Introduction:
While abundant research has been undertaken, the disease of osteoporosis is not fully understood and bone fractures cannot be eliminated by existing treatments. Interestingly, although osteoporosis reduces the bone mass and structural strength, latest research has found that the remaining bone tissue is significantly stronger and stiffer [1]. This unexpected finding has been corroborated to some extent by micro-CrT studies which demonstrated an increase in the tissue-level mineral content [1], which was counter to the overall mineral density owing to a loss of bone mass. Although these studies were intriguing, the mechanisms by which such changes initiate are unknown.

Osteoblasts are responsible for mineralizing the bone matrix and are also capable of transducing mechanical signals into biochemical stimuli to alter bone mass [2]. Therefore these cells are likely to play a role in either the increase in tissue mineralization or reduction in bone mass, or both, during osteoporosis. Indeed osteoblasts possess receptors for estrogen [3], suggesting that cellular function may be affected when estrogen production is deficient during osteoporosis. However, to date in vitro studies have largely concentrated on quantifying the effects of estrogen deficiency on osteoclast activity. Recent studies suggest that estrogen deficiency primarily alters osteoblast activity and that osteoclast resorption may be a secondary effect mediated by osteoblasts [4]. Other researchers have shown that osteoblastic cells from osteoporotic patients display impaired response to mechanical stress in vitro [5]. However, many details of the physiology of osteoblastic cells during osteoporosis are still vague and it is unknown whether tissue-level alterations in mineral during estrogen deficiency [1] are owing to altered mineral production by osteoblasts.

In this study we test the hypothesis that osteoblastic matrix mineralization is altered at the onset of estrogen deficiency. In vitro cell culture experiments are carried out to compare the mineral production of osteoblastic cells subject to estrogen deficiency and estrogen treatment.

Methods:
To compare the effects of estrogen deficiency and varying levels of estrogen on mineral production of osteoblastic cells, in vitro cell culture experiments were carried out. Briefly osteoblast-derived osteosarcoma cells (SaOS-2) were maintained in cell culture medium (αMEM, 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 µg/ml streptomycin) until subconfluence in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were passaged, split and further cultured under separate conditions: (1) Control cells were grown under control conditions (αMEM, 10% FBS) where matrix mineralization was enhanced using ascorbic acid (50 µg/ml); (2) Cells were grown under conditions where varying concentrations (1, 10, 100 nmol/L) of commercially available 17β-estradiol (E2, Sigma) were included in the culture media; (3) to stimulate estrogen deficiency a commercially available antagonist to the estrogen receptor β (Fulvestrant, Sigma) was included in the culture media at a concentration known to exhibit antiestrogenic effects in adult female rats; (4) Cells were treated with a combination of the estrogen antagonist and estrogen treatment (10 nmol/L). All cells were seeded at a density of 1x10⁵ cells/cm² and replicate cultures were used for each condition.

At specific time points (0, 4, 7 days) cells were fixed in 4% paraformaldehyde in phosphate buffered saline. Mineralization was determined using the von Kossa silver nitrate staining method by pretreating with saturated lithium carbonate solution, and incubating with 5% silver nitrate solution for 30 minutes under a bright light. Nuclear fast red was used to counterstain the nuclei of the cells. Cellular morphology and proliferation was compared between groups.

Results:
Control cells proliferated slowly from Day 0 but continued to grow until reaching confluency. On Day 0 there was no detectable mineralization, but by Days 4 and 7 the presence of multiple mineralized nodules was confirmed by black deposits detected by von Kossa staining [Fig. 1(a)].

Cells cultured with 1nmol/L E2 rapidly proliferated from Day 0, although total cell numbers were comparable to control by Day 7. Furthermore mineralization was observed at Days 1, 4 and 7, but mineralized nodules were smaller and more numerous than those produced by cells cultured under control conditions [Fig. 1(b)].

Higher concentrations of E2 (10, 100nmol/L) inhibited both the growth and mineralization by SaOS-2 cells in vitro; by day 7 there were significantly less cells than both the control and 1nmol/L E2 treated cultures, and mineralized nodules were sparsely distributed [Fig. 1(c,d)].

Cells treated with the estrogen receptor antagonist (Fulvestrant) proliferated initially, however by Days 4 and 7 there was a rapid decrease in cell numbers. Large mineralized nodules were frequently observed, demonstrating a rapid increase in mineralization of the matrix due to estrogen deficiency inducing apoptosis [11]. These cells treated with both Fulvestrant and E2 exhibited similar growth profiles to the Fulvestrant treated cells, but few mineralized nodules were observed [Fig. 1(f)].

Discussion and conclusion:
In this study we report that both estrogen treatment and estrogen deficiency dramatically alter the growth and matrix mineralization by SaOS-2 cells in vitro. Cells treated with 1nmol/L E2 proliferate rapidly similar to control cells, whereas increased levels of E2 significantly inhibits cell growth. Previous studies have reported that E2 alters osteoblastic cell growth [7] and that primary osteoblasts treated with 10nmol/L E2 have extremely low levels of mineral deposition [8]. High concentrations of estradiol also inhibit osteoblast proliferation and induce apoptosis [9]. Our results indicate that E2 may exhibit similar effects on osteoblasts, but that this occurs in a dose dependent manner whereby low levels of E2 promote proliferation and mineralization.

In the estrogen-deficient cellular environment (Fulvestrant), rapid proliferation of SaOS-2 cells occurred within the 1st day, but by Day 7 there were few cells remaining. Therefore cells may have undergone apoptosis following prolonged estrogen deficiency. It has previously been shown that treatment of human osteoblasts with Fulvestrant in non-osteogenic medium results in downregulation of estrogen receptor expression and may induce adipogenesis [10]. However the current studies suggest that in an osteogenic environment, estrogen deficiency may induce apoptosis of the cells and alter mineral production. Furthermore the resultant mineral increase may be related to cell death, whereby dying osteoblastic cells hypermineralize their matrix [11].

These experiments provide direct evidence that estrogen deficiency affects proliferation and mineral production by osteoblastic cells in vitro. These findings support the hypothesis that, although estrogen deficiency has a potent effect on osteoclast behaviour, osteoblast activity is also altered to produce more mineral at the tissue level. Whether these changes occurs prior to osteoclast resorption in vivo is as yet unknown.


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