The Synergistic Effect of Zinc, Coumestrol, Genestein and Daidzein on Proinflammatory Cytokines Production and Receptor Activator of NFκB Ligand Expression that Implicates in Bone Resorption

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Abstract

Several observations have showed the synergistic effect of nutrients elements and phytoestrogens on bone resorption elimination. Zn is one of the trace elements found to increase the stimulatory effect of phytoestrogens including genestein, coumestrol and daidzein on bone formation; however the synergistic modulation of Zn, genestein, coumestrol and daidzein on proinflammatory-producing T-cells and receptor activator of NFκB ligand (RANKL) expression that implicated in osteoclast formation is still open area to debate. This study found that Zn enhanced the inhibitory effect of genistein, daidzein and coumestrol on TNF-α expression; however the same effect was shown with daidzein on IL-1β expression while there is no further effect found with other compounds. Zn is only in combination with genistein decreaseIL-6 expression; moreover Zn had additional effect on the inhibitory effect of all test phytoestrogens on RANKL expression in dose-dependent manner. In conclusion, Zn alone or in combination with phytoestrogens has an inhibitory effect on the expression of pro-inflammatory cytokines and RANKL that play an important role in the promotion of bone resorption.

Keywords: Zinc, Phytoestrogens, T cells, Pro-inflammatory cytokines

Introduction

Recently it has become clear that the interactions between the immune system and bone have a key role in osteoporosis after aging. Estrogen plays an important role in the balance of bone remodeling and its deficiency causes bone loss and fractures after aging especially in women [1]. Moreover, estrogen deficiency increases T-lymphocytes population and TNF production by activated T-cells that act a key factor promote osteoclastogenesis [2,3]. Receptor activator of nuclear factor kappa-B (NFκ-B) ligand (RANKL) (also called osteoprotegerin ligand (OPGL)) is a member of the tumor necrosis factor superfamily and plays an important role in bone remodeling and osteoclast formation.TNF acts a major key in bone resorption and can induce osteoclast formation through several mechanisms involving RANKL-inducing mechanism which in turn directly promote osteoclastogenesis [4]. In addition, estrogen deficiency can modulate the production of several cytokines by T-cells such as TGF-β and IFN-γ [5]. Recent studies found that ovarictomy mice with T-lymphocytes-deficient nude did not induce bone resorption, while the wild type of overctomy mice with T-cells induce bone loos [6,7]. Osteoclast formation is controlled by appropriate changes in osteoblast derived RANKL and osteoprotegerin (OPG) expression [8]. Resorptive stimuli increase RANKL expression and decrease levels of the RANKL antagonist OPG [9]. This enables the initiation of osteoclast formation. Changes in immune status can disrupt this balance and excessive osteoclastic bone loss which causes several disorders such rheumatoid arthritis and osteomyelitis [10].

Genestein, daidzein and coumestrol are phytoestrogens (PEs) which are a diverse group of plant derived compounds with a structure and function similar to oestradiol, have been examined as an alternative therapeutic agent. Diets with high phytoestrogen content are associated with a more robust skeleton [11,12]. Phytoestrogens directly inhibit the response of osteoclast precursors to RANKL and TNF-α [13, 14, 15, 16], and genistein also reduce osteoblast RANKL expression and elevate OPG [17]. Interestingly, phytoestrogens also reduce lymphocytes number and activity for instance genistein and daidzein reduce T cells number and thymus weight in ovariectomised mice [18, 19]. Cytokines such as TNF-α, IL-1 and IL-6 and RANKLS as well [20]. Thus, the anti-steoclastic action of phytoestrogens involved in an inhibitory effect on T cells number and cytokine production. Furthermore, Phytoestrogens that is found could further protect against post-menopausal bone loss through the modulation of pro-inflammatory cytokines expression produced by T-cells. However the effect of Zinc on T cell derived cytokines is not well established.

Zn deficiency was found to affect on the immune system function including increases in proinflammatory cytokines in the patients. The previous study observed under Zn deficiency, TNF-α and IL-1β expression in HL-60 cells via redox-mediated mechanism and also recommended to use Zn supplemented as a treatment in several inflammatory diseases [21] and TNF-α and IL-6 as well [22]; Zn that is found can modulate pro-inflammatory cytokines expression via a mechanism involving effect on NF-κB, a transcriptional gene is important for the biosysnthesis of proinflammatory cytokines [22]. Moreover, a low level of Zn was found after aging which can cause a disruption in in inflammatory response result in several diseases including atherosclerosis [23]. Previously, zinc has been found induced osteoblast differentiation, mineralization and bone matrix formation and reduced RANKL/OPG ratio expressed by mature osteoblast [24] with the no effect on osteoclast formation. Pro-inflammatory cytokines produced by T cells such as TNF-α and IL-1β play an important role in osteoclast differentiation. However, zinc has no direct effect on osteoclast formation but it may modulate T-produced pro-inflammatory cytokines involved in osteoclast formation. Thus, the effect of zinc alone or in combination with PEs on cytokine expression produced by T cells was not studied yet. Therefore this study investigated the effect of the zinc alone or in combination with PEs on T cell-producing pro-inflammatory cytokines and RANKL expression that promote bone resorption.

Material and Methods

Media and reagents

Jurkat E6.1 T cells a human leukemic T cell line (ECACC ,UK) cultured in phenol red free RPMI medium supplemented with 10% foetal calf serum (Autogen Bioclear, UK) 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin. Culture incubated at 37ºC in 5% CO_2 in a humidified incubator till using in experiments [25].

Real time quantitative PCR analysis of inflammatory cytokine expression

T cells were cultured for four days with genistein (10^{-6}-10^{-9} M), daidzein (10^{-6}-10^{-9} M) or coumestrol (10^{-6}-10^{-9} M) in the presence of Con A (10 µg/ml). Total RNA was extracted and reversed transcribed with M-MLV reverse transcriptase, and real time-PCR performed on a Step One PCR system (Applied Biosystems, UK) using the DNA-binding dye SYBR green as a fluorophore. A total of 2 µl of external plasmid standard or cDNA was added to a final reaction volume of 25 µl containing 0.05 U/µl Taq, SYBR green and specific primers (0.2 µM). Primers used are shown in (Table 1). The concentration of DNA plasmid stock was determined by OD at 260 nm. The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR green during the extension time. Reaction conditions were 94ºC for two minutes, followed by 35 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds. At the end of each PCR run a melt curve analysis was performed to show the absence of non-specific bands. For each sample mRNA level was expressed in comparison to control group normalized against β-actin. by the instrument's software. Samples were analysed in triplicate.

The data were analyzed based on the differences between the reference (control group) and the treatment groups using a comparative Ct analysis according to the following formula:

$\Delta C_{t\ sample} = C_{t\ sample} - C_{t\ reference\ gene}$

$\Delta\Delta Ct = \Delta C_{t\ sample} - \Delta C_{t\ reference\ control}$

Amount of target (RQ) = $2^{-(\Delta\Delta Ct)}$

Where Ct is a threshold cycle.

Results

This study found that TNF-α expression augmented in the presence of ConA while it is decreased in the presence of Zn (Figure 1). Phytoestrogens compounds; genistein (10^{-5}-10^{-7} M) (Figure 1A), coumestrol 10^{-5} M (Figure 1C) and daidzein (10^{-5}, 10^{-7} M) (Figure 1B) decreased TNF-α expression, however zinc significantly enhanced down-regulation of TNF-α mRNA expression of PEs in dose-dependent manner; however, coumestrol at 10^{-6} and 10^{-7} M in the presence of Zn increased this expression.

The expression of IL-1β was significantly increased by stimulated T-cells and this expression is not affected in the presence of zinc alone or in combination with phytoestrogens (Figure 2 A, B, C). However, zinc suppressed the stimulatory effect of daidzein (10^{-5} M) on IL-1β expression (Figure 2B) and this concentration is similar to that decreased osteoclast formation as found previously [15].

The effect of zinc on IL-6 expression was opposite to its effect on TNF-α and IL-1β expression. IL-6 expression is significantly enhanced by stimulating T-cells while this expression is not affected by zinc treatment alone (Figure 3). while the combination of zinc and genistein (10^{-5}-10^{-7}M) decreased IL-6 production versus stimulated T-cells but still higher than control group (Figure 3A), while the treatment of zinc and daidzein (10^{-5} M) decreased the stimulatory effect of daidzein on IL-6 expression (Figure 3B) referring to the suppression effect of zinc and no further effect on other concentrations but still lower in comparison to control group. Similar effect had been shown with zinc in combination with coumestrol (10^{-}

^{5}M) while the presence of zinc increased IL-6 expression with coumestrol (10^{-6} M) (Figure 3C).

Genistein (10^{-5}-10^{-7} M) also decreased receptor activator of NFκB ligand (RANKL) expression but this expression is more suppressed in the presence of zinc at all concentration that is used in this study (Figure 4A). Moreover, coumetrol (10^{-5}-10^{-7} M) decreased RANKL expression with higher levels in the presence of zinc than coumestrol alone (Figure 4C) refers to the additional inhibitory effect of zinc on this expression which can modulate osteoclast formation. In addition, daidzein at anti-osteoclastic dose (10^{-5}M) suppressed RANKL expression in the presence of zinc due to the inhibitory effect of this element in combination with PEs on RANKL expression (Figure 4B).

Discussion

T cells are evaluated to produce a wide range of pro and anti-inflammatory cytokines that play a critical role in the initiation of inflammatory diseases such as rheumatoid arthritis and post-menopausal bone resorption. TNF-α is a key cytokine that trigger osteoclast differentiation. Several studies found that osteoclastogenic cytokines -producing T cell plays an important role in the progression of osteoporosis [26] such as TNF-α, IL-1β, IL-6 and RANKL and the latest expressed on T-cell surface and all of these factors can induce osteoclast formation. Accumulated observations determined the implication of T-cells in inducing bone loss after menopause due to the absence of estrogen [27,19,28]. Furthermore, it was found that after aging the function of lymphocytes was increased which associated with high levels of proinflammatory cytokines [23]. In addition, it was found that T-cells are implicated in bone resorption and disorders due to its ability to produce RANKL on its surface that plays an important role in osteoclast formation.

Previous study found that PEs alone reduced TNF-α, IL-1β, IL-6 and RANKL expression by T-cells, while in opposite way increased osteoblast differentiation [20, 24]. Moreover, zinc has been shown to induce osteoblast differentiation and bone matrix formation alone or in combination with PEs [24]. Thus, this study aimed to determine if any further effect of zinc on T-cells; the current study revealed that zinc can reduce bone resorption through effect on pro-inflammatory cytokines and RANKL expression in T cells that promote osteoclastogenesis. This study found a direct effect of zinc alone on the cytokines expression especially on TNF-α and RANKL; moreover PEs compounds that are used in this study reduced the expression of theses cytokines while the addition of zinc enhanced the inhibitory effect of PEs on the mRNA expression of the examined cytokines.

Zinc significantly showed an augmentative effect on the suppressive effect of genistein, daidzein and coumestrol on the mRNA expression of proinflammatory cytokines-producing T cells including TNF-α, IL-1β and IL-6 which are trigger osteoclast differentiation in dose-dependent manner and this is consistent with previous study that found T cells enhanced the production of pro-inflammatory cytokines that induces bone loss after inducing osteoclast formation [29].

In addition, the results of this study showed that ConA did not induce RANKL expression significantly versus control group, although the results revealed that zinc alone suppressed RANKL expression induced by stimulated T-cells with ConA and in comparison to control group. The inhibitory effect of genistein, daidzein and coumestrol on TNF-α and RANKL mRNA expression was significantly enhanced in the presence of zinc, while there is no further effect has been shown on IL-1β and IL-6 expression, and all of the results conducted that the main mechanism that mediated the synergistic effect of zinc with Pes including increases the anti-osteoclastic action of PEs through decrease TNF-α and RANKL expression; main keys that switch on osteoclast differentiation. However, the high levels of IL-6 induced by daidzein were reduced in the presence of zinc and this included a new effect

of daidzein on IL-6 expression that also could trigger osteoclast formation, and no effect of zinc was seen on IL-1β at all.

This study observed the synergistic effect of zinc with PEs that reduced the level expression of osteoclastogenic cytokines-producing T cells which in turn decrease osteoclast formation; moreover, zinc alone reduced the expression of TNF and RANKL and thus it is could participate partially to reduce osteoclast formation and bone resorption.

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Table(1):Primer sequences

Genes	5′-3′ Forward primer	3′-5′ Reverse primer
Human *β-Actin*	GCGCGGCTACAGCTT CACCA	TGGCCGTCAGGCAGCTCGTA
Human *TNF-α*	GCTCCAGTGGCTGAA CCGCC	AGCACATGGGTGGAGGGGCA
Human *IL-6*	TCAATGAGGAGACTTGCCTGG TGA	TCTGCAGGAACTGGATCAGGACTT
Human *IL-1β*	ACGCTCCGGGACTCA CAGCA	TGAGGCCCAAGGCCACAGGT
Human *RANKL*	ACAGGCCTTTCAAGGAGCTGTGC	ACCAGATGGGATGTCGGTGGC

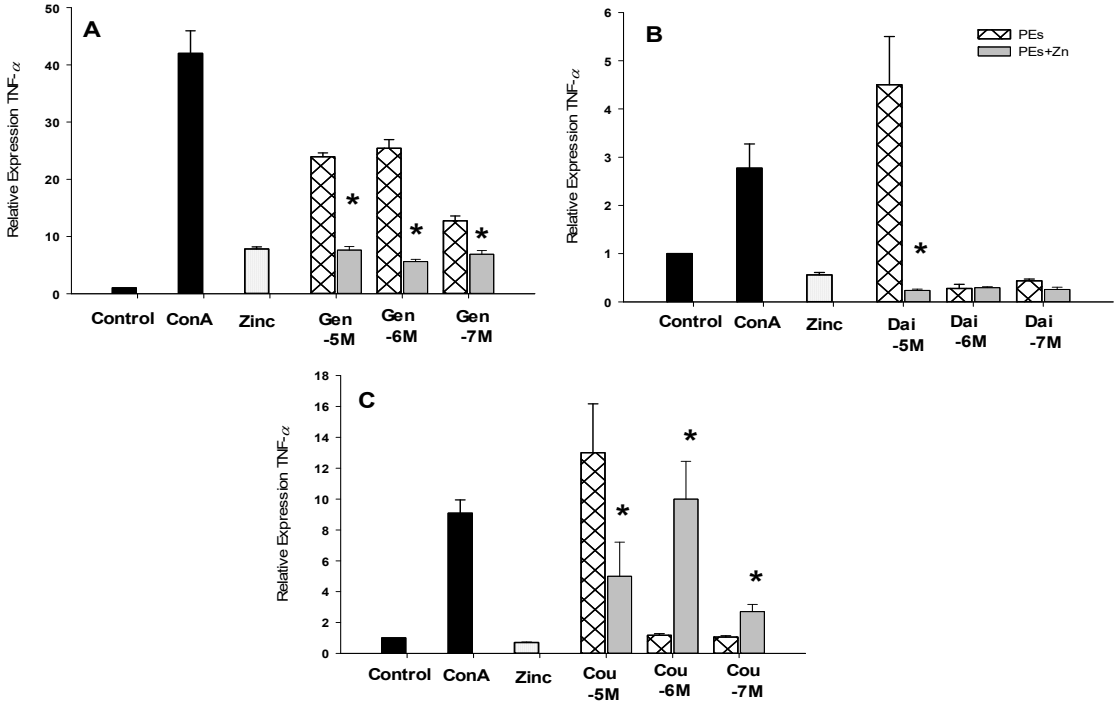

Figure (1): The synergistic effect of zinc with phytoestrogens (PEs) compounds A: genestein, B: daidzein, C: coumestrol on TNF-α gene expression. * Values are significantly different (P<0.05) from phytoestrogens treatment alone.

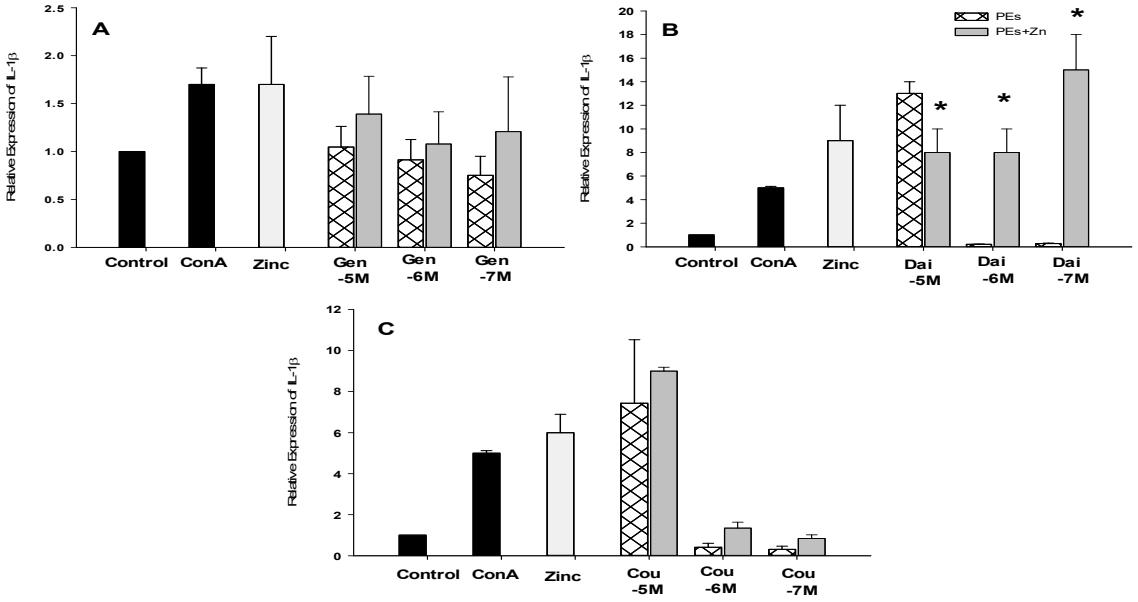

Figure (2): The synergistic effect of zinc with phytoestrogens (PEs) compounds A: genestein, B: daidzein, C: coumestrol on IL-1β gene expression. * Values are significantly different (P<0.05) from phytoestrogens treatment alone.

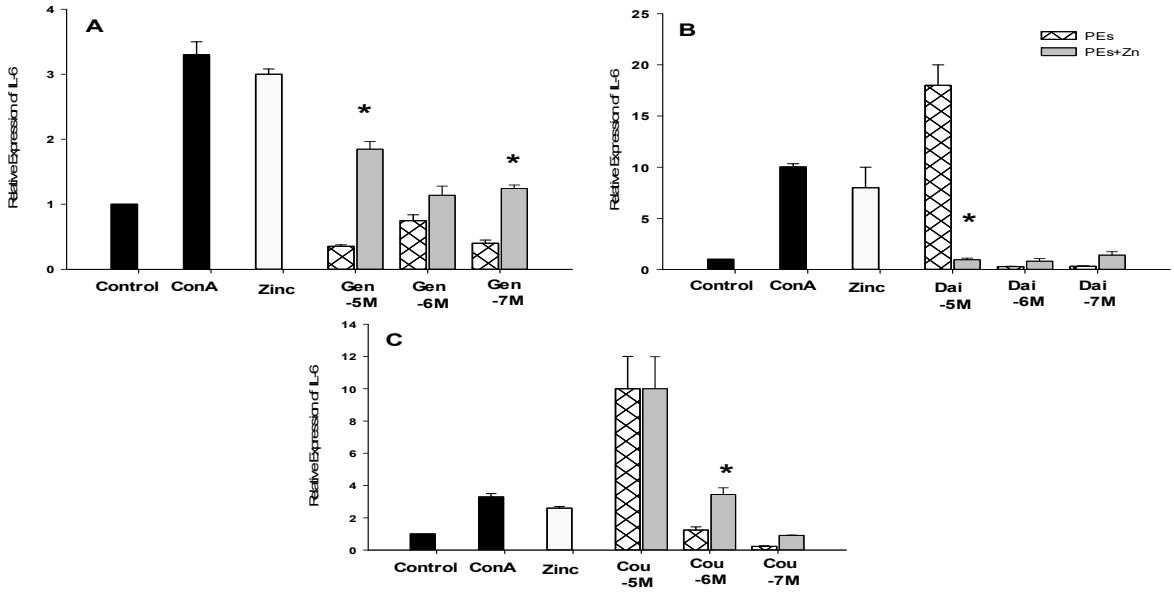

Figure (3): The synergistic effect of zinc with phytoestrogens (PEs) compounds A: genestein, B: daidzein, C: coumestrol on IL-6 gene expression. * Values are significantly different (P<0.05) from phytoestrogens treatment alone.

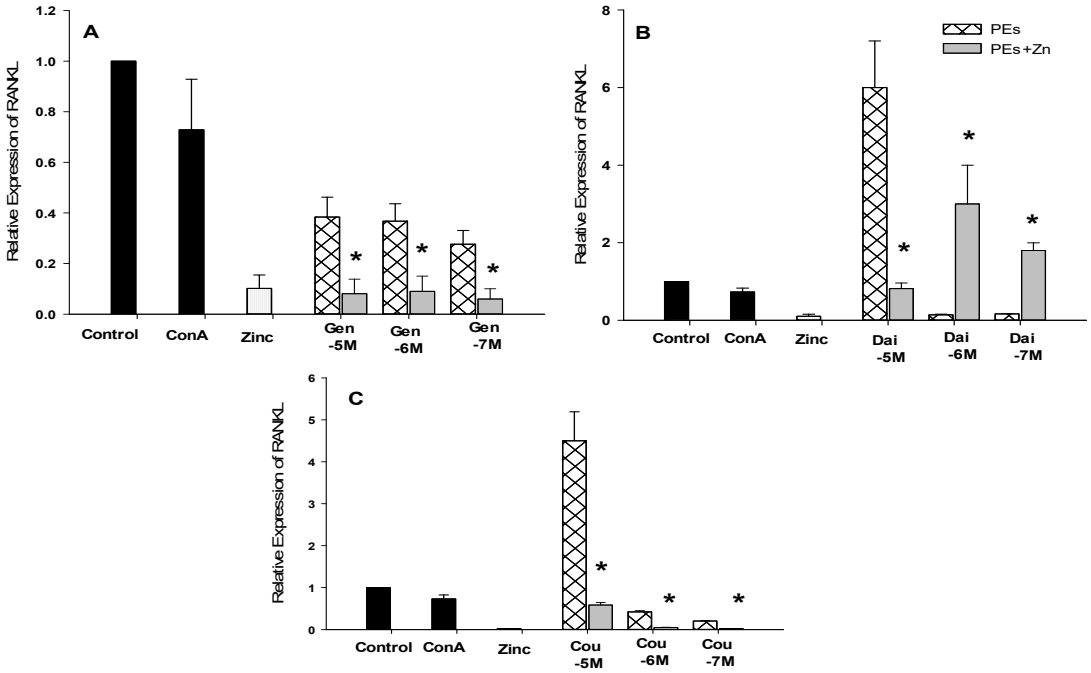

Figure (4): The synergistic effect of zinc with phytoestrogens (PEs) compounds A: genestein, B: daidzein, C: coumestrol on receptor activator oof NFκB ligand (RANKL) gene expression. * Values are significantly different (P<0.05) from phytoestrogens treatment alone.

التاثير التازري للزنك مع الجنستين، الكومسترول والدايدزين في انتاج الوسائط الخلوية البادئة للالتهاب وتعبير المستقبل المنشط لرابط العامل النووي كابا المرتبطين بتنخر العظام

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الخلاصة

بينت الكثير من الدراسات التاثير التازري لكل من العناصر الغذائية مع الفايتوستروجينات في تقليل تنخر العظام. يعد الزنك واحدا من اهم هذه العناصر إذ وجد انه يزيد من القدرة التحفيزية للفايتوستروجينات في زيادة نسبة تكوين العظام، إن التاثير التازرري لعنصر الزنك مع الجنستين، الكومسترول والداديدزين في التعبير الجيني للوسائط الحركية البادئة للالتهاب وRANKL هو قيد المناقشة لحد الان. بينت الدراسة ان الزنك بمفرده او مع مركبات الفايتوستروجينات المستعملة في البحث انه زاد من القابلية التثبيطية للفايتوستروجينات لانتاج TNF-α بينما وجد التاثير نفسه في انتاج IL-1β فقط مع الدايدزين. ايضا فان الزنك مع الجنستين قلل من التعبير الجيني للوسيط IL-6 وايضا زيادة في القدرة التثبيطية للفايتوستروجينات على التعبير للجيني للـ RANKL في وسط معتمد على الجرعة. نستنتج من الدراسة الحالية ان الزنك بمفرده او مع مركبات الفايتوستروجينات زاد من تثبيط التعبير الجيني للوسائط الحركية البادئة للالتهاب والتي تؤدي دورا مهما في زيادة تنخر العظام وان تناول هذه العناصر مع بعضها ممكن ان يقلل من تنخر العظام.
الكلمات المفتاحية: الزنك، الفايتوستروجينات، الخلايا التائية، الوسائط الحركية البادئة للالتهاب.