The Effect of the Combination of Vitamin K_2 and Genistein, Coumestrol and Daidzein on the Osteoblast Differentiation and Bone Matrix Formation

تاثير التداخل لفيتامين K_2 مع الجنستين، الكومسترول والديادزين عل تمايز خلايا الارومة العظمية وتكوين المادة العظمية

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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 K_2 which can be associated with bone protective activities. The effect of vitamin K_2 alone and in combination with genistein, coumestrol and daidzein on osteoblast differentiation and mineralization were tested. Significantly, vitamin K_2 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 K_2 enhanced the stimulatory effect of type I collagen and osteocalcin expression. Vitamin K_2 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 K_2 can be more effective factor in the presence of phytoestrogens on the improvement of bone formation after menopause.

Keywords: vitamin K_2, phytoestrogens, osteoblast, bone resorption, bone matrix

الملخص

يعتبر فيتامين K_2 احد العناصر المهمة التي تتصاحب مع حماية العظم. تمت في هذه الدراسة تقييم تاثير هذا الفيتامين لوحده او بوجود المركبات الفلافونيدية الجنستين ، الكومسترول والديادزين على فعالية الخلايا البادئة للعظم وترسيب المعادن . بينت الدراسة ان فيتامين K_2 بوجود المركبات الفلافونيدية قد حفزت معنويا ترسيب المعادن في العظم والتي تزيد من صلابته بينما لم يوجد اي تاثير على فعالية انزيم Alkaline Phosphatase. ايضا فان هذا الفيتامين مع المركبات الفلافونيدية قد حفز التعبير الجيني للجينات التي لها دور في بناء البروتينات العظمية الكولاجين والكالسين العظمي بينما لم توجد اي زيادة في ه ذا التعبير بوجود المركبات الفلافونيدية . كما لم يوجد تاثير على التعبير الجيني للجينات التي تعبر عن مستقبلات التي لها دور في تمايز الخلايا الناقضة للعظم وعن جينات تمايز الخلايا البادئة للعظم . بينت هذه الدراسة ان فيتامين K_2 لوحده او بوجود مركبات الفايتوستروجينات يكون اكثر تأثيرا على نمو العظم وخاصة في فترة سن اليأس.

الكلمات الدالة: فيتامين k_2، فيتوستروجين، بناء العظم، ارتشاف العظم، مصفوفة العظم

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 (K_1 and K_2) 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 K_2 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 K_1 acts as antioxidant agent, whereas vitamin K_2 (MK-4) proposed as a protective agent for neural cells from apoptosis by decreasing oxidative stress [15]. Flavones found to contain soybeans, menaquinon-7 (K_2) and cryptoxanthin, which have been revealed to stimulate osteoblastic bone formation and inhibit osteoclatic bone resorption *in vitro*. It was found that the supplementation with vitamin K_2 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 K_2 (MK-7) with seven isoprene unites is abundant in fermented soybeans (*natto*) and has anabolic effect on bone calcification. In contrast, vitamin K_2 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^{-5}M) 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 K_2 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^{-6}M), 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 K_2 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 K_2 on osteoblast differentiation and activity. Therefore, the current study examined the effect of genistein, coumestrol and daidzein in the presence of vitamin K_2 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 K_2 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 K_2 (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 K_2. 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 Runt-related transcription factor 2 (*Runx2*) forward AGACCCCAGGCAGGCACAGT, reverse GCGCCTAGGCACATCGGTGA; human osterix forward GCACCCTGGAGGCAACTGGC, reverse GAGCTGGGTAGGGGGCTGGA; human type I collagen forward CCTGGCAGCCCTGGTCCTGA, reverse CTTGCCGGGCTCTCCAGCAG; human receptor activator of NF-kB ligand [26] forward ACAGGCCTTTCAAGGAGCTGTGC, reverse ACCAGATGGGATGTCGGTGGC; human osteoprotegerein (*OPG*) forward AATCGCACCCACAACCGCGT reverse AGCAGGAGACCAAAGACACTGCA; human *β-actin* forward GCGCGGCTACAGCTTCACCA, reverse TGGCCGTCAGGCAGCTCGTA.

Statistical analysis

Differences between groups were assessed using Fisher's one-way ANOVA (SPSS Inc., Chicago, IL, USA). A difference of $P \leq 0{\cdot}05$ 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).

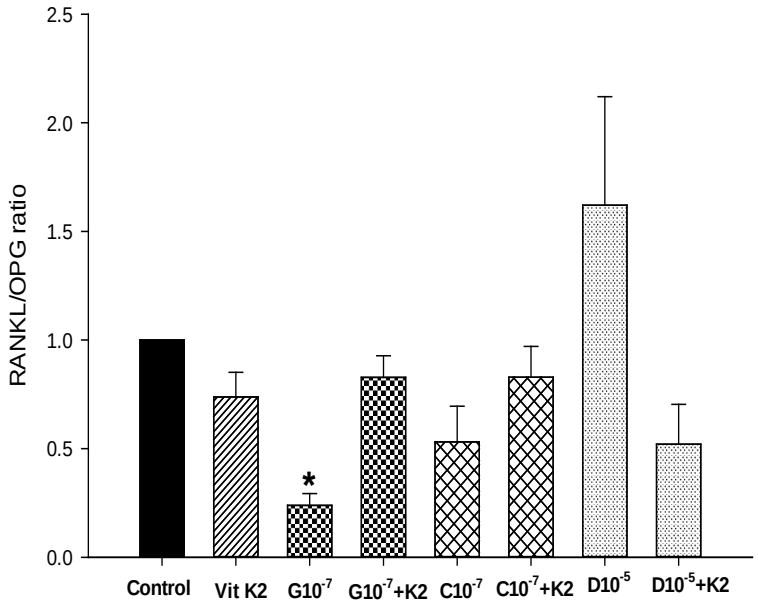

Fig. (1): The effect of vitamin K_2 on the expression of nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) Ratio RANKL/OPG ratio). $G10^{-7}$= genistein 10^{-7}M, $C10^{-7}$= coumestrol 10^{-7}M, $D10^{-5}$= daidzein 10^{-5}M.

(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.

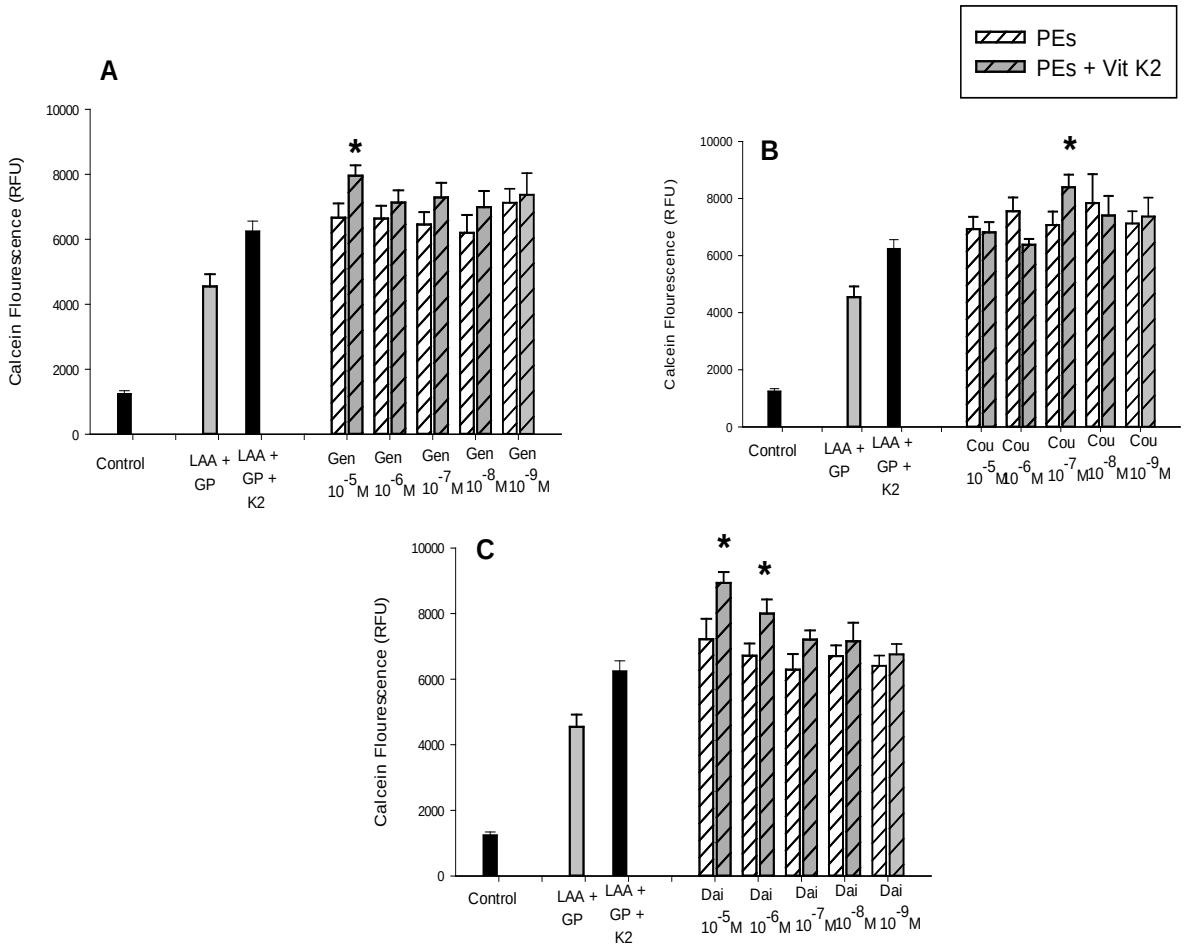

Fig. (2): Synergistic effect of vitamin K_2 with phytoestrogens on bone mineralization.
*** Values are significantly different from phytoestrogens treatment alone.**
Gen= genistein, Cou= coumestrol, Dai= daidzein.

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).

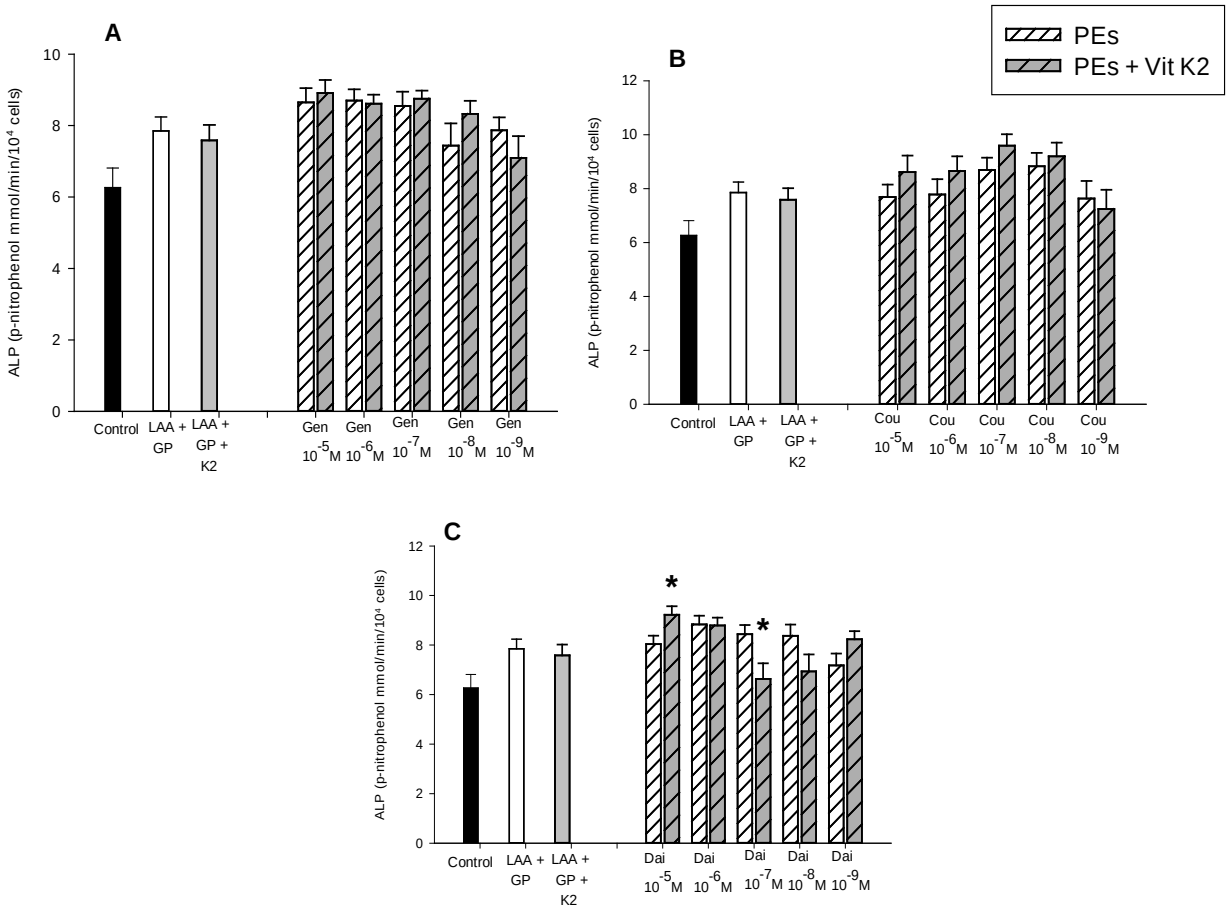

Fig. (3): Synergistic effect of of vitamin K_2 with phytoestrogens on ALP activity.

*** P≤ 0.05 versus phytoestrogens treatment alone. Gen= genistein, Cou= coumestrol, Dai= daidzein.**

Effect of vitamin K_2 on *type I collagen, osteocalcin, Runx2* and *osx* gene expression

Vitamin K_2 alone decreased *type I collagen* or *osteocalcin* expression, but vitamin K_2 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 K_2 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 K_2 on the expression organic components of bone matrix in combination with genistein, coumestrol and daidzein

	Type I collagen mRNA copies		Osteocalcin mRNA copies	
	Mean	**SE**	**Mean**	**SE**
Control	**154110.8**	**12003.**	**770**	**78.1**
Vitamin K_2 (10^{-5}M)	**70212.8**	**5452.9**	**441 [a]**	**36.3**
Coumestrol (10^{-7}M)	**1069198 [a]**	**75038**	**1868.4 [a]**	**217.5**
Coumestrol (10^{-7}M) and K_2	**1398595.7 [a b]**	**122951**	**2892.8 [a b]**	**386.6**
Daidzein (10^{-5}M)	**62457.5 [a]**	**3934.9**	**1190.1 [a]**	**263.4**
Daidzein (10^{-5}M) and K_2	**438989.8 [a b]**	**43731.2**	**1865.1[a b]**	**181.5**
Genistein (10^{-7}M)	**97275.6 [a]**	**3368.2**	**1607 [a]**	**209**
Genistein (10^{-7}M) and K_2	**67680 [b]**	**7037.4**	**685 [a b]**	**62**

a P≤0.05 versus control, b P≤0.05 versus relevant PEs alone.

Table (2): The effect of vitamin K_2 on RUNX2 and osterix expression in combination with genistein, coumestrol and daidzein

	Runx2 mRNA copies		Osterix mRNA copies	
	Mean	SE	Mean	SE
Control	1521.3	295	27355	6643.9
Vitamin K_2 (10^{-5}M)	22538 [a]	1025.6	69965.7 [a]	18836.3
Coumestrol (10^{-7}M)	28582 [a]	858.8	139140.7 [a]	10415.5
Coumestrol (10^{-7}M) and K_2	25911 [a]	480.6	124928 [ab]	9519.6
Daidzein (10^{-5}M)	99419 [a]	7357.9	138813.2 [a]	25524.5
Daidzein (10^{-5}M) and K_2	103024 [ab]	11717	147171.3 [ab]	9677.3
Genistein (10^{-7}M)	27610 [a]	975.9	115361.2 [a]	10062.9
Genistein (10^{-7}M) and K_2	31192 [a]	1091	128211.2 [ab]	8281.4

a P≤0.05 versus control group.

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 K_2 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 K_2 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 K_2 significantly increased the mineralization peak induced by genistein, coumestrol and daidzein concluded that vitamin K_2 has protective effect on non-organic matrix bone formation. The stimulatory effect of vitamin K_2 on mineralization was noted also on organic bone matric, *type I collagen* and *osteocalcin* expression in combination with phytoestrogens, indicating that the vitamin K_2 can enhance the anabolic effect of phytoestrogen in appropriate concentration and increased bone matrix formation.

In opposite, vitamin K_2 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 K_2 addition, although ALP activity still significant versus control group. The present result suggested that the augmentative effect of vitamin K_2 on mineralization did not mediate through ALP activity.

This study aimed to examine the mechanism by which phytoestrogens and zinc or vitamin K_2 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 K_2 alone increased *Runx2* and *Osx* 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 oseoblastic 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 K_2 on RUNX2 and Osx, it is also increased the peak of osteocalcin and type I collagen levels. Additionally, vitamin K_2 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 K_2 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 K_2 mediated in biphasic way, vitamin K_2 can enhance mineralization, type I collagen and osteocalsin 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].

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.

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