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Orlaith Brennan

Royal College of Surgeons in Ireland, obrennan1@rcsi.ie

Fergal J. O'Brien

Royal College of Surgeons in Ireland, fjobrien@rcsi.ie

Laoise M. McNamara

NUI Galway, Ireland

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

Estrogen alters mineral production

Orlaith Brennan^{1,2}, Fergal J O'Brien^{1,2} and Laoise McNamara³

- 1 Department of Anatomy, Royal College of Surgeons in Ireland, Dublin 2, Ireland
- 2 Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
- 3 Department of Mechanical and Biomedical Engineering, NUI Galway, Ireland

Corresponding Author

Laoise McNamara

Department of Mechanical and Biomedical Engineering,

NUI Galway,

Galway,

Ireland

Phone: +353-91-492251

Fax: +353- 91-563991

E-mail: laoise.mcnamara@nuigalway.ie

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, 17 β -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 μ g/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 dexamethasone. 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 (F₁) or 10µM (F₂), (3) to inhibit estrogen activity, cells were treated with either F₁ or F₂ 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% CO₂ 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% cetylpyridium 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 (Ca²⁺) 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 permeabilised 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 \pm 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 F₁+E2 was significantly reduced relative to the control group ($p < 0.05$). By day 21, osteocalcin expression in F₁+E2 and F₂+E2 and also in F₂ 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 F₁+E2 and F₂+E2 ($p < 0.0001$) and also in F₁ ($p < 0.05$) 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 F₁+E2 and F₂+E2, there was a significantly lower osteopontin expression than the

controls ($p < 0.001$). At day 28 E2 remained significantly higher and F₁+E2 and F₂+E2 were significantly less, as was the case with F₁ ($p < 0.05$).

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 deficient 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 F₁ 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.

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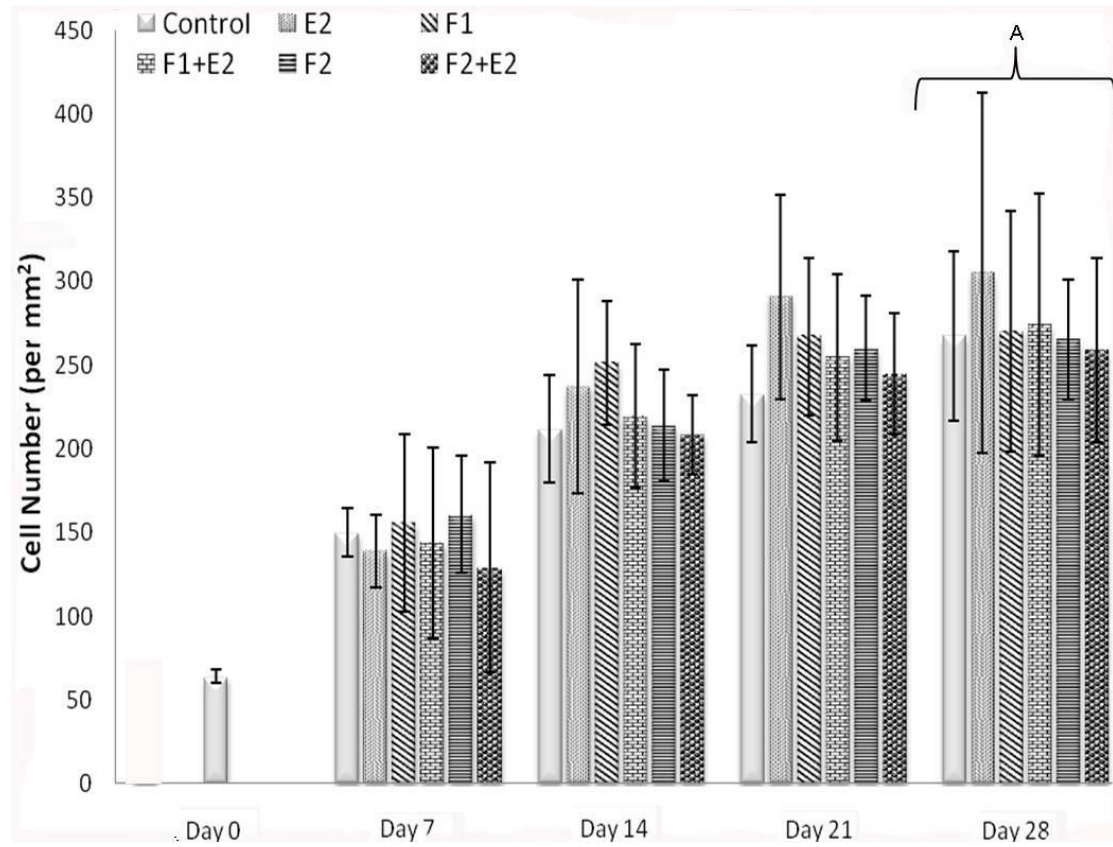


Figure 1: Cell number measured using DAPI showed a significant increase between day 0 and day 28 in all groups (A, $p < 0.001$). All groups showed a significant increase in cell number between days 0 and 7 and days 7 and 14. There was no significant difference in cell number between the groups at any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

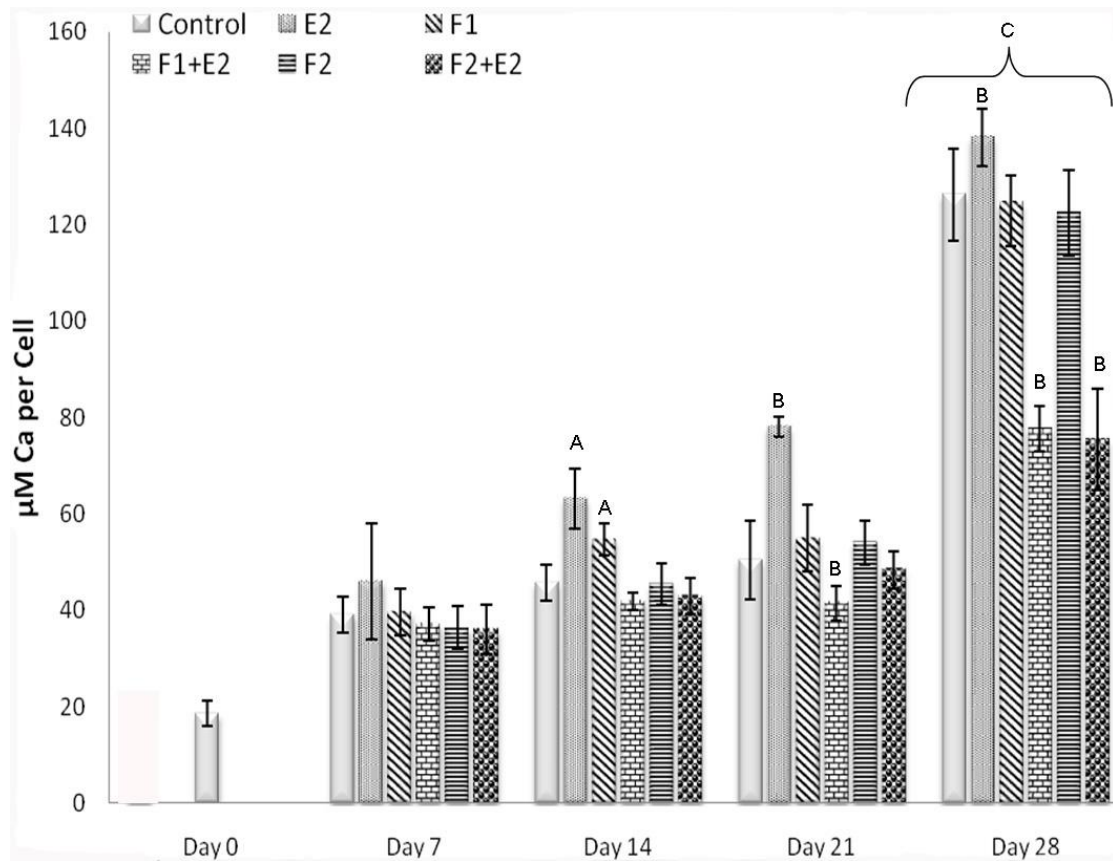


Figure 2: Analysis of cell's ability to produce mineral showed an increase over time in all groups with significantly more mineral by day 28 (C, $p < 0.0001$) regardless of the conditions. Notable increases in mineral production were in the estrogen enhanced group at day 14 (A, $p < 0.001$) and days 21 and 28 (B, $p < 0.0001$). Mineral production was also significantly increased in F₁ at Day 14 ($p < 0.001$). Significant reductions in mineral were seen at days 21 and 28 in F₁+E2 and by day 28 in F₂+E2 (B, $p < 0.0001$).

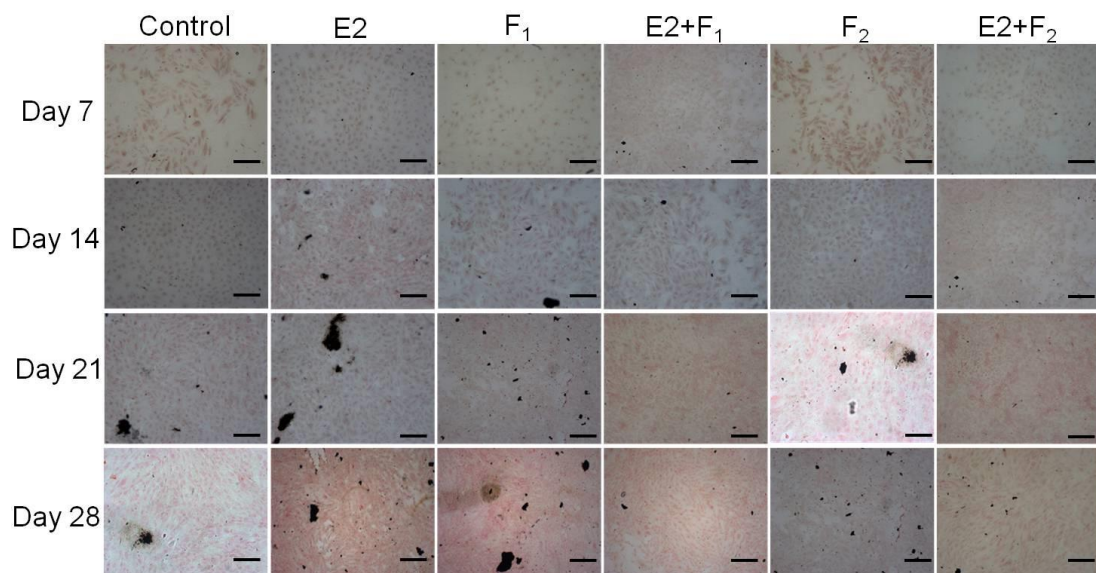


Figure 3: von Kossa and nuclear fast red stained MC3T3 cells (4X magnification; scale bar = 100µm) cultured for 7,14, 21 and 28 days in various media conditions. An increase in cell number can be seen in all groups over time and increased mineralisation over time in the osteogenic and estrogen enriched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

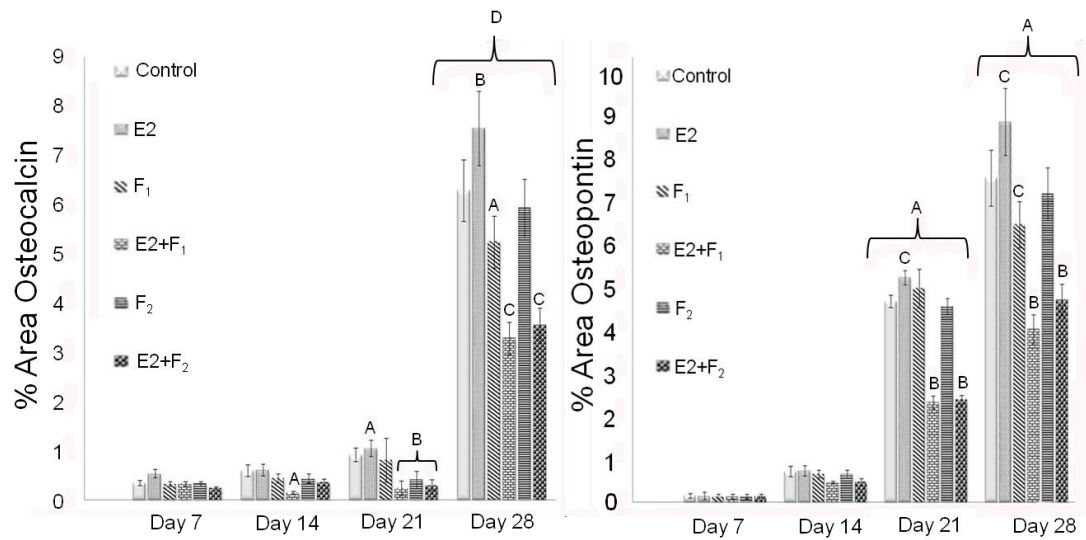


Figure 4: Osteocalcin expression was significantly increased in all groups by day 28 (D, $p < 0.0001$). At day 14 expression in $F_1 + E_2$ was significantly reduced (A, $p < 0.05$). By day 21 osteocalcin expression was significantly lower in $F_1 + E_2$, $F_2 + E_2$ and in F_2 (B, $p < 0.001$) and remained higher in E_2 (A, $p < 0.05$). At the final time point, expression continued to be significantly greater in E_2 (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 $F_1 + E_2$ and $F_2 + E_2$ (B, $p < 0.001$) and were higher in E_2 (C, $p < 0.05$). At the final time point osteopontin expression continued to be significantly higher in E_2 (C, $p < 0.05$) while expression remained lower in $F_1 + E_2$ and $F_2 + E_2$ (B, $p < 0.001$) and a decrease in F_1 (C, $p < 0.05$) was seen.