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ESTROGEN PLUS ESTROGEN RECEPTOR ANTAGONISTS ALTER MINERAL PRODUCTION BY OSTEOBLASTS IN-VITRO

Estrogen alters mineral production

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Abstract

In early postmenopausal women, estrogen withdrawal is associated with increased bone turnover leading to bone loss and increased risk of fracture. Recent studies have suggested that the remaining bone tissue is significantly stronger, stiffer and has an increased tissue-level mineral content. Such changes may occur to compensate for bone loss or as a direct result of estrogen deficiency. To date many details of the physiology of osteoblastic cells during estrogen deficiency are vague. In this study we test the hypothesis that osteoblastic matrix mineralisation is altered at the onset of estrogen deficiency. In vitro cell culture experiments were carried out up to 28 days to compare the mineral production of MC3T3-E1 osteoblastic cells subject to estrogen deficiency (fulvestrant), enhanced estrogen supplementation (17-β estradiol) or a combination of both. Mineralisation was detected using von Kossa staining and was quantified with alizarin red absorbance readings. The expression of osteocalcin and osteopontin proteins, markers of osteoblast differentiation and mineralisation, was monitored using immunohistochemistry. Our results demonstrate that estrogen enhancement improves matrix mineralisation by MC3T3 cells in vitro. Furthermore this study found a significant reduction in the level of mineralisation when cells were treated with a combination of estrogen and fulvestrant. In an estrogen deficient environment mineralisation by osteoblastic cells was not altered. These findings suggest that altered tissue mineralisation following estrogen deficiency is not a direct result of estrogen deficiency on osteoblasts. Rather, we propose that altered tissue mineralisation may be a compensatory mechanism by bone to counter bone loss and reduced strength.

Key Words: Estrogen; Osteoblast; Bone Mineralization; Osteoporosis

Introduction

Osteoporosis is a disease, which causes bone loss, fractures and leads to severe pain, deformity and in certain cases secondary complications occur that result in death [1]. Postmenopausal osteoporosis (Type I) is the most common form of this disease and is believed to initiate when estrogen production is deficient following menopause [2]. During normal physiology bone is continually renewed by the co-ordinated activity of osteoclast and osteoblast cells [3]. However, following estrogen deficiency, osteoclasts remove excess bone without adequate formation by osteoblasts and this leads to significant bone loss [4]. Ultimately bone fractures occur under minimal trauma in the bones of the hip, wrist and spine.

In early postmenopausal women and ovariectomised rats and sheep, estrogen withdrawal is associated with increased bone turnover resulting in a decrease in whole bone and tissue biomechanical properties [5-9]. Interestingly, although osteoporosis reduces bone mass and structural strength, a recent study found that the remaining bone tissue is significantly stronger and stiffer [10]. This unexpected finding has been corroborated to some extent by micro-CT studies which demonstrated an increase in the tissue-level mineral content which was counter to the overall reduction in bone mineral density owing to a loss of bone mass [10]. More recent studies have provided further evidence of an increase in tissue mineral during osteoporosis [11, 12]. Such changes may occur to compensate for bone loss, or alternatively these changes may occur prior to bone resorption and be causative. However, although it is intriguing to speculate on such events, it remains that the mechanisms by which an increase in mineralisation is initiated are unknown.

Estrogen is instrumental in maintaining appropriate bone turnover and helping to prevent osteoporosis in women [13-16]. It has been well established that estrogen regulates the normal bone resorption process by inducing a paracrine signal in osteoblasts leading to decreased formation of mature osteoclasts [17] and increasing osteoclast apoptosis [18,19]. When estrogen levels are deficient the number of hematopoietic progenitors increases [20, 21] thereby increasing the numbers of mature osteoclasts forming [20-23]. One recent study suggests that the underlying basis of the protective effect of estrogen involves preventing osteoblast apoptosis and recruitment of osteoclasts [24]. However, although osteoclasts are the primary target for conventional drug treatments [25], inhibiting osteoclast activity alone does not completely prevent osteoporotic fractures from occurring. A greater understanding of estrogen and bone biology, specifically the effects of estrogen deficiency on bone cells, is necessary to develop successful treatments for bone metabolic disorders.

Osteoblasts are responsible for mineralising bone matrix by depositing mineral crystals on the organic matrix, which bind and nucleate in the presence of noncollagenous proteins such as alkaline phosphatase and osteocalcin [26,27], which are also produced by osteoblasts. Therefore tissue-level changes in mineral content during osteoporosis suggest that estrogen deficiency may alter the normal matrix mineralisation by osteoblastic cells. Indeed osteoblasts possess estrogen receptors [28] and studies have shown that in vitro treatment with an estrogen compound, 17ß-estradiol (E2), enhances proliferation and differentiation of osteoblasts [29, 30], regulates the growth of mineralised bone nodules and prevents apoptosis [31]. Research has shown that osteoblastic cells from osteoporotic patients display impaired response to mechanical stress in vitro [32]. Estrogen deficiency also induces osteocyte apoptosis [33, 34], which might result in hyper-mineralisation of the surrounding tissue [35-37]. These studies indicate that a change in osteoblastic activity may occur when estrogen production is deficient during osteoporosis. Furthermore recent studies suggest that estrogen's regulation of osteoclast activity may be a secondary effect governed by osteoblasts [38]. However to date the role of osteoblasts during estrogen deficiency is not fully understood and merits further research.

Fulvestrant, or ICI 182,780, is an estrogen receptor (ER) antagonist used to treat postmenopausal women who are suffering from estrogen receptor-positive metastatic breast cancer. It is reported to have no agonist effects [39] and works both by down-regulating and by degrading the estrogen receptor leading to an inhibition of estrogen signalling through the estrogen receptor [40-42]. Fulvestrant is an analogue of estradiol which competes with endogenous estrogen for binding to the estrogen receptor [43] with a binding affinity that is 89% that of 17-β estradiol [44]. Previously fulvestrant has been used in vitro to mimic estrogen deficiency in human osteoblasts [45], however, the exact role of fulvestrant on osteoblast activity is unknown.

In this study we test the hypothesis that osteoblast number, non collagenous protein production and matrix mineralisation are altered at the onset of estrogen deficiency. In vitro cell culture experiments were carried out to compare the mineral production of osteoblastic cells subject to estrogen treatment and supplemented with the estrogen receptor antagonist, fulvestrant.

Materials and Methods

Cell culture

MC3T3-E1 murine calvarial osteoblasts were maintained in α-modified MEM supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100U/ml penicillin and 100 µg/ml streptomycin. Cells were grown until sub-confluence and then plated at a density of 2×10^4 cells per ml in 6-well plates with 2ml

of osteogenic media where matrix mineralisation was enhanced using 50µg/ml ascorbic acid, 10mM/L β-glycerophosphate and 10nM/L dexomethasome. All treatments were initiated after 24 hrs of incubation (day 0) when cells were cultured under separate osteogenic conditions; (1) cells were treated with 100nM of commercially available 17β-estradiol (E2) (Sigma), (2) cells were treated with a commercially available estrogen antagonist ICI 182780 (Fulvestrant, Sigma) at either 0.1 µM (F_1) or 10µM (F_2), (3) to inhibit estrogen activity, cells were treated with either F_1 or F_2 in estrogen enhanced osteogenic media, (4) cells were grown in osteogenic media (control). Tissue culture mediums were replenished with fresh media every 3-4 days. All cells were maintained at 37 °C in a humidified 5% CO_2 environment for a period of 7, 14, 21 or 28 days. Each experiment was performed in duplicate wells with 3 repeats (n=6).

Quantification of cell number

To determine cell number, cells were fixed in 10% neutral buffered formalin for 15 minutes before being rinsed twice with phosphate buffered saline (PBS). The cells were then incubated in 1 µg/ml DAPI diluted in PBS for 30 minutes. They were then washed and mounted using Vectashield Mounting Media. Slides were examined using epifluorescence microscopy and the cell number determined.

Quantification of mineralisation

Mineralised matrix was detected using alizarin red staining, for quantitative analysis, and von Kossa staining for qualitative analysis. For alizarin red staining the cells were fixed with 10% neutral buffered formalin for 10 minutes and then rinsed twice with deionised water before being stained with 2% alizarin red for 5 minutes. For quantification, cells stained with alizarin red were de-stained with 10% cethylpyridium chloride for 20 minutes before 100µl of the extracted stain was transferred to a 96-well plate and the absorbance at 570nm was determined using an atomic absorption spectrophotometer (Wallac Victor, PerkinElmer Life Sciences). Using a standard curve the absorption reading was converted into calcium ion concentration (Ca^{2+}) and the results were normalised to the cell number to estimate calcium concentration per cell.

For von Kossa staining the cells were initially fixed in 4% paraformaldehyde for 10 minutes. They were treated with 5% silver nitrate for 60 minutes under bright light, followed by two washes with PBS and treatment with 5% sodium thiosulfate for 5 minutes. The reaction was stopped by washing the cells with deionised water. Cells were counter stained with nuclear fast red (Sigma) for 10 minutes before being rinsed in deionised water. The cells were imaged at 10X magnification under brightfield microscopy (Eclipse 90i, Nikon) to identify the phosphate deposits in the bone nodules.

Immunohistochemistry for non-collagenous protein expression

Osteocalcin (OCN) and osteopontin (OPN) protein expression were examined by immunohistochemistry using primary anti-osteocalcin rabbit polyclonal IgG (antibody sc-30045, Santa Cruz Biotechnologies Inc, USA) and anti-osteopontin IgG rabbit polyclonal IgG (sc-20788, Santa Cruz Biotechnologies Inc, USA) according to the manufacturer's instructions. Briefly, cells were fixed in 1% formalin for 10 minutes before being rinsed with PBS. The cells were then permeablilised with tween and incubated overnight at 4°C with the primary antibodies diluted at 1:200. The following day, cells were incubated at room temperature for 2 hours with a goat anti-rabbit IgG-FITC diluted at 1:200. The cells were then mounted using Vectashield mounting media (Vector Laboratories, USA). Staining was detected using fluorescence microscopy (Eclipse 90i, Nikon). ImageJ image processing (NIH Freeware, USA) was then used to determine the percentage area of the image which was stained.

Statistics

Results are expressed as a mean ± standard deviation. Statistical evaluations of differences between groups over time were carried out using multiple analyses of variance and t-tests (SPSS Inc, Chicago, IL). For all statistical tests, a value of $p \leq 0.05$ was considered to be a statistically significant difference.

Results

Cell Number:

Results from the DAPI count showed that cell number increased significantly in the osteogenic group between day 0 and day 7 ($p<0.005$) and also between days 7 and 14 ($p<0.05$) (Figure 1). There was no subsequent increase in cell number at days 21 and 28. In the estrogen enhanced group, cell number also rose significantly by day 7 ($p<0.005$) and between days 7 and 14 ($p<0.005$). As with the osteogenic group cell number did not significantly increase between days 14 and 21 or between days 21 and 28. A similar pattern was observed in F_1 , F_2 , F_1+E2 and F_2+E2 groups with a significant increase in cell number at day 7 ($p<0.05$). Cell number was again significantly greater by day 14 than day 7 ($p<0.05$) in these groups. No further increases were seen between time points in these groups. There was no significant difference in cell number between the groups at any of the time points.

Alizarin Red:

In the osteogenic group (control) the calcium concentration/cell increased significantly over time from day 0 to day 28 ($p<0.0001$) (Figure 2). Specifically, the Ca^{2+} concentration per cell increased significantly from day 0 to day 7 ($p<0.0001$), day 7 to 14 ($p<0.0001$), day 14 to day 21 ($p<0.005$) and day 21 to day 28 ($p<0.0001$). In the 17-β estradiol enhanced group (E2), significant increases in mineral production were also measured between days 0 and 28 ($p<0.0001$). Incrementally there was an increase between days 0 and 7 ($p<0.001$) and between days 7 and 14, 14 and 21, and days 21 and 28 ($p<0.0001$). In the group treated with the lower fulvestrant concentration (F_1) it was found that mineralisation also increased significantly from day 0 to day 28 ($p<0.0001$). There were significant incremental increases in mineralisation between days 0 and 7, days 7 and 14, and days 21 and 28 ($p<0.0001$). At the higher fulvestrant concentration, F_2, there was again a significant increase in mineralisation between days 0 and 28 and also between each time point ($p<0.0001$).

MC3T3s which were cultured with estrogen plus fulvestrant (F_1+E2 and F_2+E2) also showed an increase in mineral production by day 28 relative to day 0 ($p<0.0001$). In F_1+E2 mineralisation increased significantly between days 0 and 7 ($p<0.0001$), day 7 and 14 ($p<0.0001$) and days 21 and 28 ($p<0.0001$). However there was no increase in mineralisation between days 14 and 21 ($p=0.3$). In MC3T3 cells cultured with F_2+E2 again a significant increase in mineralisation over the duration of the study was found ($p<0.0001$). With significant increases measured during each interval, between days 0 and 7 ($p<0.0001$), days 7 to 14 ($p<0.0005$), days 14 and 21 ($p<0.005$) and between days 21 and 28 ($p<0.0001$).

Comparison between the groups at each time point found that at day 7 there was no significant difference in the level of mineralisation between the groups. By day 14, mineralisation was significantly greater in the estrogen enhanced group and also in the lower fulvestrant concentration, F_1, than the osteogenic group ($p<0.001$). At day 21, mineralisation continued to be significantly greater in E2 than in the osteogenic group ($p<0.0001$). At day 21 mineralisation was also significantly lower in F_1+E2 than in the osteogenic group ($p<0.0001$). By the final time point, day 28, mineralisation continued to be greater in E2 than the osteogenic group ($p<0.0001$). There was no significant difference in mineralisation between either F_1 or F_2 and the osteogenic control. Notably, at this point mineralisation was significantly less in F_1+E2 and F_2+E2, than in the osteogenic group ($p<0.0001$).

Von Kossa staining:

The Von Kossa staining supports the results from the alizarin red quantification. The level of mineralisation, as indicated by mineralised nodules in Figure 3, increases over time with the largest number of mineralised nodules evident at day 28 in all conditions. A greater number of nodules are visible in the E2 group than any other group. The least number of nodules are present in the MC3T3-E1 samples which were cultured with estrogen plus fulvestrant.

Osteocalcin and Osteopontin:

Osteocalcin expression and osteopontin expression were reported as the % area of fluorescence. Figure 4a shows that osteocalcin expression did not increase significantly between days 0 and 7 or days 7 and 14 in any of the groups. There was also no increase between days 14 and 21 in any of the groups with the exception of E2 ($p<0.05$). However, between days 21 and 28 there was a significant increase ($p<0.0001$) in all groups. In the estrogen plus fulvestrant groups, osteocalcin expression did not significantly change between days 0 and 7, however, between days 7 and 14 there was a significant reduction in expression ($p<0.05$). No change in expression was measured between day 14 and day 21, and again there was a significant increase between days 21 and 28 ($p<0.0001$).

At day 7 there were no significant changes in osteocalcin expression in any of the experimental groups relative to control. At day 14, expression in F_1+E2 was significantly reduced relative to the control group ($p<0.05$). By day 21, osteocalcin expression in F_1+E2 and F_2+E2 and also in F_2 was significantly reduced relative to the control group ($p<0.001$). However, expression was significantly higher in the estrogen enhanced group than the controls ($p<0.05$). At day 28, osteocalcin expression was again significantly reduced in F_1+E2 and F_2+E2 ($p<0.0001$) and also in F_1 ($p<0.05$) while expression remained highest in the estrogen enhanced group ($p<0.001$).

Osteopontin expression did not significantly increase between days 0 and 7 in any of the experimental conditions (Figure 4b). There was a significant increase in expression in all groups between days 7 and 14 ($p<0.05$), days 14 and 21 ($p<0.001$) and also between days 21 and 28 ($p<0.001$). No significant difference in expression was measured between groups at days 7 or 14. At day 21, osteopontin expression was significantly greater in the estrogen enhanced group than the control group ($p<0.05$). Meanwhile in F_1+E2 and F_2+E2, there was a significantly lower osteopontin expression than the

controls ($p<0.001$). At day 28 E2 remained significantly higher and F_1+E2 and F_2+E2 were significantly less, as was the case with F_1 ($p<0.05$).

DISCUSSION

In this study we report that estrogen treatment alone increases matrix mineralisation by MC3T3 cells in vitro, while blocking the estrogen receptors through the use of fulvestrant alone did not influence mineralisation by these osteoblastic cells. Interestingly, culturing cells in estrogen plus fulvestrant significantly reduced mineralisation by MC3T3 cells. Osteocalcin protein expression corroborated the mineralisation results while osteopontin expression also highlighted the differentiation of osteoblasts. DAPI staining showed that there was no significant difference in cell number between the groups at any of the time points. Thus, changes in protein expression and mineral levels between the groups can be attributed to changes in the ability of the osteoblasts to produce mineral rather than due to changes in the number of cells present.

The current study has a number of limitations. It may not be correct to assume that fulvestrant treatment accurately represents in vivo estrogen deficiency as the cells were not previously treated with high levels of estrogen prior to treatment with fulvestrant. Nonetheless the current studies provide an insight into bone cell biology in an estrogen deficienct environment. Another limitation of this study may be the use of MC3T3 cells, a murine immortalised cell line, to infer estrogen related changes in human bone cells. MC3T3-E1 cells are an osteoblast cell line which express high amounts of alkaline phosphatase, produce mineral and terminally differentiate into osteocytes [46] , and for these reasons are accepted as an appropriate model of primary osteoblasts when cultured with osteogenic media [47,48] . Finally, it was not possible to test the hypothesis that increased mineralisation occurs as a compensatory mechanism by bone to counter bone loss following estrogen deficiency using in vitro cell culture experiments. Future studies are required that employ an in vivo model to test this hypothesis and fully understand the origin of changes in bone tissue mineralisation during estrogen deficiency.

The control cells behaved consistently with previous studies whereby the osteoblastic cells proliferated rapidly up until day 14 after which there was a period of extracellular matrix development up until day 21, production of OCN and OPN, followed by a period of mineralisation [49]. Cells treated with estrogen increased in number rapidly and produced significantly more mineral, osteocalcin and osteopontin than any other group. Studies have suggested that estrogens have an anabolic effect that directly influences osteoblasts [50,51]. The current study supports this as the increased osteopontin

expression seen at day 21 is a characteristic marker of osteoblast differentiation [49]. Estrogen also up-regulates the mRNA expression of osteogenic genes for alkaline phosphatase, collagen I, transforming growth factor-beta1 and bone morphogenic protein-2 [52]. In this study we also show that estrogen increased osteocalcin expression and mineralisation.

The current study found that treatment with fulvestrant alone at either 0.1µM or 10µM did not result in significant changes in cell number or mineralisation when compared to the control group. One previous study also showed that addition of fulvestrant to osteoblasts did not cause any change in cell proliferation, osteoblastic markers or mineralisation when compared to control cultures [29]. Although mineralisation was not significantly different between the fulvestrant treated groups and the controls, both osteocalcin and osteopontin expression were significantly reduced in F_1 at day 28. It has previously been shown that fulvestrant can reduce bone volume in rats [53] and treatment of human osteoblasts with fulvestrant in non-osteogenic conditions results in downregulation of estrogen receptor expression and can induce adipogenesis [45]. The results from the current study suggest that attachment of the fulvestrant molecule, an estrogen antagonist, to the osteoblast estrogen receptor is not sufficient to initiate an increase in mineralisation. As the cells were expanded and cultured in alpha MEM which contains phenol red, a weak estrogen [54], blocking the estrogen receptors with fulvestrant is depriving the cells of estrogen. These findings therefore suggest that estrogen deficiency might not directly alter the normal mineralisation process by osteoblastic cells.

Therefore tissue level changes in mineral content in osteoporotic bone might arise to compensate for bone loss i.e. changes in mineralisation occur indirectly as a result of structural changes in the bone rather than as a direct result of estrogen deficiency. It is known that osteoblasts and osteocytes can transduce mechanical signals into biochemical stimuli to alter bone mass [26,55]. Following osteoporotic bone loss these cells are likely to be subjected to elevated loading. Therefore, it may be that the mechanical properties of remaining tissue are altered to compensate for the overall reduction in bone strength to some extent, perhaps when osteocytes embedded in the remaining tissue experience elevated loading.

When cells were grown with both estrogen and fulvestrant there was a significant decrease in the amount of mineral produced by the osteoblasts in comparison to those treated in osteogenic media and with either estrogen or fulvestrant alone. That mineralisation was significantly less than the control group is interesting. Although selective-estrogen receptor downregulators, such as fulvestrant, are reported to lack estrogen agonist activity, results of preclinical studies have shown that fulvestrant may

actually have agonist and antagonist effects on bone, depending on the presence of circulating estradiol levels [58]. In ovariectomized rats, bone turnover and bone loss increased after fulvestrant therapy, whereas bone turnover and bone loss decreased in rats with intact ovaries receiving fulvestrant [53]. In the current study it would appear that in the absence of estrogen, fulvestrant has no effect on osteoblast activity whereas in the presence of estrogen, osteoblast activity is decreased. The reasons for this are not entirely clear. Until very recently no human data existed regarding fulvestrant's effect on bone turnover. Unlike other breast cancer treatments, such as tamoxifen which possess some agonist effects, fulvestrant is reported to have none [59]. However, a pilot study of 14 postmenopausal women suffering from breast cancer found that bone formation and resorption increased over time following treatment with fulvestrant [60]. As mentioned previously the decline in estrogen levels following menopause results in increased bone turnover which leads to decreased bone mass and an increased risk of fracture. From the current study it appears that osteoblast activity is inhibited by the presence of estrogen and fulvestrant in combination. In conjunction with previous studies (52,56,58) which indicate that the effects of fulvestrant may depend on the levels of circulating hormones and also that fulvestrant may have some agonist effects with respect to bone, this study suggests that there is much to learn about the effects of fulvestrant on bone cells. Certainly the current findings suggest that in the presence of estrogen, fulvestrant has a deleterious effect on bone formation.

Conclusion

This study has found that estrogen deficiency, as induced by fulvestrant, had no significant effect on mineralisation by the osteoblasts. This result suggests that the increased mineralisation seen in previous studies following estrogen deficiency is not a direct result of estrogen deficiency on the osteoblasts. Rather, it is hypothesised that it may occur as a compensatory mechanism by bone to counter the loss of bone mass following estrogen deficiency.

REFERENCES

1 Johnell O, Kanis JA, Oden A, Sernbo I, Redlund-Johnell I, Petterson C, De Laet C, Jonsson B. Mortality after osteoporotic fractures. Osteoporos Int 2004; 15:38-42

2 Riggs BL, Khosla S, Melton LJ, 3rd. Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 2002; 23:279-302

3 Parfitt AM. The cellular basis of bone remodeling: the quantum concept reexamined in light of recent advances in the cell biology of bone. Calcif Tissue Int 1984; 36 Suppl 1:S37-45

4 Eriksen EF, Hodgson SF, Eastell R, Cedel SL, O'Fallon WM, Riggs BL. Cancellous bone remodeling in type I (postmenopausal) osteoporosis: quantitative assessment of rates of formation, resorption, and bone loss at tissue and cellular levels. J Bone Miner Res 1990; 5:311-319

5 Namkung-Matthai H, Appleyard R, Jansen J, Hao Lin J, Maastricht S, Swain M, Mason RS, Murrell GA, Diwan AD, Diamond T. Osteoporosis influences the early period of fracture healing in a rat osteoporotic model. Bone 2001; 28:80-86

6 Turner CH, Forwood MR. On animal models for studying bone adaptation. Calcif Tissue Int 1994; 55:316-317

7 Wronski TJ, Dann LM, Scott KS, Cintron M. Long-term effects of ovariectomy and aging on the rat skeleton. Calcif Tissue Int 1989; 45:360-366

8 Kennedy OD, Brennan O, Rackard SM, Staines A, O'Brien FJ, Taylor D, Lee TC. Effects of ovariectomy on bone turnover, porosity, and biomechanical properties in ovine compact bone 12 months postsurgery. J Ortho Res 2009; 27:303-309

9 Brennan O, Kennedy OD, Lee TC, Rackard SM, O'Brien FJ. Biomechanical properties across trabeculae from the proximal femur of normal and ovariectomised sheep. J Biomech 2009; 42:498-503

10 McNamara LM, Ederveen AG, Lyons CG, Price C, Schaffler MB, Weinans H, Prendergast PJ. Strength of cancellous bone trabecular tissue from normal, ovariectomized and drug-treated rats over the course of ageing. Bone 2006; 39:392-400

11 Brennan M, Gleeson JP, Browne M, O'Brien FJ, Thurner PJ, McNamara LM. Site Specific Increase in Heterogeneity of Trabecular Bone Tissue Mineral during Estrogen Deficiency. e Cells and Materials 2011; 15:396-406

12 Busse B, Hahn M, Soltau M, Zustin J, Puschel K, Duda GN, Amling M. Increased calcium content and inhomogeneity of mineralization render bone toughness in osteoporosis: mineralization, morphology and biomechanics of human single trabeculae. Bone 2009; 45:1034-1043

13 Khosla S, Melton LJ, 3rd, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. J Clin Endocrinol Metab 1998; 83:2266-2274

14 Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 1996; 11:1043-1051

15 Rozenberg S, Kroll M, Pastijn A, Vandromme J. Osteoporosis prevention and treatment with sex hormone replacement therapy. Clin Rheumatol 1995; 14 Suppl 3:14-17

16 Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 1994; 331:1056-1061

17 Oursler MJ, Osdoby P, Pyfferoen J, Riggs BL, Spelsberg TC. Avian osteoclasts as estrogen target cells. Proc Natl Acad Sci U S A 1991; 88:6613-6617

18 Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. Nat Med 1996; 2:1132-1136

19 Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP, Brown M. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. EMBO J 2008; 27:535-545

20 Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 1992; 257:88-91

21 Jilka RL, Passeri G, Girasole G, Cooper S, Abrams J, Broxmeyer H, Manolagas SC. Estrogen loss upregulates hematopoiesis in the mouse: a mediating role of IL-6. Exp Hematol 1995; 23:500-506

22 Rosen CJ. Pathogenesis of osteoporosis. Baillieres Best Pract Res Clin Endocrinol Metab 2000; 14:181-193

23 Brockstedt H, Kassem M, Eriksen EF, Mosekilde L, Melsen F. Age- and sex-related changes in iliac cortical bone mass and remodeling. Bone 1993; 14:681-691

24 Bradford PG, Gerace KV, Roland RL, Chrzan BG. Estrogen regulation of apoptosis in osteoblasts. Physiol Behav 2009; 99:181-185

25 Kloosterboer HJ, Ederveen AG. Pros and cons of existing treatment modalities in osteoporosis: a comparison between tibolone, SERMs and estrogen (+/- progestogen) treatments. J Steroid Biochem Mol Biol 2002; 83:157-165

26 el Haj AJ, Minter SL, Rawlinson SC, Suswillo R, Lanyon LE. Cellular responses to mechanical loading in vitro. J Bone Miner Res 1990; 5:923-932

27 Marks SC, Popoff SN. Bone cell biology: The regulation of development, structure, and function in the skeleton. Am J Anat 1988; 183:1-44

28 Batra GS, Hainey L, Freemont AJ, Andrew G, Saunders PT, Hoyland JA, Braidman IP. Evidence for cell-specific changes with age in expression of oestrogen receptor (ER) alpha and beta in bone fractures from men and women. J Pathol 2003; 200:65-73

29 Qu Q, Perala-Heape M, Kapanen A, Dahllund J, Salo J, Vaananen HK, Harkonen P. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 1998; 22:201-209

30 Riggs BL, Melton LJ, 3rd. Bone turnover matters: the raloxifene treatment paradox of dramatic decreases in vertebral fractures without commensurate increases in bone density. J Bone Miner Res 2002; 17:11-14

31 Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 2002; 298:843-846

32 Sterck JG, Klein-Nulend J, Lips P, Burger EH. Response of normal and osteoporotic human bone cells to mechanical stress in vitro. Am J Physiol 1998; 274:1113-1120

33 Brennan O, Kennedy OD, Lee TC, Rackard SM, O'Brien FJ. Effects of estrogen deficiency and bisphosphonate therapy on osteocyte viability and

microdamage accumulation in an ovine model of osteoporosis. J Orthop Res 2011; 29:419-424
34 Tomkinson A, Reeve J, Shaw RW, Noble BS. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. J Clin Endocrinol Metab 1997; 82:3128-3135
35 Frost HM. Micropetrosis. J Bone Joint Surg Am 1960; 42-A:144-150
36 Boyde A. The real response of bone to exercise. J Anat 2003; 203:173-189
37 Kingsmill VJ, Boyde A. Mineralisation density of human mandibular bone: quantitative backscattered electron image analysis. J Anat 1998; 192 (Pt 2):245-256
38 Michael H, Harkonen PL, Vaananen HK, Hentunen TA. Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption. J Bone Miner Res 2005; 20:2224-2232
39 Osborne CK, Wakeling A, Nicholson RI. Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. Br J Cancer 2004; 90 Suppl 1:S2-6
40 Kansra S, Yamagata S, Sneade L, Foster L, Ben-Jonathan N. Differential effects of estrogen receptor antagonists on pituitary lactotroph proliferation and prolactin release. Mol Cell Endocrinol 2005; 239:27-36
41 Wakeling AE. The future of new pure antiestrogens in clinical breast cancer. Breast Cancer Res Treat 1993; 25:1-9
42 Wakeling AE. Similarities and distinctions in the mode of action of different classes of antioestrogens. Endocr Relat Cancer 2000; 7:17-28
43 Curran M, Wiseman L. Fulvestrant. Drugs 2001; 61:807-813; discussion 814
44 Wakeling AE, Bowler J. Steroidal pure antioestrogens. J Endocrinol 1987; 112:R7-10
45 Foo C, Frey S, Yang HH, Zellweger R, Filgueira L. Downregulation of beta-catenin and transdifferentiation of human osteoblasts to adipocytes under estrogen deficiency. Gynecol Endocrinol 2007; 23:535-540
46 Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983; 96:191-198
47 Menard C, Mitchell S, Spector M. Contractile behavior of smooth muscle actin-containing osteoblasts in collagen-GAG matrices in vitro: implant-related cell contraction. Biomaterials 2000; 21:1867-1877
48 Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 1992; 7:683-692
49 Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 1993; 14:424-442
50 Bellido T, Girasole G, Passeri G, Yu XP, Mocharla H, Jilka RL, Notides A, Manolagas SC. Demonstration of estrogen and vitamin D receptors in bone marrow-derived stromal cells: up-regulation of the estrogen receptor by 1,25-dihydroxyvitamin-D3. Endocrinology 1993; 133:553-562
51 Bu YH, Peng D, Zhou HD, Huang QX, Liu W, Luo XB, Tang LL, Tang AG. Insulin receptor substrate 2 plays important roles in 17beta estradiol induced bone formation. J Endocrinol Invest 2009; 38:682-689
52 Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D. Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J Cell Biochem Suppl 2001; Suppl 36:144-155

[53] Gallagher A, Chambers TJ, Tobias JH. The estrogen antagonist ICI 182,780 reduces cancellous bone volume in female rats. Endocrinology 1993; 133:2787-2791

[54] Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 1986; 83:2496-2500

[55] McGarry J, Klein-Nulend J, Prendergast PJ. The effect of cytoskeletal disruption on pulsatile fluid flow-induced nitric oxide and prostaglandin E(2) in osteocytes and osteoblasts. Biochem Biophys Res Commun 2005; 330:341-348

[56] Frost HM. In vivo osteocyte death. J Bone Joint Surg Am 1960; 42-A:138-143

[57] Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001; 104:719-730

[58] Heshmati HM, Khosla S, Robins SP, O'Fallon WM, Melton LJ, 3rd, Riggs BL. Role of low levels of endogenous estrogen in regulation of bone resorption in late postmenopausal women. J Bone Miner Res 2002; 17:172-178

[59] Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326:852-856

[60] Agrawal A, Hannon RA, Cheung KL, Eastell R, Robertson JF. Bone turnover markers in postmenopausal breast cancer treated with fulvestrant--a pilot study. Breast 2009; 18:204-207

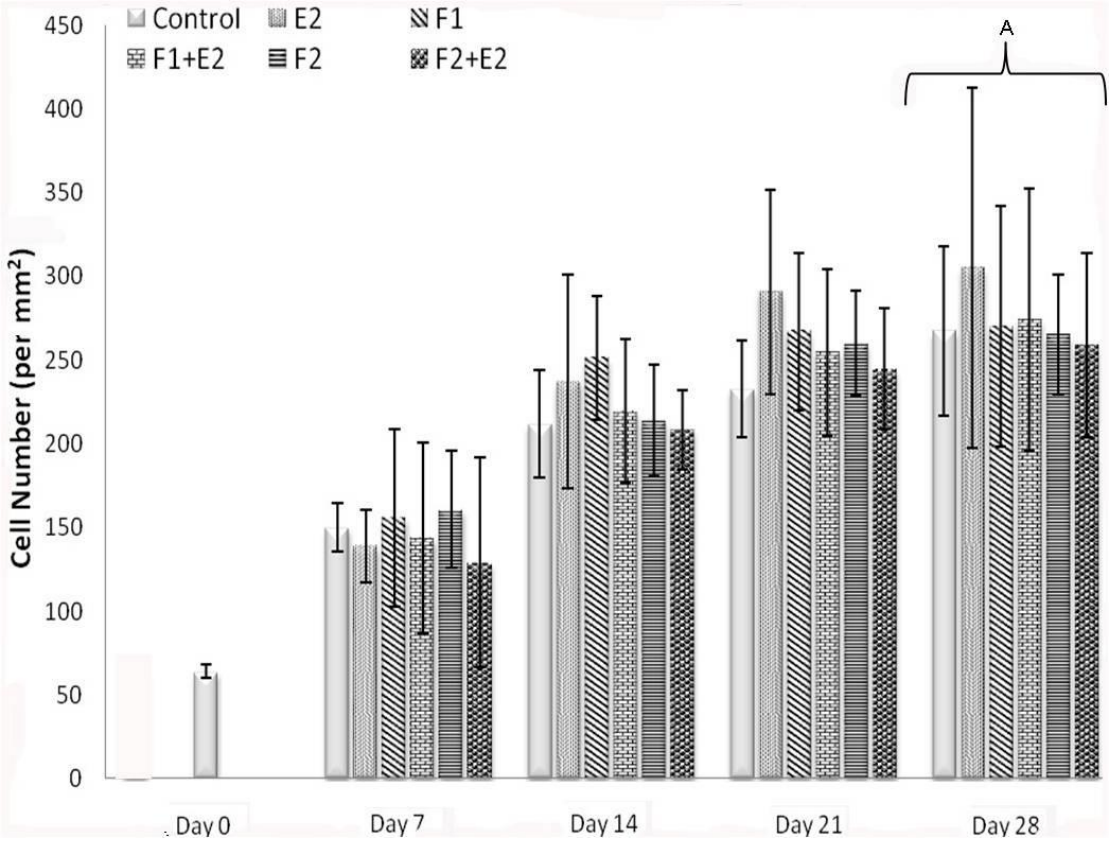

Figure 1: Cell number measured using DAPI showed a significant increase between day 0 and day 28 in all groups (A, $p<0.001$). All groups showed a significant increase in cell number between days 0 and 7 and days 7 and 14. There was no significant difference in cell number between the groups at any time point.

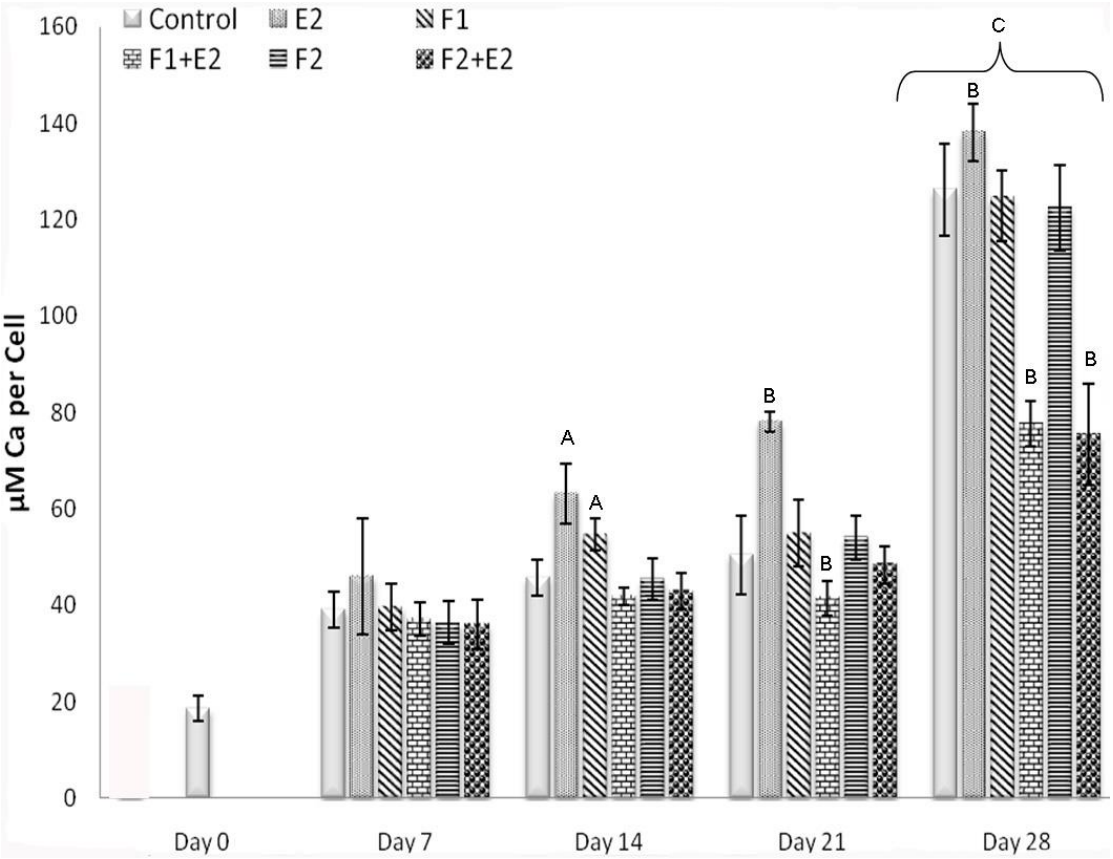

Figure 2: Analysis of cell's ability to produce mineral showed an increase over time in all groups with significantly more mineral by day 28 (C, $p<0.0001$) regardless of the conditions. Notable increases in mineral production were in the estrogen enhanced group at day 14 (A, $p<0.001$) and days 21 and 28 (B, $p<0.0001$). Mineral production was also significantly increased in F_1 at Day 14 ($p<0.001$). Significant reductions in mineral were seen at days 21 and 28 in F_1+E2 and by day 28 in F_2+E2 (B, $p<0.0001$).

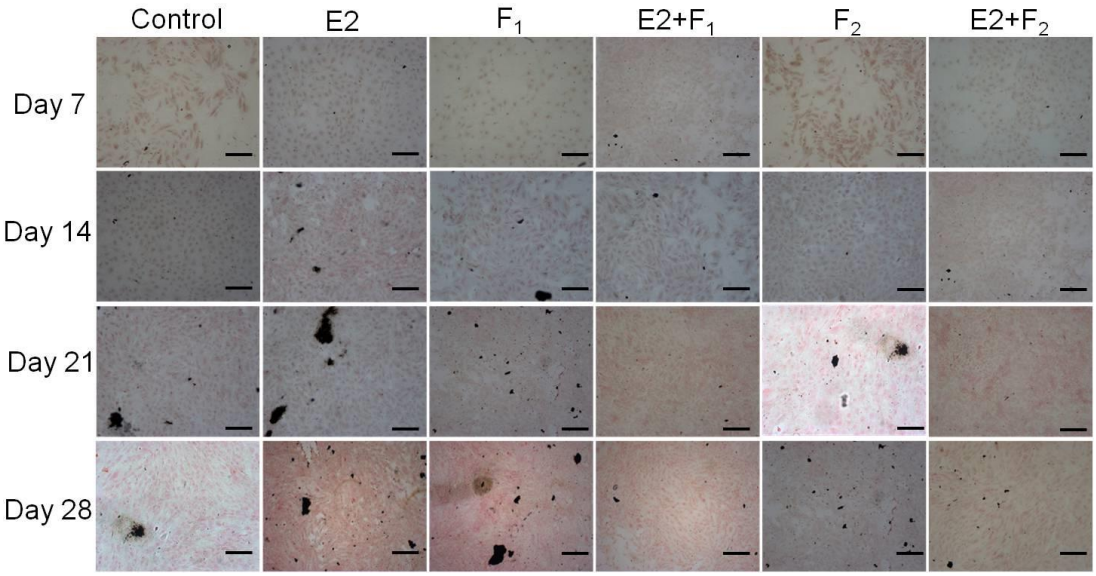

Figure 3: von Kossa and nuclear fast red stained MC3T3 cells (4X magnification; scale bar = 100µm) cultured for 7,14, 21 and 28 days in various media conditions. An increase in cell number can be seen in all groups over time and increased mineralisation over time in the osteogenic and estrogen enriched groups is evident.

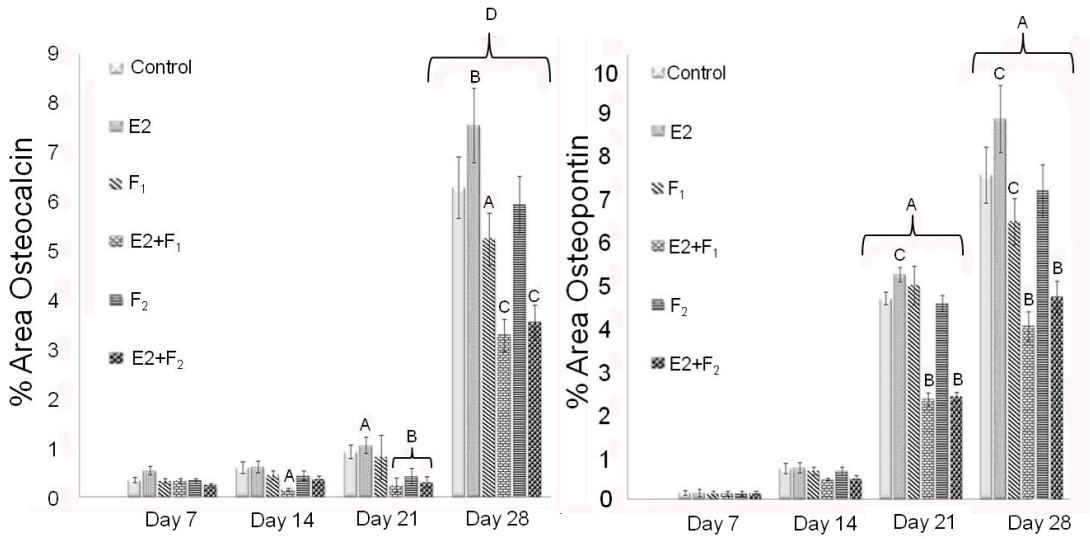

Figure 4: Osteocalcin expression was significantly increased in all groups by day 28 (D, $p<0.0001$). At day 14 expression in F_1+E2 was significantly reduced (A, $p<0.05$). By day 21 osteocalcin expression was significantly lower in F_1+E2, F_2+E2 and in F_2 (B, $p<0.001$) and remained higher in E2 (A, $p<0.05$). At the final time point, expression continued to be significantly greater in E2 (B, $p<0.001$). Osteopontin expression was significantly increased in all groups by days 21 and day 28 (A, $p<0.0001$). At day 21 expression levels were significantly lower in F1+E2 and F_2+E2 (B, $p<0.001$) and were higher in E2 (C, $p<0.05$). At the final time point osteopontin expression continued to be significantly higher in E2 (C, $p<0.05$) while expression remained lower in F_1+E2 and F_2+E2 (B, $p<0.001$) and a decrease in F_1 (C, $p<0.05$) was seen.