Age-Dependent Responsiveness of Human Female Cultured Bone Cells to Estrogenic Compounds

Dalia Somjen1*, Sara Katzburg, Alvin M. Kaye1, Fortune Kohen2 and Gary H. Posner3

1Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239
2Department of Biological Regulation, The Weizmann Institute of Research, Rehovot 76100, Israel and
3Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA

Abstract

Human cultured female osteoblasts (Ob) respond age-dependently to estradiol-17β (E2) and to phytoestrogens by increased DNA synthesis (DNA) and creatine kinase specific activity (CK). ERα mRNA expression is higher than ERβ mRNA in Obs. Pre-menopausal Ob (prOb) reveals higher expression of ERα than post-menopausal Ob (poOb), but similar intracellular and membranual E2 bindings. ERα mRNA is stimulated in prOb and inhibited in poOb by different estrogens. ERα mRNA in prOb is stimulated by 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ specific agonist) and 4,4’,4”-[4-Propyl-(1H)-pyrazol-1,3,5-triy] tris-phenol (PPT; ERα specific agonist) and raloxifene (Ral) and inhibited by genistein (G) and daidzein (D) while in poOb only E2 and DPN inhibited it. All phytoestrogenic carboxy-derivatives stimulated ERα mRNA in both Ob, while the protein bound- carboxy were ineffective. All compounds except carboxy-biochainin A (BA) had no effect on ERβ. There is higher expression of 1α 25 hydroxy- vitamin D (1OHase) mRNA in poOb, whereas 1,25(OH)2D3 (1,25) production is similar, but all compounds increased 1OHase mRNA and 1,25 to higher extent in poOb. Obs express 15 and 12 lipooxygenase (12LO and 15LO) mRNA and produce 12 and 15 HETE (12H and 15H). 12LO expression is higher and 15LO is lower in prOb, while 12H is higher in prOb and 15H is similar in both. Both LOs are increased to higher extent in poOb by all estrogens except Ral and PPT. 12H is increased by DPN, PPT and carboxy-derivatives similarly in both Obs, while 15H is increased by biochainin A (BA), Ral and carboxy-derivatives. DNA synthesis and CK are stimulated by all compounds in both Obs, but to higher extent in poOb. The 1,25 less-calcemic analog JK 1624F2-2 (JKF) up-regulated DNA and CK response to all estrogens except BA and the carboxy-derivatives in both Obs. JKF stimulated intracellular binding of E2 similarly by all compounds except BA, but inhibited its membranal binding in both Obs. In conclusion Obs respond age-dependently to estrogens in a yet unknown mechanism and the beneficial outcome for human female bones is not yet clear.

Keywords: Osteoblasts; Estrogens; Vitamin D; ERs; LO: HETE

Introduction

Mammalian osteoblasts express specific intracellular and membranual receptors for estrogens [1-5] and respond to estrogens [6-11].

The age dependent- responses were studied by different researchers but still debatable, in human osteoblasts in vitro [12-17].

In our studies we analyzed the response of cultured female human derived osteoblasts (Ob) by different parameters to different estrogens, including estradiol 17β (E2), 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ specific agonist) and 4, 4’, 4’’-[4-Propyl-(1H)-pyrazol-1,3,5-triy] tris-phenol (PPT; ERα specific agonist), raloxifene (Ral) and phytoestrogens including their synthetic carboxy-derivatives and protein- bound carboxy-derivatives. Whether these findings imply also to human female bone response to hormonal treatments in vivo is yet not established.

We found that there is a higher expression of ERα mRNA and lower expression of ERβ mRNA in pre- than in post- menopausal Ob (prOb and poOb respectively), with no significant age difference in intracellular and membranual estrogen binding activities.

Ob also express of 1α 25 hydroxy vitamin D hydroxylase (1OHase) mRNA to higher extent in prOb compared to poOb, but no age-dependent difference in 1OHase activity namely 1,25(OH)2D3 (1,25) production. Ob express also 12 and 15 lipooxygenase (LO) mRNA and produce 12 and 15 HETE (H). 12LO mRNA is more expressed in prOb than poOb, but higher expression of 15LO mRNA in poOb than prOb. The production of 15H is the same in both, whereas the production of 12H is higher in prOb.

Human female derived cultured bone cells (Ob) show age-dependent changes in DNA synthesis (DNA) and CK specific activity (CK) in response to different estrogenic compounds [18].

The non- hypercalcemic vitamin D analog JK 1624F2-2 (JKF) up-regulated the response to E2 and some phytoestrogens in both Ob to the same extent.

The aim of the present review is to summarize our findings using cultured human female Ob derived from either pre- or post-menopausal women carried out in our laboratory on:

α. Modulation of ERα and ERβ mRNA expression.

β. Modulation of 1OHase mRNA expression.

γ. Modulation of 1OHase activity (1, 25 production).

δ. Modulation of 12LO and 15 LO mRNA expression.

ε. Modulation of 12LO and 15 LO activity 12H and 15H production.

*Corresponding author: Prof. Dalia Somjen, Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Centre and The Sackler Faculty of Medicine Tel-Aviv University, Tel-Aviv 64239, Israel, Tel: 97236973812; Fax: 972369734321; E-mail: dalia@sasmc.health.gov.il

Received August 29, 2011; Accepted October 14, 2011; Published October 19, 2011

Citation: Somjen D, Katzburg S, Kaye AM, Kohen F, Posner GH (2011) Age-Dependent Responsiveness of Human Female Cultured Bone Cells to Estrogenic Compounds. Endocrinol Metabol Syndrome 5:1. doi:10.4172/2161-1017.51-001

Copyright: © 2011 Somjen D, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Materials and Methods

Reagents

CK assay kit, E2, genistein (G), daidzein (D), biochanin A (BA) were from Sigma Chemicals Co. (St. Louis, MO). DPN and PPT were from Tocris biosciences (Bristol, BS11 OQI. UK). Ral was from Eli Lilly and Company. All other reagents were of analytical grade.

Carboxy derivatives and protein bound carboxy- derivatives of phytoestrogens were synthesized by us [19].

Cell cultures

Human female bone cells from pre- (<50 years old) and post-menopausal women (>55 years old) were prepared from bone explants obtained from biopsies of hip fractures from healthy patients, by a non-enzymatic method [14,20]. The bone-derived cells were identified as osteoblasts by major osteoblastic characteristics [13].

Hormonal treatment

1. Cultured cells were treated with control (vehicle; 0.01 % ethanol), 30nM E2 or DPN (420nM) and PPT (390nM) or phytoestrogenic compounds or Ral (3000nM) for 24h, followed by harvesting either for CK preparation and assay, or for DNA [14,20].

2. Pre-treatment with JKF was performed by 3 daily additions of 1nM followed by 24h treatment with E2 (30nM) or different phytoestrogens or Ral (3000nM) as described [21].

3. Cultured cells were incubated for 1 hour with serum-free medium, followed by addition for 10 minutes of control (vehicle; 0.01 % ethanol), 30nM E2, or 420nM DPN and 390nM PPT or 3000nM different phytoestrogens or 3000nM Ral followed by harvesting for DNA [14,20].

4. Cultured cells were treated with control (vehicle; 0.01 % ethanol), 30nM E2, or 420nM DPN and 390nM PPT or 3000nM different phytoestrogens or Ral daily for three days, followed by harvesting for mRNA extraction and assay for different mRNA expression [18].

Creatine kinase preparation and assay

Cells were scraped off and homogenized by freezing and thawing and extracts were obtained for CK activity measurement [14,23]. Protein was assayed by Coomassie brilliant blue dye binding, using BSA as standard [24].

DNA synthesis assay

Cells were grown until sub- confluence, treated with control (vehicle; 0.01 % ethanol) and various hormones or agents as indicated. Twenty-two hours following the exposure to these agents, [3H] thymidine was added for 2h. Cells were then treated with 10% ice-cold trichloro-acetic acid (TCA) for 5min and washed twice with 5% TCA and cold ethanol. The cellular layer was dissolved in 0.3ml of 0.3N NaOH, samples were aspirated and [3H] thymidine incorporation into DNA was determined [14,23].

Determination of mRNA for ERα and ERβ or 1OHase or 12 and 15LO by real time PCR

RNA was extracted from cultured bone cells and subjected to reverse transcription. The reaction was carried out using ERα and ERβ or 1-OHase or 12LO and 15LO cDNA as standards, compared to RNAse P [22].

Determination of 1, 25 (OH)2D3 formation

1, 25 (OH)2D3 formation in cultured skeletal cells was assayed as previously described [25].

Determination of 12 or 15HETE formation

12 or 15HETE formation in cultured bone cells was assayed as described [25].

Competitive binding assay for intracellular estrogenic binding sites in human female-derived osteoblasts

Cells with and without pre- treatment with JKF, were incubated for 60 min at 37°C with [3H] E2 with and without excess of unlabelled compounds. Binding was terminated by four successive washes with ice- cold binding medium, and cellular content of [3H] E2 was measured [18].

Competitive binding assay for membranal estrogenic binding sites in human female-derived osteoblasts

Cells with and without pre- treatment with JKF, were incubated for 60 min at 37°C with [Eu] E2-BSA with and without excess of unlabelled protein- bound compounds as described. Binding was terminated by four successive washes with ice- cold binding medium and cellular content of [Eu] E2-BSA was measured [22].

Statistical analysis

The significance of differences between experimental and control means was evaluated using Student’s t-test in hich n=5 number of cultures.

Results

Age-dependent modulation of the expression of estrogen receptors in human female cultured bone cells

Human cultured bone cells derived from either prOb or poOb express both estrogen receptors (ERs); the ERα and ERβ mRNA with about 100 times higher abundance of ERα than ERβ in these cells (0.092+0.00075 compared to 0.0075+0.00072 in prOb and 0.0075+0.00072 compared to 0.0104+0.0011 in poOb). ERα mRNA expression in prOb is higher than in poOb (0.092+0.00075 compared to 0.0075+0.00072) whereas ERβ is similar in both Ob (0.0094+0.00012 compared to 0.0104+0.0011).

Three daily treatments of the different Obs with E2 or phytoestrogens resulted in modulation of the expression of both ERs (Figure 1a). ERα was stimulated in prOb by all compounds except the ERα agonist PPT, but inhibited in poOb by all compounds except D and PPT. On the other hand E2 and BA inhibited ERβ in both Ob, Ral, ERβ agonist DPN and PPT stimulated it in prOb whereas DPN inhibited it in poOb and D, G and Ral stimulated it in poOb whereas PPT had no effect (Figure 1a).

All carboxy derivatives of phytoestrogens up regulated ERα expression in both Ob to higher extent in prOb. On the other hand ERβ was stimulated in both Ob by cBA, inhibited by cG and no effect by cD (Figure 1b). All protein bound carboxy derivatives of phytoestrogens were not effective in any Ob (Figure 1b).
Modulation of expression and activity of vitamin D-1α 25 hydroxylase (1OHase) mRNA in human female cultured bone cell

Human bone-derived cells in culture derived from either pre- or post-menopausal females express vitamin D-1α 25 hydroxylase (1OHase) mRNA with higher abundance in prOb than in poOb (0.0075+0.00014 in prOb compared to 0.0041±0.00038 in poOb). The cells also produce 12HETE (12H and 15H) and 12H to higher extent (1.68±0.047 in prOb compared to 1.03±0.034 pg/ml in poOb) and 15H to higher extent in poOb (1.28±0.011 in prOb compared to 1.48±0.065pg/ml in poOb).

12LO and 15LO mRNA expression was modulated by different hormones (Figure 3a and Figure 3b). In prOb and poOb 12LO mRNA expression is stimulated by all estrogenic compounds except PPT and Ral. Of note is that in cells from poOb D and BA stimulated it to higher extent than in poOb (Figure 3a). In prOb and poOb 15LO mRNA expression is stimulated only by DPN and PPT but not by Ral. Of note is the fact that in cells from poOb E2 and D stimulated it and BA stimulated it in poOb to higher extent (Figure 3a). All carboxy derivatives of the phytoestrogens did not stimulate12LO in both Ob (Figure 3b).

Figure 1: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM) or b. carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on ERα and ERβ mRNA expression. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: * P <0.05; ** P <0.01; ***P<0.001. ##P<0.05; ###p<0.01 compared with prOb.

Figure 2: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM) or b. carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on 1OHase mRNA expression and 1,25 formation. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: * P <0.05; ** P <0.01. #P<0.05 compared with prOb.
Modulation of age-dependent DNA and CK response of human female bone cells to estrogenic compounds by vitamin D less-calcemic analog

Human bone-derived cells in culture derived from either pre- or post-menopausal females respond to the different estrogenic compounds by stimulation of DNA and of CK (Figure 5a). Pre-treatment of the cells for 3 days with the less-calcemic vitamin D analog JKF (1nM/day) led to increased CK response to all estrogens except to BA (Figure 5b) and to the carboxy-derivatives of the phytoestrogens in both Ob (Figure 5c). Similar results were obtained when the changes in DNA were monitored (Figure 5d and Figure 5b).

Modulation of age-dependent intracellular and membranai E2 binding of human female bone cells to estrogenic compounds by vitamin D less-calcemic analog

Human bone-derived cells in culture derived from either pre- or post-menopausal females bind specifically estrogen to both intracellular and membranai binding sites. Pre-treatment of the cells with the less-calcemic vitamin D analog JKF (1nM/day) led to increased intracellular binding by all estrogens except to BA in both Ob (Figure 7a). On the other hand this pre-treatment led to decreased membranai binding by all estrogens in both Ob (Figure 7b).

and all the protein bound carboxy derivatives of the phytoestrogens had slight effect on both Ob (Figure 3b). All carboxy derivatives of the phytoestrogens stimulated 15LO in both Ob but to higher extent in prOb (Figure 3b) and all the protein bound carboxy derivatives of the phytoestrogens had similar effect on both Ob (Figure 3b).

In prOb and poOb 12H formation is stimulated only by BA, DPN and PPT (Figure 4a). Of note is the fact that in poOb BA stimulated it to higher extent than in poOb (Figure 4a). In prOb and poOb 15H formation is stimulated only by BA and Ral (Figure 4a). Of note is the fact that in cells from prOb Ral stimulated it to higher extent (Figure 4a). All carboxy derivatives and their protein bound derivatives of the phytoestrogens stimulated 12H in both Ob (Figure 4b) while only cBA and cD-Ov had higher effect in prOb (Figure 4b). All carboxy derivatives of the phytoestrogens and their protein bound derivatives stimulated 15H in both Ob (Figure 4b) and only the protein bound derivatives of the phytoestrogens had higher effect in poOb (Figure 4b).

Age-dependent DNA and CK response of human female bone cells to estrogenic compounds

Human bone-derived cells in culture derived from either pre- or post-menopausal females respond to the different estrogenic compounds by stimulation of DNA synthesis (DNA) and the specific activity of creatine kinase (CK), a marker for intra cellular hormonal responsiveness (Figure 5a). In both parameters the stimulation by the different estrogenic compounds was higher in prOb except for BA (Figure 5a).
Discussion

We have previously shown age-dependent response of skeletal cells and organs to estrogenic compounds such as E2, phytoestrogens from different sources and SERMs, exemplified by raloxifene (Ral), in bone marrow and bone in vivo and in their derived cells in culture in vitro. We measured different intracellular effects such as cell proliferation determined as [3H] thymidine incorporation into DNA (DNA) and energy metabolism measured by the specific activity of creatine kinase BB (CK) as well as ERs mRNA expression, 1OHase mRNA expression and activity and LO mRNA expression and activity. In the present study we decided to examine whether these results imply also to the age-dependent response of Ob derived from human derived cultured female bone cells originated from pre- or post-menopausal women (prOb and poOb respectively).

We found that the expression of ERα in Ob is higher in prOb than in poOb, whereas ERβ is the same in both Ob [18]. This might explain the higher responsiveness of “younger” Ob compared to the “older” Ob. ERα is up-regulated by all estrogenic compounds in prOb (Figure 1a) whereas in poOb all compounds except D and PPT down-regulated ERα (Figure 1a). This means that not only that the level of ERα is lower in poOb, it is down-regulated by exposure to estrogenic compounds, which might be very problematic to the bone. ERβ on the other hand is down-regulated by exposure to estrogenic compounds, might be very problematic to the bone. The effect of pre-treatment for 3 days of prOb and poOb with JKF (1nM) and then 24H treatment with E2 (30nM), D, G, BA and Ral (3000nM) or DPN (420nM) was stimulated in poOb by D, G and Ral only, whereas it was inhibited in prOb (Figure 1a). If ERβ is mediating catabolic effects on bone, this might be again beneficial for the prOb. All carboxy-derivatives of the

Figure 5: a. Stimulation of CK (lower panel) and DNA (upper panel) in prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM). Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Control means were 33.6±6.8 for prOb and 23.8±2.0nmol/min/mg protein for poOb. Experimental means compared to control means: *, P<0.05; **, P<0.01. #P<0.05 compared with the level in prOb.

Figure 6: a. The effect of pre-treatment for 3 days of prOb and poOb with JKF (1nM) and then 24H treatment with E2 (30nM), D, G, BA and Ral (3000nM) or c. carboxy derivatives (300nM) for DNA. Cells were obtained, cultured, treated and assayed as described. Control means were 30.6±3.8 for prOb and 22.9±1.5nmol/min/mg protein for poOb. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, P<0.05; **, P<0.01; #P<0.01, ##P<0.05; ###P<0.01 compared with prOb.
Figures 2 and 4 to estrogenic compounds was modified by pre. These findings might explain some of the difference in vitamin D metabolism in the post-menopausal women which is yet to be examined at least in age-dependent animal models. In both age groups only BA, DPN and PPT up-regulated the 12LO activity resulted in increased 12H formation (Figure 4a) and only BA and RA up-regulated the 15LO activity resulted in increased 15H formation (Figure 4a). All carboxy- and protein-bound carboxy- derivatives of the phytoestrogens up-regulated regulation of both 12 and 15H in both Ob to different extent (Figure 4b). Whether this is beneficial or hazardous to the bone is not yet know.

We found that all estrogenic compounds except BA increased in both age groups Ob cell proliferation and energy metabolism (Figure 5a) but to higher extent in prOb. Again these findings indicate that “young” Ob are more active and responsive to the hormonal treatment than the “older” Ob.

The vitamin D less-calcemic analog JKF up-regulated similarly in both Ob the response of DNA and CK to the different estrogenic compounds except to BA (Figure 5b and Figure 6a). On the other hand the response to the carboxy- derivatives of the phytoestrogens was not affected by JKF (Figure 5c and Figure 6b). Ob from both age groups bind specifically E2 both to intracellular and membranal receptors similarly. JKF up-regulated competitive binding of all compounds except BA to the intracellular receptors in both Ob (Figure 7a). On the other hand JKF down-regulated competitive binding of all compounds except RA to the membranal receptors in both Ob (Figure 7b).

Of interest is the fact that the age-dependent response of bone cells in vitro to estrogenic compounds was modified by manipulation of the endocrine environment by vitamin D compounds to the same extent.

In conclusion cultured bone cells in vitro from human female pre- and post-menopausal bones respond in some of the parameters age-dependently to estrogenic by changes in intracellular and membranal parameters, in a yet unknown mechanism.

Whether or not this implies also to human bone physiology in vivo is yet to be established.

References
1. Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, et al. (1988) Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. Science 241:81-84.
2. Eriksson EF, Colvard DS, Berg NJ, Graham ML, Mann KG, et al. (1988) Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84-86.
3. Dayani N, Corvol MT, Robin P, Yechenine B, Moncharmont B, et al. (1988) Estrogen receptors in cultured rabbit articular chondrocytes: influence of age. J Steroid Biochem 31:351-356.
4. Colvard DS, Eriksson EF, Keeling PE, Wilson EM, Lubahn DB, et al. (1989) Identification of androgen receptors in normal human osteoblast-like cells. Proc Natl Acad Sci U S A 86:854-857.
5. Dalia Somjen, Sara Katzburg, Orly Sharona, Alvin M Kayeb, Batya Gayerc.
Fortune Kohenc, et al. (2004) Modulation of response to estrogens in cultured human female bone cells by a non-calcemic Vitamin D analog: changes in nuclear and membranal binding. J Steroid Biochem Mol Bio 89-90: 393-395.

6. Gray TK, Flynn TC, Gray KM, Nabell LM (1987) 17 beta-estradiol acts directly on the clonal osteoblastic cell line UMR106. Proc Natl Acad Sci U S A 84: 6267-6271.

7. Sömjen D, Weisman Y, Harell A, Berger E, Kaye AM (1989) Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone. Proc Natl Acad Sci U S A 86: 3361-3365.

8. Ernst M, Schmid C, Froesch ER (1988) Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Natl Acad Sci USA 85: 2307-2310.

9. Fukayama S, Tashjian AH Jr (1989) Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124: 397-401.

10. Takano-Yamamoto T, Rodan GA (1990) Direct effects of 17 beta-estradiol on trabecular bone in ovariectomized rats. Proc Natl Acad Sci U S A 87: 2172-2176.

11. Turner C.H (1991) Do estrogens increase bone formation? Bone 12: 305-306.

12. Kaye AM, Weisman Y, Harell A, Sömjen D (1990) Hormonal stimulation of bone cell proliferation. J Steroid Biochem Mol Biol 37: 431-435.

13. Berger E, Bleiberg I, Weisman Y, Lifschitz-Mercier B, Leider-Trejo L, et al. (2001) The hormonal milieu in early stages of bone cell differentiation modifies the subsequent sex-specific responsiveness of the developing bone to gonadal steroids. J Bone Miner Res 16: 823-831.

14. Katzburg S, Ornoy A, Hendel D, Lieberherr M, Kaye AM, et al. (2001) Age and gender specific stimulation of creatine kinase specific activity by gonadal steroids in human bone-derived cells in culture. J Endocrinol Invest 24:166-172.

15. McMillan J, Fateh-Sedeh S, Sylvia VL, Bingham V, Zhong M (2006) Sex-specific regulation of growth plate chondrocytes by estrogen is via multiple MAP kinase signaling pathways. Biochim Biophys Acta 1763: 381-392.

16. Boling EP (2001) Gender and osteoporosis: similarities and sex-specific differences. J Gend Specif Med 4: 36-43.

17. Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C (2010) Regulation of adult bone turnover by sex steroids. J Cell Physiol 224: 305-310.

18. Somjen D, Katzburg S, Kaye AM, Posner GH. Age-dependent Responsiveness of Human Female Bone Cells to Vitamin D Analog and PTH. In preparation.  

19. Somjen D, Kohen F, Lieberherr M, Gayer B, Scheijer E, et al. (2005) Membranal effects of phytoestrogens and carboxy derivatives of phytoestrogens on human vascular and bone cells: new insights based on studies with carboxy-biochanin A. J Steroid Biochem Mol Biol 93: 293-303.

20. Katzburg S, Lieberherr M, Ornoy A, Klein BY, Hendel D (1999) Isolation and hormone responsiveness of primary cultures of human bone-derived cells: gender and age differences. Bone 25: 667-673.

21. Katzburg S, Hendel D, Waisman A, Posner GH, Kaye AM, et al. (2004) Treatment with non-hypercalcemic analogs of 1,25-dihydroxyvitamin D3 increases responsiveness to 17beta-estradiol, dihydrotestosterone or raloxifene in primary human osteoblasts. J Steroid Biochem Mol Biol 88: 213-219.

22. Somjen D, Kohen F, Lieberherr M, Gayer B, Scheijer E, et al. (2005) The effects of phytoestrogens, and their carboxy derivatives on human vascular and bone cells: new insights based on studies with carboxy-biochanin A. J Steroid Biochem Mol Biol 93: 293-303.

23. Sömjen D, Kohen F, Lieberherr M (1997) Nongenomic effects of an anti-idiotypic antibody as an estrogen mimetic in female human and rat osteoblasts. J Cell Biochem 65: 53-66.

24. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

25. Somjen D, Katzburg S, Stern N, Kohen F, Sharon O, et al. (2007) 25 Hydroxy-vitamin D3 1α Hydroxylase expression and activity in cultured human osteoblasts and their modulation by parathyroid hormone, estrogenic compounds and dihydrotestosterone. J Steroid Biochem Mol Biol 107: 238-244.