a modification of Hale’s methodology [25]. After 15 days of incubation culture, the cells incubated with medium containing 1 µg/ml calcein for four hours at 37ºC, and fluorescence measured by a cytofluor II fluorescence multiwell plate reader (Perseptive Biosystem, USA).

Real time quantitative PCR analysis

Saos-2 cells were incubated with PEs with or without vitamin K₂. Total RNA was extracted using a Sigma genelute RNA isolation kit and reverse-transcribed with Moloney Murine Leukemia Virus RT using random nonamer primers. Real-time PCR was performed on a StepOne PCR system (Applied Biosystems) using the DNA-binding dye SYBR green for detection of PCR products. Primers for genes were as follows: human Runx-related transcription factor 2 (Runx2) forward AGACCCCCAGCGGAGACGAGT, reverse GCGGGCTACAGCCATCGGTGA; human osterix forward GCACCTGGAGGCAACTGGC, reverse GAGCTGGTAGGGGCTCGTG; human type I collagen forward CCTGGCAGCGCCTCTGCTGA, reverse CTTGCCGGCCTCCAGCGAG; human receptor activator of NF-κB ligand [26] forward ACACGGCTTTTCAAGGAGGCTGTC; human osteoprotegerine (OPG) forward AAATGCCACCCCAACCGCTG reverse AGCAGGAGACCAAGACACTGCA; human β-actin forward GCAGGCGCTACAGCTCCTCA, reverse TGGGCCGTCAGGCCAGGCTCGTA.

Statistical analysis

Differences between groups were assessed using Fisher’s one-way ANOVA (SPSS Inc., Chicago, IL, USA). A difference of P≤ 0.05 was considered statistically significant.
Results and Discussion

Effect of vitamin K$_2$ on RANKL/OPG ratio and Mineralization

This study found that vitamin K$_2$ alone or with genestein and coumestrol had no direct effect on RANKL/OPG ratio expression, increased the inhibitory effect of daidzein on this ratio, however this was not significant when compared to control or daidzein alone Figure (1).

![Graph showing the effect of vitamin K$_2$ on RANKL/OPG ratio](image)

Fig. (1): The effect of vitamin K$_2$ on the expression of nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) Ratio RANKL/OPG ratio. G10$^{-7}$= genistein 10$^{-7}$M, C10$^{-7}$= coumestrol 10$^{-7}$M, D10$^{-5}$= daidzein 10$^{-5}$M.

(Vitamin K$_2$ alone had no significant effect on mineralization; the addition of vitamin K$_2$ significantly enhanced the mineralization induced by genistein (10$^{-5}$ M) Figure (2A), coumestrol (10$^{-7}$M) Figure (2B), and daidzein (10$^{-5}$M, 10$^{-6}$M) Figure (2C) in comparison to test compounds alone, indicating the positive effect of vitamin K$_2$ on inorganic bone minerals deposition.
Fig. (2): Synergistic effect of vitamin K$_2$ with phytoestrogens on bone mineralization.

* Values are significantly different from phytoestrogens treatment alone.

Gen= genistein, Cou= coumestrol, Dai= daidzein.

Effect of vitamin K$_2$ on ALP activity
The results found that vitamin K$_2$ had no effect on ALP activity in combination with coumestrol or genistein, although these compounds alone and in combination with vitamin K$_2$ increased ALP expression versus control group Figure (3A, B). However, the addition of vitamin K$_2$ enhanced ALP expression with daidzein (10$^{-5}$ M) Figure (3C).
Effect of vitamin K2 on type I collagen, osteocalcin, Runx2 and osx gene expression

Vitamin K2 alone decreased type I collagen or osteocalcin expression, but vitamin K2 addition augmented the stimulatory effect of coumestrol and daidzein on type I collagen and osteocalcin expression and had no effect appeared in combination with genistein on type I collagen expression and also reduced the stimulatory effect of genistein on osteocalcin expression to the comparable level of control group Table (1). Vitamin K2 alone increased Runx2 and osterix expression; however this vitamin did not enhance the stimulatory effect of genistein, coumestrol and daidzein on both gens expression. However, All tested compounds have been shown to induce Runx2 and Osterix expression versus control group, coumestrol (10^{-7} M), daidzein (10^{-5} M) and genistein (10^{-7} M) Table (2).

Table (1): The effect of vitamin K2 on the expression organic components of bone matrix in combination with genistein, coumestrol and daidzein

<table>
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<tr>
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<th>Type I collagen mRNA copies</th>
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<tr>
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<td>Genistein (10^{-7}M) and K2</td>
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a P≤0.05 versus control, b P≤0.05 versus relevant PEs alone.

Fig. (3): Synergistic effect of of vitamin K2 with phytoestrogens on ALP activity.

* P≤ 0.05 versus phytoestrogens treatment alone. Gen= genistein, Cou= coumestrol, Dai= daidzein.
Bone remodeling is a process including bone resorption and bone formation that generates a skeleton optimized to mechanical and mineral requirements. Several studies observed the positive effect of dietary factors such as phytoestrogens on bone cell differentiation and activity. However the cellular mechanism mediating the action of the combination of PEs and vitamin K2 on osteoblasts is still open area to debate. This study clarifies the cellular mechanism through the appropriate combination of these factors may augment osteoblast differentiation. Bone resorption is regulated by osteoblast signals that stimulate osteoclast formation from monocytic precursors. Calcium fall in serum act a resorptive stimuli that increase RANKL expression and decreased in OPG (soluble decoy receptor for RANKL) expression on osteoblast surface [27]. Vitamin K2 has long been considered as potent protective factor against osteoporosis, hepatocarcinoma, and atherosclerosis [10, 11]. Interestingly, several clinical trials detected the positive correlation between bone fracture and vitamin K deficiency [12-14].

In this study, Vitamin K2 significantly increased the mineralization peak induced by genistein, coumestrol and daidzein concluded that vitamin K2 has protective effect on non-organic matrix bone formation. The stimulatory effect of vitamin K2 on mineralization was noted also on organic bone matrix, type I collagen and osteocalcin expression in combination with phytoestrogens, indicating that the vitamin K2 can enhance the anabolic effect of phytoestrogen in appropriate concentration and increased bone matrix formation.

In opposite, vitamin K2 has not been shown to enhance ALP activity induced with phytoestrogens except with daidzein at (10^{-5} M) concentration which this activity significantly increase after vitamin K2 addition, although ALP activity still significant versus control group. The present result suggested that the augmentative effect of vitamin K2 on mineralization did not mediate through ALP activity.

This study aimed to examine the mechanism by which phytoestrogens and zinc or vitamin K2 augment osteoblast function, current study determined the expression of key regulators of osteoblast differentiation. Osteoblastogenesis is a sequential process involving transcription factors that lead to stimulate mesenchymal precursors to form mature osteoblasts [28]. The initial stage of osteoblast differentiation is controlled by the expression Runx2 and Osterix.

Vitamin K2 alone increased Runx2 and Osterix expression but there is no additive effect of this vitamin on the anabolic effect of anti-osteoclastic dose of genistein, coumestrol and daidzein on early key expression responsible for osteoblastic expression RANKL/OPG ratio) This results is consistent with ALP activity that explained there is no effect on early stage on osteoblast differentiation while the major effect was detected on bone matrix formation, type I collagen and osteocalcin expression which is consistent with mineralization results, which may be the mechanism mediated the positive effect of this vitamin on bone formation.

In addition to the effect of vitamin K2 on RUNX2 and OXs, it is also increased the peak of osteocalcin and type I collagen levels. Additionally, vitamin K2 was noticed directly induced both organic and non-organic bone formation and no effect appeared on early stage and ALP activity of osteoblast differentiation and osteoclastic stimuli RANKL and OPG expression which may be the mechanism mediated the anabolic effect of this vitamin on bone formation.

These findings conducted that vitamin K2 can be more effective in combination with tested phytoestrogens through different mechanisms involving increasing of osteoblasts differentiation and mineralization. The current study found that the anabolic effect of vitamin K2 mediated in biphasic way, vitamin K2 can enhance mineralization, type I collagen and osteocalcin formation, an organic bone matrix, and no effect on RANKL/OPG ratio. Thus, appropriate combination of these two factors may alter bone formation as found in vivo [29, 30].

<table>
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<th>Table (2): The effect of vitamin K2 on RUNX2 and osterix expression in combination with genistein, coumestrol and daidzein</th>
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</table>

a P<0.05 versus control group.
These results strengthen the data for the use of combinations of PEs with vitamin K$_2$ in the treatment of skeletal disorders and these dietary factors can improve bone formation after menopause through different mechanisms.

References


