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Effects of Ovarian Steroids on Osteoblast Viability and Mineralization

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Authors' contributions

This work was carried out in collaboration between all authors. Author LHM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LFCB, JESL, ALOB and LHM managed the analyses of the study. Authors LFCB, JESL, NFR and VMG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Understanding the interactions between bone metabolism and ovarian and plasma changes induced by polycystic ovary syndrome (PCOS) is essential for the comprehension of the pathophysiological mechanisms involving these organs. Ovarian steroids are associated with activities related to bone synthesis and resorption. Knowledge of the effects of the main ovarian steroids on the viability of osteoblasts in culture, mimicking the conditions of PCOS, can assist in understanding the functions of the cells in this endocrine disease that affects more than 10% of women of reproductive age. This work evaluated the viability of undifferentiated and differentiated osteoblasts in an osteogenic medium, together with phosphatase activity and mineralization of the extracellular matrix. The cells were cultured for different periods of time in the presence of varied concentrations of progesterone, testosterone, estradiol, and the combination of all three hormones.

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The influence of the presence of fetal bovine serum in the culture medium was also determined. The results suggested that the culture technique employed could be used for *in vitro* studies of the effects of polycystic ovary syndrome on osteoblastic activities, before and after cell differentiation.

Keywords: Cell culture; osteoblast; polycystic ovary syndrome; cell viability; ovarian steroids.

1. INTRODUCTION

Bone is a metabolically active tissue whose constant remodeling is dependent on the activity of osteoclasts for bone resorption and osteoblasts for synthesis of the bone matrix [1,2,3]. Bone remodeling involves the continuous removal of bone followed by the synthesis of new bone matrix and subsequent mineralization. It is required for calcium homeostasis and that repair of injuries associated with stress, age, and enodcrine interactions [4,5,6].

The sex steroids act on bone metabolism. Androgens are importante in the physiological mechanisms that control bone and muscle activities in women, exerting direct and/or indirect action on bone remodeling. Direct actions are a result of the binding of testosterone (T) to androgen nuclear receptors of bone cells. Indirect actions involve the conversion of T to estradiol (E2). This conversion requires the activity of the enzyme CYP19A1 (Cytochrome P450 19A1), present in the mitochondrial matrix of bone cells, which aromatizes androstenedione and T to estradiol [7]. Clinical evidence has found increased bone mass as a result of the actions of androgens in women with hirsutism, polycystic ovarian disease, and androgen-secreting ovarian tumor [8,9,10]. In addition, androgen deficiency may cause reductions in trabecular and cortical bone density [11].

Estrogen deficiency observed in women at menopause or in case of ovarian failure stimulates the activity of osteoclasts, causing increased bone resorption and decreased bone mineral density. Early estrogen therapy can minimize vertebral damage by inhibiting the resorption, thereby reducing the number and activity of osteoclasts [12]. Estrogens and androgens decrease the number of bone remodeling cycles, regulating transcription of the genes responsible for osteoclastogenesis, replication, and differentiation of mesenchymal cells [13].

The functional role of progestogens in the prevention of bone loss is less well understood than the roles of androgens and estrogens. Progesterone (P4) receptors have been identified in osteoclasts and osteoblasts from rats and humans [14]; however, it is not clear whether the activities of P4 in bone are direct or indirect [7]. The use of cyclic progestin prevents bone loss in women with premenopausal amenorrhea or subclinical ovulatory disorders [15].

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrinopathy with adverse metabolic characteristics such as insulin resistance, hyperinsulinemia [16,17], obesity, anovulation, hyperandrogenism and abnormal ovarian morphology [17,18,19]. Approximately 5-10% of women of reproductive age are affected by PCOS [20], and between 38% and 88% of these women are overweight or obese [21,22,23,24]. The clinical and biochemical conditions of PCOS can be affected by obesity and are related to various metabolic events such as insulin resistance accompanied by compensatory hyperinsulinemia, increasing the risk of developing type 2 diabetes between 5-and 8-fold in women with PCOS. Furthermore, the risks of developing hypertension and coronary heart disease are also higher in obese women with PCOS [5,21,23], while oxidative stress, dyslipidemia, inflammation and fibrinolysis increase the risk of stroke in women with PCOS [25]. It is estimated that the prevalence of the risk factors for each of these diseases is approximately two times higher in women with PCOS compared to women who do not have the syndrome [26].

Current treatments for PCOS are not restricted to reproductive approaches; they also include the promotion of cardiovascular health and disease prevention measures, with non-pharmacological measures including nutritional counseling and regular physical activity. Although the effects of long-term treatment strategies for PCOS have not been fully elucidated [27], lifestyle and dietary changes [28,29], regular physical exercise and weight loss [29,30,31,32], cessation of smoking, stress management, and moderate alcohol consumption [29] have been identified as important steps in the treatment of PCOS.

Women with PCOS have higher incidences of excess weight, obesity, dyslipidemia, hyperinsulinemia, and type II diabetes, and consequently present greater risk of cardiovascular disease and bone disorders. Furthermore, modification of bone metabolism due to hyperandrogenemia and insulin resistance can lead to changes in the composition of structures ranging from skeletal muscle to adipose tissue. Our laboratory is working on induced PCOS in animal models [33] in order to investigate the interrelationships among this important ovarian endocrine disease and adipose, skeletal muscle, and bone tissues [34]. The aim of this study was to use a cell culture model mimicking polycystic ovary syndrome to study the effects of this endocrinopathy on osteoblast cell viability, alkaline phosphatase activity, and extracellular matrix mineralization.

2. MATERIALS AND METHODS

2.1 Cell Culture

The OSTEO-1 osteoblast line [34], obtained from ICB-USP, was used for the cell cultures. Our laboratory has already used these cells in more than ten experiments. Six separate cell cultures were used in the present study. Each well of 96-well culture plate was inoculated with 5 x 10^3 cells in 100 µL of modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA), without or with supplementation using 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained at 37ºC under a humid atmosphere of 5% CO_2 and 95% air for 12 h, 24 h, 36 h (data not shown), 48 h, and 72 h [35]. Tests were performed to determine the optimum culture time, which was set at 72 h. The medium was replaced every 72 h and stored at -20°C for later analysis of alkaline phosphatase.

2.2 Steroid Hormones

Progesterone, testosterone, and estradiol (Sigma-Aldrich, St. Louis, MO, USA) were used at different concentrations: control (without hormones), 10^{-8} M and 10^{-7} M (mimicking normal plasma concentrations in adult female rats), and 10^{-6} M and 10^{-5} M (mimicking plasma concentrations in adult female rats with induced PCOS) [33]. The steroids were added both independently, and in combination (all in the same well) to the wells of culture plates containing 5 x 10^3 cells and 100 µL of culture medium, and the plates were maintained under the culture conditions described previously.

2.3 Steroid Hormones and Osteogenic Differentiation

Differentiation was induced using an osteogenic medium, following the procedures outlined by Maniatopoulos et al. [36]. Eagle's medium was supplemented with 10% FBS, 50 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10^{-8} M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA). Each well of 24-well culture plates was inoculated with 5 x 10^5 cells in 1000 µL of medium. The steroid hormones (P4, T, and E2) were added (alone or in combination) to the culture medium at the same concentrations described above: control (without hormones), 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M. The plates were incubated at 37°C under a humidified atmosphere of 5% CO_2 and 95% air for 7 days and 14 days. Day 0 (zero) was taken to be the day on which the cells showed confluence (between 80% and 100%) at the bottom of the wells of the culture plate. The medium was replaced every 72 h and stored at -20ºC for later analysis of alkaline phosphatase.

2.4 Cell Viability

Cell viability was assessed after 12 h, 24 h, 36 h (data not shown), 48 h, and 72 h of incubation. The MTT colorimetric assay was used, in which the reduction of 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) produces formazan (a blue crystalline product), by the action of mitochondrial dehydrogenase in viable cells. The quantity of these product is directly proportional to the blue coloration, enabling estimation of the number of mitochondria and consequently, the number of viable cells in the culture, hence providing and indirect measure of cell viability. After removal of the culture medium from the wells, 50 µL of MTT (0.5 mg/mL) was added to each well and the plate was incubated for 4 h at 37ºC in 5% CO_2. Subsequently, 100 µL of acid isopropanol was added to each well in order to fully solubilize of the precipitate formed, and the absorbance of the solution was measured at 570 nm [37] using a microplate reader (Polaris, Celer Biotecnologia, Belo Horizonte, MG, Brazil). The optical density (OD) was expressed as cell viability using the index: cell viability (%) = (100 x)/control. The value for the control was taken as 100%, with values above and below

100% indicating increased and decreased cell viability, respectively.

2.5 Alkaline Phosphatase Activity

Alkaline phosphatase activity was determined from measurements of the release of thymolphthalein from thymolphthalein monophosphate, using a commercial kit (Labtest Diagnostica, Belo Horizonte, MG, Brazil). Aliquots of 50 µL of the culture medium were used. Absorbance was measured at 590 nm and the ALP activity was calculated based on the value for a standard.

2.6 Detection and Quantification of Biological Mineralization

The method described by Gregory et al. [38] was used to detect extracellular matrix mineralization. Briefly, at the end of days 7 and 14 of the culture, plate wells containing osteogenic cells were washed with cold PBS (Sigma-Aldrich, St. Louis, MO, USA) and filled with formaldehyde (10% v/v) (Merck, Kenilworth, NJ, USA) for 30 min. Subsequently, the wells were washed with deionized water and 1 mL of Alizarin Red (Sigma-Aldrich, St. Louis, MO, USA) solution was added. After 30 min, the excess Alizarin Red solution was removed, the wells were washed with water, and the plates were kept at room temperature for the wells to dry.

Quantification of extracellular matrix mineraliztion was performed by adding 450 µL of acetic acid solution (10% v/v) to each well that had been previously stained with Alizarin Red. The plates were kept on a shaker for 30 min at room temperature, after which, 400 µL aliquots of the contents of each well were transferred to Eppendorf tubes, followed by addition of 150 µL of NH_4OH. Finally, the contents of the Eppendorf tubes were transferred to 96-well plates and the absorbances were measured at 405 nm using a microplate reader [38].

2.7 Statistical Analysis

The Sigma Stat software (Systat Software, Point Richmond, CA, USA) was used for statistical analyses. Means and standard deviation were calculated for each experimental variable and statistical significance was set up $p<0.05$. Data collected were compared by means of one-way repeated measure analysis of variance (ANOVA), follwed by Turkey's multiple comparisions pos hoc test.

3. RESULTS

3.1 Effects of Fetal Bovine Serum and Culture Time on Viability of the OSTEO-1 Cells

The viability of the OSTEO-1 osteoblasts (Fig. 1) cultured for 72 h was higher than that of the control group (0 h), in both the absence and presence of FBS. The viability of cells cultured for 72 h in the presence of FBS was higher than the cell viability of cells grown without serum for the same culture time.

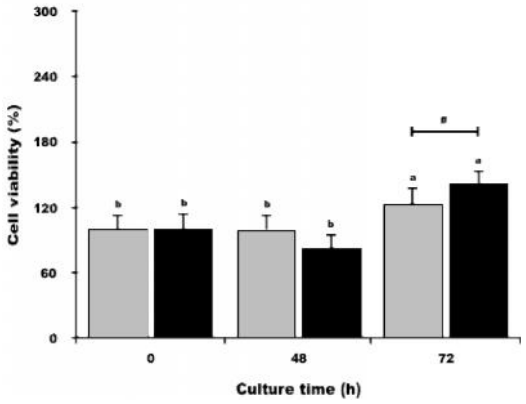


Fig. 1. Effects of absence (gray bars) and presence (black bars) of bovine fetal serum on viability of osteoblast – OSTEO-1 cultured until 72 hours

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences within the group among the times; # indicate differences among the groups. p<0.05

3.2 Effects of Steroids P4, T, and E2 on Viability of the OSTEO-1 Cells Cultured for 72 h

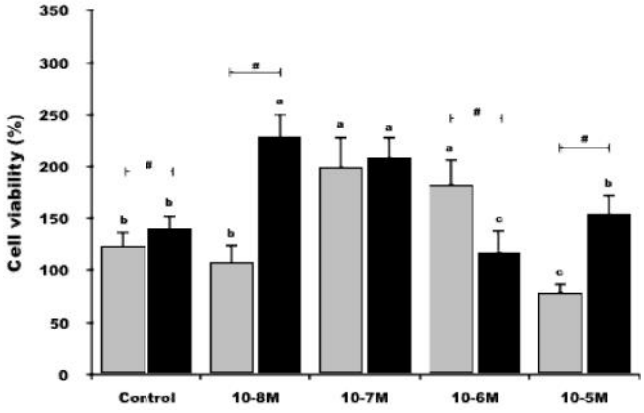
Higher viability was shown by the cells maintained in culture for 72 h without FBS in the presence of progesterone (Fig. 2A) at concentrations of 10^{-7} M and 10^{-6} M, while lower viability was observed in the presence of P4 at 10^{-5} M. In the presence of 10% FBS, cell viability was higher at P4 concentrations of 10^{-8} M and 10^{-7} M. The lowest cell viability was observed for cells cultured with P4 at 10^{-6} M. In comparison of the groups with and without serum, at P4 concentrations of 10^{-8} M, 10^{-5} M, and 0 M (control), higher viability was found for the cells cultured in the presence of serum. At a P4 concentration of 10^{-6} M, the serum-free cell group showed the highest cell viability.

The viability of the OSTEO-1 cells maintained in culture for 72 h in the presence of testosterone (Fig. 2B) and absence of serum varied according to the concentration used. The highest viability was observed with 10^{-7} M of T and the lowest cell viability was observed for cells cultured with 10^{-5} M of T. When the cells were cultured in the presence of 10% FBS, the cell viability decreased with increasing T concentrations. Comparing the groups with and without serum, the serum groups showed higher viability, compared to those cultured without serum.

The effect of estradiol on the viability of the OSTEO-1 cells is shown in Fig. 2C. In the absence of 10% FBS, the highest and lowest cell viabilities were observed in the presence of 10^{-5} M and 10^{-8} M of E2, respectively. In the presence of 10% serum, the highest and lowest cell viabilities were observed in the presence of 10^{-6} M and 10^{-8} M of E2, respectively. Comparing the groups with and without 10% FBS, viability was greater for the control group and the serum groups at concentrations of 10^{-7} M and 10^{-6} M of E2. In the case of the groups treated with 10^{-5} M of estradiol, the serum-free group presented the highest viability.

The viability of the OSTEO-1 cells cultivated for 72 h was also influenced by addition of all the steroids together (Fig. 2D). In the absence of 10% fetal bovine serum, cell viability was highest in the presence of all the hormones at concentrations of 10^{-7} M and lowest with all the hormones at concentrations of 10^{-5} M. When fetal bovine serum was added to the culture medium, higher viabilities were observed in the cultures with hormone concentrations of 10^{-8} M and 10^{-7} M, while lower cell viability was observed in the presence of 10^{-5} M of the hormones. Comparison of the groups with and without FBS showed that cell viability was higher in the groups with serum in the case of the control group, and the cells cultured using hormone concentrations of 10^{-8} M and 10^{-6} M. At hormone concentrations of 10^{-7} M and 10^{-5} M, the viability was higher for the cells cultured without serum.

(A)



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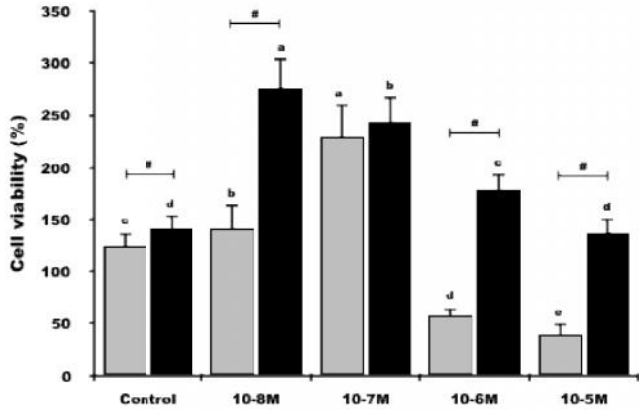


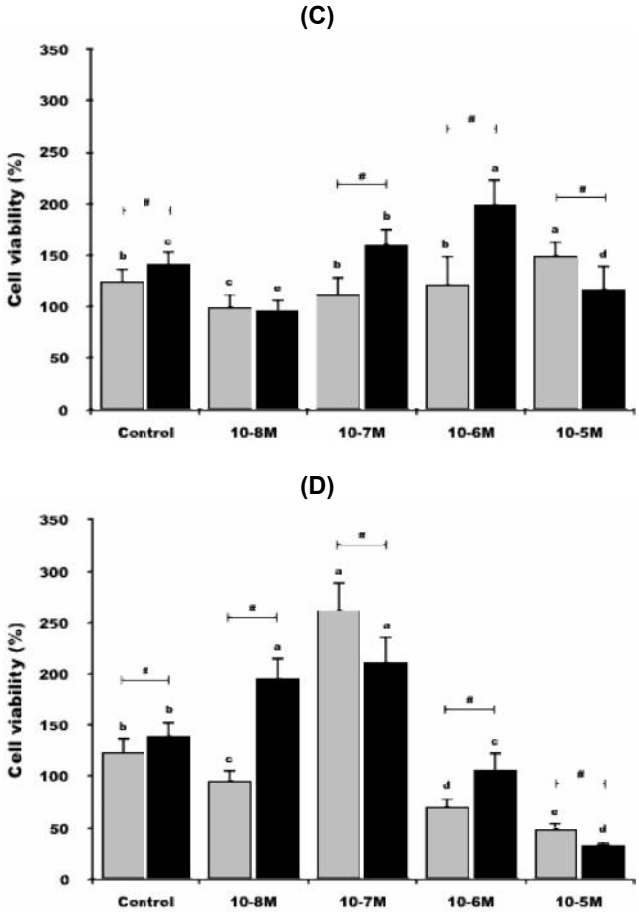


Fig. 2. Effects of different concentrations (0 (control), 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M) of progesterone (A), testosterone (B), estradiol (C) and all hormones together (D) on osteoblast – OSTEO-1 viability cultured in the absence (gray bars) and presence (black bars) of bovine fetal serum for 72 hours

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences within the group among the concentrations; # indicate differences among the groups. p<0.05

3.3 Effects of Culture Time and the P4, T, and E2 Steroid Hormones on Viability of Osteoblasts Cultured in Osteogenic Medium

For the culture time employed here, the cells cultured in osteogenic medium for 14 days showed lower viability than those cultured for 0 and 7 days (Fig. 3). The viability of osteoblasts cultured for 7 days and 14 days was influenced by progesterone (Fig. 4A). For cells cultured for 7 days, greater viability was observed in the presence of the highest concentration (10^{-5} M) of P4, while lower viabilities were observed for cells cultured at concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M of P4. When the cells were cultured for 14 days, all P4 concentrations used reduced the cell viability, with the concentration of 10^{-6} M resulting in the greatest reduction.

When the osteoblasts were cultured for 7 days in the presence of testosterone (Fig. 4B), hormone concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M resulted in decreased cell viability. All T concentrations used decreased the viability of osteoblasts cultured for 14 days, and the lowest viability was observed in the presence of 10^{-5} M of the hormone.

When the cells were cultured with estradiol for 7 days, the highest and lowest viabilities were foud for concentrations of 10^{-5} M and 10^{-6} M, respectively (Fig. 4C). Lower viabilities were obtained for cells cultured for 14 days with

estradiol at concentrations at 10^{-7} M, 10^{-6} M, and 10^{-5} M.

When the combined hormones were added to the osteogenic medium (Fig. 4D), concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M decreased the viability of cells cultured for 7 days, while cells cultured for 14 days showed decreased viability at all concentrations of the hormones with the greates reduction at 10^{-5} M.

Finally, for the control groups as well as the groups with individual and combined addition of hormones, cell viability was lower in the groups cultured for 14 days, compared to the groups cultured for 7 days.

3.4 Effects of Culture Time and the P4, T, and E2 Hormones on Alkaline Phosphatase Activity

The alkaline phosphatase activity changed according to the time during which the osteoblasts were cultured (Fig. 5). Higher alkaline phosphatase activity was observed for the group cultured for 7 days, compared to the groups cultured for 0 and 14 days.

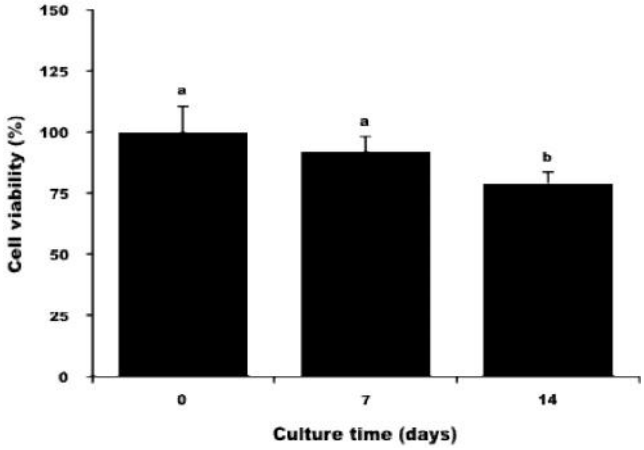
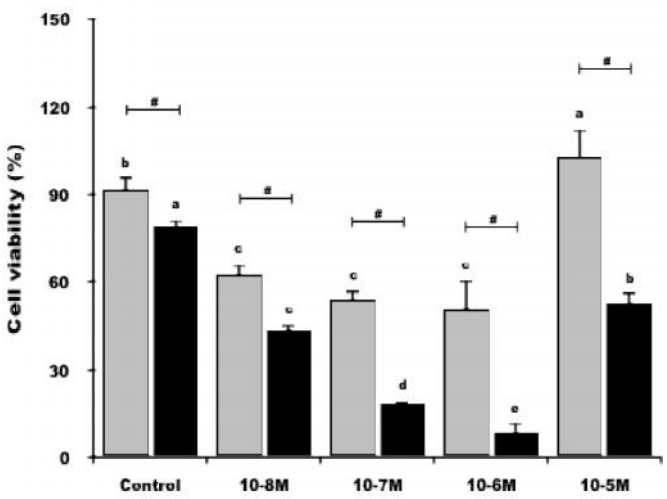


Fig. 3. Effects of culture time on viability of osteoblast cultured in osteogenic medium until 14 days

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences among the times. p<0.05

(A)



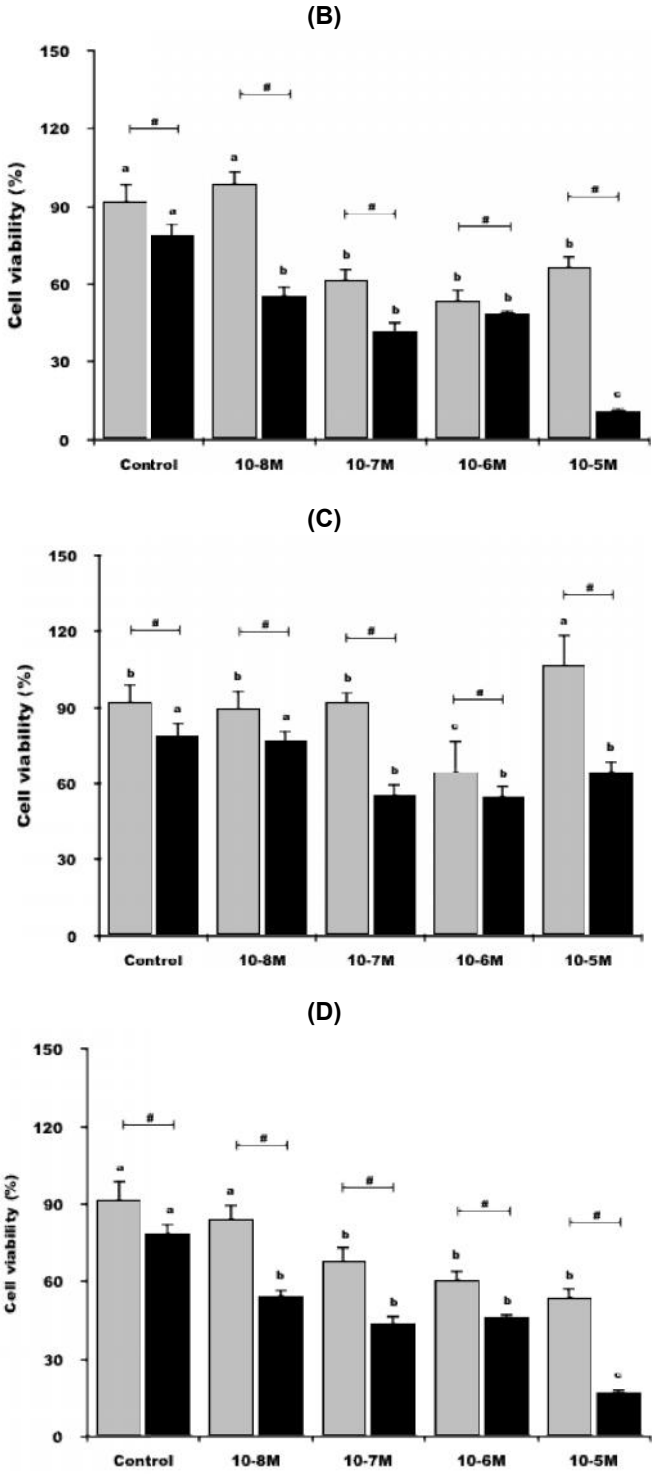


Fig. 4. Effects of different concentrations (0 (control), 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M) of progesterone (A), testosterone (B), estradiol (C) and all hormones together (D) on viability of osteoblast cultured in osteogenic medium for 7 days (gray bars) and 14 days (black bars)

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences within the group among the concentrations; # indicate differences among the groups. p<0.05

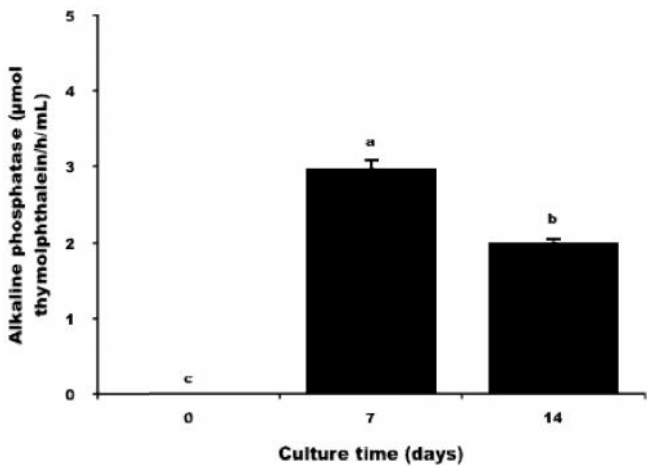


Fig. 5. Alkaline phosphatase activity in osteoblast cultured in osteogenic medium for 0, 7 and 14 days

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences among the times. p<0.05

The groups cultured for 7 and 14 days showed an influence of progesterone on the alkaline phosphatase activity (Fig. 6A). For osteoblasts cultured for 7 days, the lowest alkaline phosphatase activity was observed with P4 at a concentration of 10^{-7} M. When the cells were cultured for 14 days, the lowest alkaline phosphatase activity was observed for the cells cultured with P4 at a concentration of 10^{-8} M. In terms of the effect of culture time, the 7-day osteoblast cultures showed higher alkaline phosphatase activity than the 14-day cultures, both without P4 (control) and with P4 at all concentrations except 10^{-7} M.

For both culture times, the alkaline phosphatase activity was also influenced by testosterone (Fig. 6B). Testosterone concentrations of 10^{-8} M and 10^{-6} M increased phosphatase activity in the cells cultured for 7 days, while lower alkaline phosphatase activities were observed when T was added at concentrations of 10^{-7} M and 10^{-5} M. When the cells were cultured for 14 days, a T concentration of 10^{-8} M significantly decreased the alkaline phosphatase activity. In terms of the effect of the culture time, alkaline phosphatase activity was lower for the 14-day cultures, compared to the 7-day cultures.

The effect of E2 on alkaline phosphatase activity is shown in Fig. 6C. Lower phosphatase activity was observed when the cells were cultured for 7 days with E2 concentrations of 10^{-8} M and 10^{-5} M. For cells cultured for 14 days, alkaline phosphatase activity was higher with E2 concentrations of 10^{-8} M and 10^{-5} M. The alkaline phosphatase activity was higher for cells cultured for 7 days, compared to those cultured for 14 days.

Finally, the effects on alkaline phosphatase activity of adding all the hormones to the osteoblast cultures simultaneously are shown in Fig. 6D. For the 7-day cultures, lower alkaline phosphatase activity was observed for 10^{-8} M concentration of the hormones. In the case of cells cultured for 14 days, the lowest alkaline phosphatase activity was observed using the hormones at concentrations of 10^{-8} M. The alkaline phosphatase activity was higher for cells cultured for 7 days, compared to those cultured for 14 days.

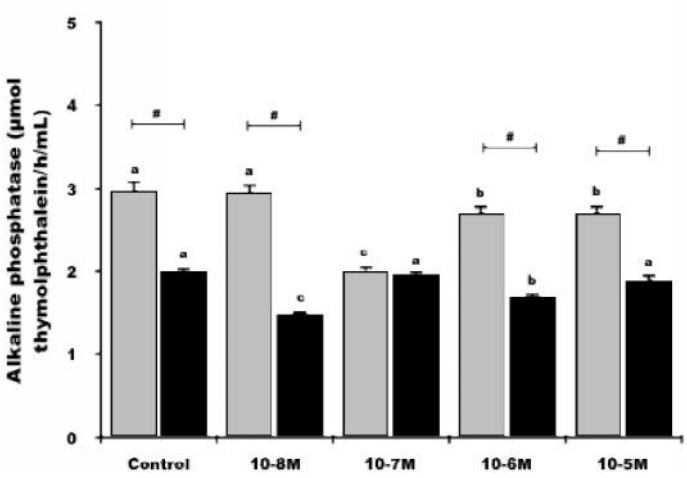
3.5 Effects of Culture Time and the P4, T, and E2 Hormones on Biological Mineralization

The osteoblasts cultured in osteogenic medium for 7 and 14 days showed mineralization of the extracellular matrix (Fig. 7). When progesterone was added to the medium, there were decreases in mineralization at progesterone concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M (Fig. 8A), for both culture times, compared to the cultures without addition of progesterone.

All Tconcentrations used decreased mineralization of the extracellular matrix (Fig. 8B), irrespective of culture time, compared

to the cultures without T. In cultures with testosterone at 10^{-8} M and 10^{-6} M, greater mineralization was observed for cells cultured for 14 days, compared to those cultured for 7 days.

(A)



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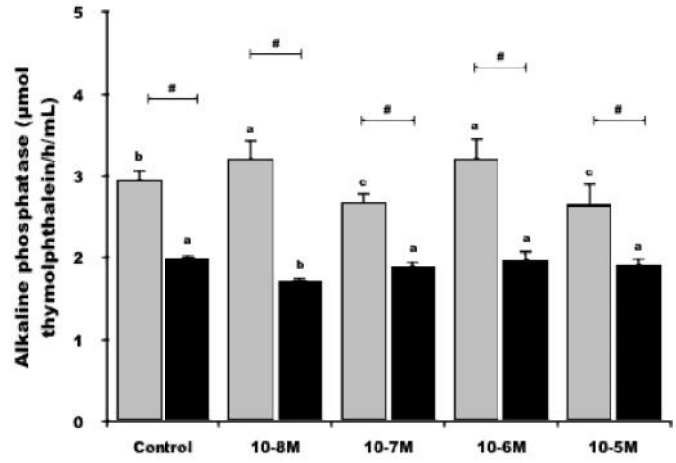
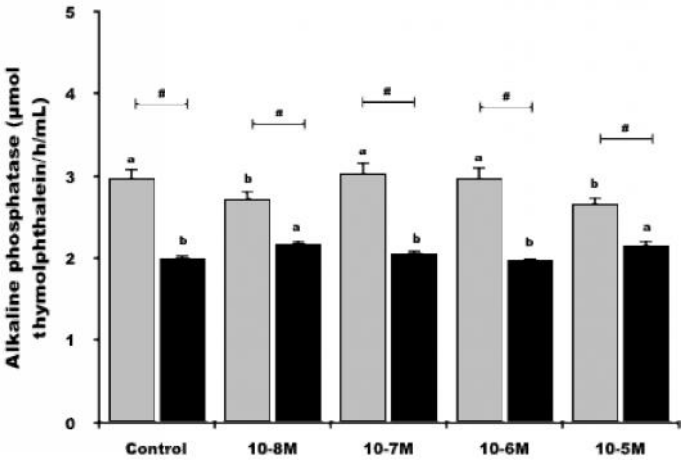


(C)



(D)

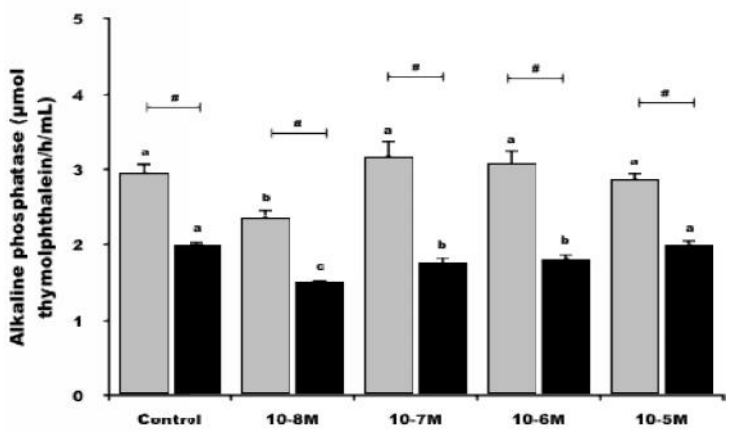


Fig. 6. Effects of different concentrations (0 (control), 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M) of progesterone (A), testosterone (B), estradiol (C) and all hormones together (D) on alkaline phosphatase activity in osteoblast cultured in osteogenic medium for 7 days (gray bars) and 14 days (black bars)

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences within the group among the concentrations; # indicate differences among the groups. p<0.05

For cells cultured for 7 days, E2 concentrations of 10^{-8} M, 10^{-6} M, and 10^{-5} M decreased extracellular matrix mineralization, while the cells cultured for 14 days only showed decreased mineralization for E2 concentrations of 10^{-6} M and 10^{-5} M (Fig. 8C), compared to the control groups. Cells cultured with E2 at 10^{-8} M showed higher mineralization in the 14-day culture, compared to the 7-day culture.

When all the hormones were added together to the media, the 7-day cultures showed decreased mineralization for hormone concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M. The 14-day cultures showed increased in mineralization for 10^{-5} M concentrations of the hormones, while decreased mineralization was observed for all other hormone concentrations (Fig. 8D). Finally, greater mineralization was observed for cells cultured for 14 days using the higher hormone concentrations (10^{-6} M and 10^{-5} M), compared to those cultured for 7 days.

4. DISCUSSION

In studies involving the *in vitro* reproduction of biological conditions, cell culture experiments are used to assist in the development of models designed to elucidate the mechanisms of control of cell and tissue functions. However, it is not always possible to reproduce, *in vitro*, conditions identical to those present in the living organism, mainly due to the numerous unknown functional mechanisms that exist *in vivo*. Therefore, the results of *in vitro* experiments have to be analyzed in specific contexts. The results of this study showed that the OSTEO-1 cells remained viable for 72 h in culture and that the presence of 10% FBS increased the viability of these cells. This viability could be associated with potential increases of cell differentiation and bone formation. Fetal bovine serum is often added to culture media as a supplement, because it contains biological components such as fatty acids, growth factors, amino acids, vitamins, and hormones, which are required for cell cultures performed following certain experimental protocols [39]. Therefore, the use of FBS in culture media, despite being necessary for the cultivation of certain cell types, can affect experimental results related to cell maintenance, division, and differentiation [40,41,42,43]. Furthermore, the use of this supplement in culture media may compromise experimental results because it is a natural substance whose composition can vary between samples [44]. The presente findings demonstrated the influence of FBS on cellular responses, especially in terms of cell viability, in the absence or presence of the steroid hormones. It was also found that when the cells were differentiated in osteogenic medium, the hormones modulated cell viability, alkaline phosphatase activity, and mineralization of the extracellular matrix.

When the OSTEO-1 cells were cultured with P4, T, and E2 (separately or in combination) in the absence and presence of serum, dual effects

were observed that were dependent on the concentrations of the hormones and FBS. The addition of P4 to the osteogenic culture medium resulted in stimulatory and inhibitory effects on cell viability for both undifferentiated [45] and differentiated cells. Such responses could be governed by factors including cell differentiation, specific receptors, and steroid- receptor interactions. The components of FBS could influence these responses by interfering in hormone-receptor interactions and activating and/or inhibiting intracellular signaling pathways. Furthermore, P4 affected alkaline phosphatase activity and mineralization of the extracellular matrix, suggesting that progesterone modulated the differentiation and functional activity of osteoblasts cultured for 14 days. The effects of progesterone on bone metabolism are controversial [46]. It has been reported to prevent bone loss in pre- and perimenopausal women [15]. The use of E2 and P4 alone, at concentrations of 10^{-10} M, was found to stimulate DNA synthesis and the growth of osteoblasts, cultured for 1 day in serum-free medium [47]. Furthermore, the metabolization of P4 can generate active products that interact with E2 receptors and modulate bone cell activity [46].

In vitro studies have demonstrated that both T and dihydrotestosterone (DHT) modulate the proliferation of osteoblast progenitor cells in different species [48,49,50,51]. Controversial effects of these hormones include stimulation or inhibition (or neither) of alkaline phosphatase activity, type I collagen synthesis, synthesis and release of osteocalcin and mineralization of the extracellular matrix of bones [52,53]. In addition, androgens decrease apoptosis of osteoclasts and osteocytes [54]. Our results demonstrated that T influenced the viability of undifferentiated osteoblasts in a dose-dependent manner. Testosterone concentrations similar to physiological levels increased cell viability, while decreased viability was observed when concentrations were increased in order to mimic PCOS. These effects were consequences of direct and/or indirect interactions of T with its receptors present in osteoblasts. Since the cells were not yet differentiated, it appeared that T was able to influence the dynamics of osteoblasts *in vitro* prior to differentiation. In addition, bone engagement could be initiated prior to differentiation of these cells, under conditions similar to PCOS, in which T concentrations are high. The results showed that when the cells became differentiated in an osteogenic medium, the highest concentration of T (10^{-5} M) decreased cell viability and mineralization of the extracellular matrix, but did not change the alkaline phosphatase activity. Therefore, the effects of high concentrations of T on osteoblasts cultured for 14 days were indicative of a reduction in osteoblastic activity and impaired bone formation *in vitro*.

Estradiol addition resulted in increased cell viability at a concentration of 10^{-6} M and decreased cell viability at 10^{-5} M, suggesting that in the case of the model studied, estradiol had a stimulatory effect on cell viability, prior to differentiation. However, after differentiation of the cells in an osteogenic medium, a higher E2 concentration resulted in decreased cell viability, increased alkaline phosphatase activity, and decreased mineralization of the extracellular matrix. Therefore, estradiol modulated the activity of osteoblasts cultured for up to 14 days. Estrogens, especially 17β-estradiol, are important for bone metabolism [55]. As women pass through menopause, E2 levels decrease and there is a reduction in bone mineral density [56]. The interaction of E2 with its receptors ERα and ERβ, detected by immunohistochemistry in osteoblasts, osteocytes, and osteoclasts [57, 58], has protective effects on the bone due to a variety of mechanisms. These include inhibition of the activity of cytokines that stimulate osteoclast activity [59,60], inhibition of the activity of nuclear factor κB (NFκB) that inhibits osteoblast activity by modulating osteoclast suppresion via the relationship with RANKL/osteoprotegerin (OPG) [57], and inhibition of osteoblast apoptosis [61]. Genetic studies using ERα knockout mice have identified reduced osteoblastogenesis and expression of osteocalcin [62]. ERβ knockout mice have shown increased trabecular bone, without any changes in the cortical bone [63].

The most interesting results in this study were obtained when all the steroid hormones were added together at higher concentrations (10^{-5} M). The lowest viability of the osteoblasts was observed under these conditions, in both the absence and presence of serum. The OSTEO-1 lineage is considered to be a pre-osteoblast cell line [34,45], and it can be inferred that the exposure of these cells to high concentrations of P4, T, and E2 together, mimicking PCOS, may compromise cell differentiation and formation of the extracellular matrix for calcium deposition, hence lowering the quality of the bone tissue. When cells were differentiated in osteogenic

medium, the combined hormones decreased cell viability in a dose-dependent manner, with the lowest viability of the osteoblasts being observed at the highest hormone concentration (10^{-5} M).

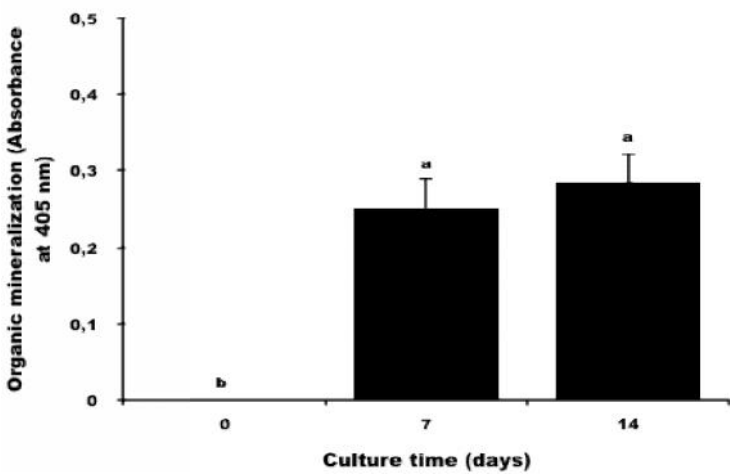
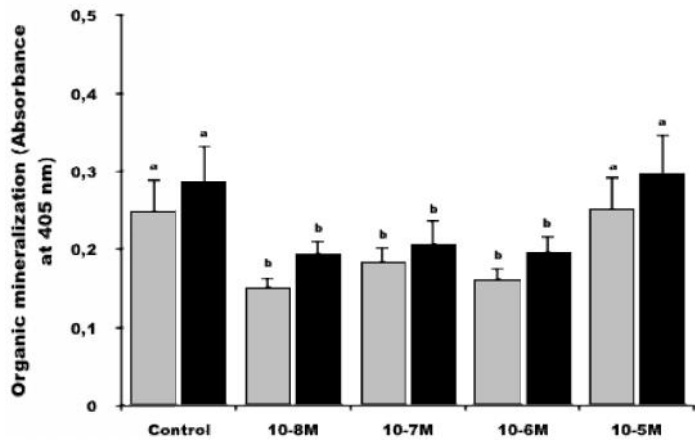
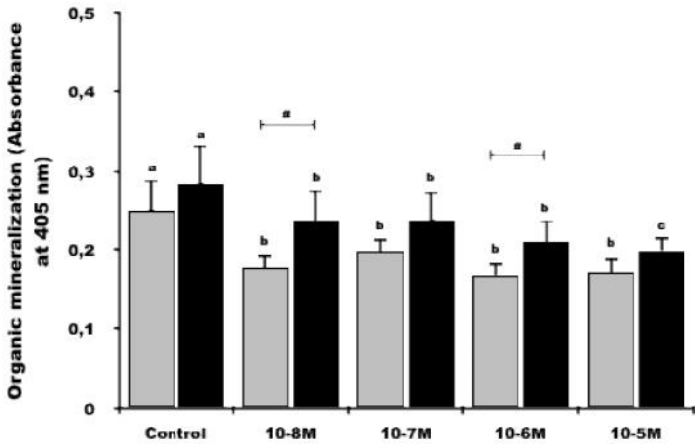


Fig. 7. Organic mineralization in osteoblast cultured in osteogenic medium for 0, 7 and 14 days
Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences among the times. p<0.05

(A)



(B)



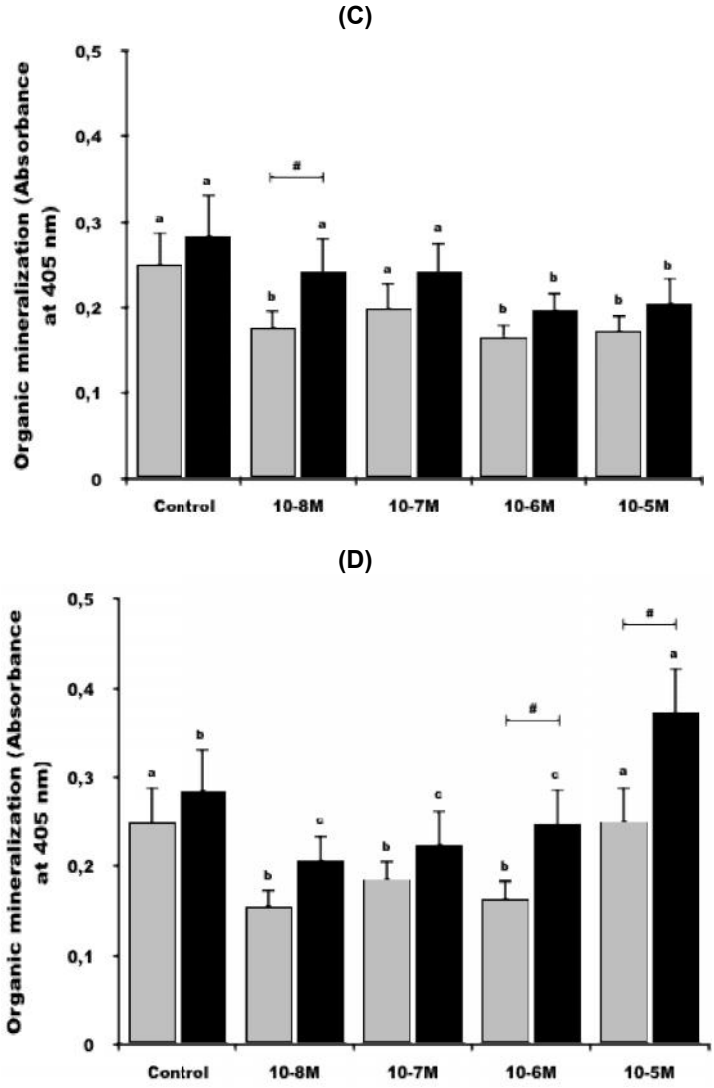


Fig. 8. Effects of different concentrations (0 (control), 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M) of progesterone (A), testosterone (B), estradiol (C) and all hormones together (D) on organic mineralization in osteoblast cultured in osteogenic medium for 7 days (gray bars) and 14 days (black bars)

Results are represented as means +/- SEM. (n= 6 independent cultures). Different superscript lowercase letters indicate differences within the group among the concentrations; # indicate differences among the groups. p<0.05

However, at this concentration there were no changes in alkaline phosphatase activity, and there was an increase in bone mineralization after 14 days of culture. Therefore, higher concentrations of the combined hormones reduced the viability of osteoblasts and stimulated mineralization of the extracellular matrix. The sex hormone could have non-genomic effects on bone cell activity. Studies have shown anti-apoptotic effects of estrogens and androgens on osteoblasts cultured *in vitro* [64]. These hormones act in the development and maintenance of the skeleton in men and women. In both sexes, plasma levels of E2 and T decrease with age, contributing to reduced bone mass and increased risk of fractures [56]. The *in vitro* results obtained in this study demonstrated that ovarian steroids at concentrations mimicking those found in PCOS [33] modulated osteoblast viability and bone synthesis. We are unawared of any other *in vitro* studies that have mimicked the effects of ovarian steroids on the activity and viability of osteoblasts in a situation similar to PCOS.

5. CONCLUSION

Taken together, the results demonstrated that the osteoblast culture model employed here enable evaluation on cell viability, alkaline phosphatase activity, mineralization of the extracellular matrix, together with the effects thereon of culture time, FBS, P4, T, and E2. Furthermore, according to studies in progress in our laboratory, this culture model can be used for *in vitro* studies of the effects of polycystic ovary syndrome on osteoblastic activities before and after cell differentiation. However, molecular and genomic studies are needed in order to identify the specific receptors and intracellular signaling pathways used by the ovarian steroids present in isolation and in combination. Additionally, studies using cell co-cultures are needed to elucidate the interactions among osteoblasts, adipocytes, and myocytes in metabolic and clinical situations similar to PCOS.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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