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ORIGINAL ARTICLE

Osteogenic Activity of Resveratrol in Human Fetal Osteoblast Cells

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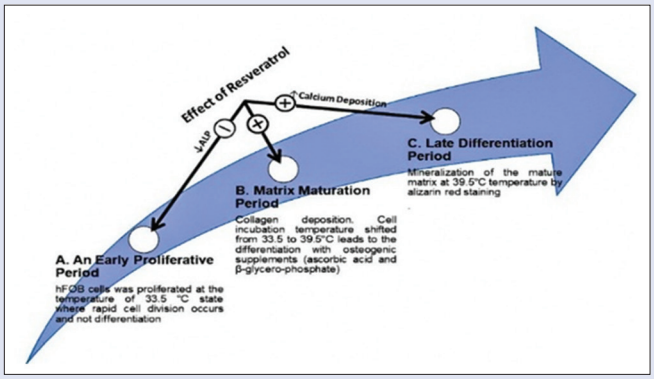
ABSTRACT

Background: Resveratrol (RSV) is a polyphenolic phytomolecule naturally present in the skin of grapes fruit. It is reported to be phytoestrogen because of its estrogenic potentials, hence it can be explored to the treatment of osteoporosis. There was no conclusive evidence for the estrogenic potential of RSV in osteoporosis. In this study, RSV is evaluated for its effects on human osteoblast cells. **Objective:** The main objective of the study was to evaluate RSV on the proliferation and differentiation of immortalized human fetal osteoblastic cells 1.19 (hFOB). **Materials and Methods:** The osteoblastic cell proliferation and differentiation potentials of RSV were tested by cell viability assay, alkaline phosphatase (ALP) activity, total protein content, and alizarin staining for the mineralization assays. **Results:** The cell viability assay indicated that RSV was found to be safe at a wider concentration range and the EC_{50} was 72.05 µM. The therapeutic concentrations as 500 nM and 1 µM were selected for the further assays. RSV at 500-nM and 1-µM concentrations treatment on immortalized human fetal osteoblastic cells 1.19 (hFOB) did not show a significant effect on ALP activity. The total cellular proteins in hFOB increased in a dose-dependent manner on RSV treatment ($P \leq 0.05$). The significant staining and the color intensity of the calcium crystals by Alizarin staining assay indicate a stimulatory effect on the mineralization phase of bone formation. **Conclusion:** In mature osteoblasts, ALP activity is expressed in early stage, whereas mineralized nodules are formed in the late stage of differentiation. Therefore, the present study suggests that RSV stimulates the process of bone formation through activation of late differentiation phase and may have positive effects on osteoblastic differentiation potential.

Key words: Alkaline phosphatase, immortalized human fetal osteoblastic cells 1.19, mineralization, osteogenesis, resveratrol

SUMMARY

- RSV is considered as a phytoestrogen due to structural similarity with 17-β estradiol. As immortalized human fetal osteoblastic cells 1.19 cell lines express very less estrogen receptor; therefore, the present study explored the osteogenic potential of RSV through estrogen-independent signaling pathway.



Abbreviations used: RSV: Resveratrol; hFOB: Immortalized human fetal osteoblastic cells 1.19; ALP: Alkaline phosphatase; SERMs: Selective estrogen receptor modulators; DMEM: Dulbecco's Modified Eagle's Medium; MTT: 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; OD: Optical density; ANOVA: One-way analysis of variance test; SEM: Standard error of the mean; pNPP: p-nitrophenyl phosphate.

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INTRODUCTION

Osteoporosis is characterized by microarchitectural deterioration of bone tissue with increased fragility and susceptibility to fractures involving the wrist, spine, hip, pelvis, ribs, or humerus.[1] Epidemiologic data in 2015 suggest that 46 million Indian women are suffering from osteoporosis are above the age of 50 years. The current life expectancy is ~ 67 years is projected to increase to 71 years by 2025 and to 77 years by 2050.[2] Current pharmacological therapies available in osteoporosis mainly include bisphosphonates, parathormone fragments and analogs, receptor activator of nuclear factor-kappa B ligand inhibitors as denosumab, and selective estrogen receptor modulators such as raloxifene.[3] However, all these treatments have limitations. The long-term use of bisphosphonates increased the risk of osteonecrosis of the joint.[4] The treatment with parathormone fragments is restricted for 24 months due to the higher incidence of osteosarcoma such as adverse events reported in humans.[5] Denosumab is a monoclonal antibody, reports safety concerns such as the increase the risk of osteonecrosis of the jaw on prolonged therapy.[6] The clinical use of selective estrogen receptor modulator (SERM) is limited in postmenopausal women as it reduces only vertebral fractures by 30%, but no efficacy in non-vertebral fractures.[7] Therefore, there is a need for a better therapeutic drug for effective treatment therapy for the management of osteoporosis.

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Resveratrol (RSV) is a naturally occurring phytomolecule reported in red wine and other plant foods such as grapes, cranberries, and ground nuts. RSV appears to be a mixed agonist/antagonist and possess activity as SERMs.[8] Apart from it, the antioxidant and anti-inflammatory properties of RSV can influence bone metabolism.[9] RSV intakes of up to 700 mg/day in animals did not exhibit any toxicity.[10] Several clinical trials on human indicate the safety profile and broad therapeutic potential.[11]

Due to its broad pharmacological potential and safety profile, RSV offers a promising therapeutic agent. Several cell line studies concluded that RSV enhanced the bone mass by promoting osteoblastic bone development or by inhibiting osteoclastic resorption[12] and suggest a promising remedy for osteoporosis. The RSV has advantages over the current clinical treatment options which act either by osteoblastogenesis or by inhibiting osteoclastogenesis. The balance of osteoblastic differentiation and osteoclastic resorption is essential to maintain the bone remodeling cycle.

Immortalized human fetal osteoblastic cells 1.19 (hFOB) cells are immortalized with a temperature-sensitive mutant of the SV40 large T-antigen gene. This gene activates at the temperature of 33.5°C and cells proliferate rapidly, whereas at the nonpermissive temperature 39.5°C, it is inactive which leads to differentiation of cells and shows mature osteoblastic characters.[13] hFOB cells express very fewer estrogen receptors. Till date, the efficacy study of RSV in hFOB cell line is not investigated. Therefore, we have assessed the *in vitro* evaluation of RSV in hFOB cells and investigated the possible osteoblastic differentiation potential through estrogen-independent signaling mechanism.

MATERIALS AND METHODS

Materials

hFOB is purchased from the American type culture collection with CRL11372. Dulbecco's Modified Eagle's Medium (DMEM), and Geneticin (G 418 disulfate salt) solution (50 mg/mL) were purchased from Sigma-Aldrich. Nutrients mixture Ham's F-12, alizarin were purchased from HiMedia. Fetal bovine serum was purchased from Invitrogen. RSV was purchased from TCI Chemicals. Cell culture flask and plates were purchased from Eppendorf.

Proliferation of immortalized human fetal osteoblastic cells 1.19

The base media for the growth of hFOB cells was a 1:1 mixture of DMEM and Ham's F12 medium DMEM. Antibiotics as 0.3 mg/mL geneticin and 10% fetal bovine serum were added to prepare complete growth medium. Cells were incubated with 5% CO_2 at 33.5°C for the proliferation. Cell culture medium was refreshed every 2 days until cells reached 75%–80% confluency and detached using 0.25% trypsin-0.5 mM ethylenediaminetetraacetic acid.

Osteogenic differentiation of immortalized human fetal osteoblastic cells 1.19

Osteogenesis of the hFOB cells was assessed in the osteogenic medium prepared by adding osteoinductive supplements (100 μg/mL of ascorbic acid and 10 mM of β-glycerophosphate) to the growth medium at the restrictive temperatures of 39.5°C. Ascorbic acid is necessary for the synthesis of osteoblastic proteins and formation collagenous matrix.[14] The β-glycerophosphate enhances the expression of the osteoblastic phenotype.[15] To study the differentiation, the culture medium was refreshed after every 3rd day. The cells were observed after 7 and 14 days and photomicrograph was taken and compared with the cells proliferated at 33.5°C.

Evaluation of cell viability by 3-(4,5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide assay

The safety profile of RSV on hFOB was assessed by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The 96-well plate was seeded with 1.0×10^5 cells. RSV at different concentrations from 3.90 to 500 μM in 0.5% DMSO and vehicle control was incubated for 48 h. After incubation 20-μL MTT (2 mg/mL) is added and incubated at 37°C for 4 h, the precipitated formazan crystals were dissolved in 100-μL DMSO and the optical density (OD) was measured at 540 nm by enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek, USA). The percentage of cell viability was determined by the equation:

% of cell survival = ([OD of the sample]/[OD of control]) × 100.

Data were analyzed using GraphPad Prism 5.0 free trial version (GraphPad Software, San Diego, CA) for half maximal effective concentration (EC_{50}) determination.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) enzymatic assay was estimated by the reported method.[16] From the preliminary studies, we have selected two RSV concentrations as 500 nM and 1 μM and evaluated for further efficacy assays. hFOB cells at 90% confluence treated with RSV at 500 nM and 1 μM for 7 days at 39.5°C in 96-well plates. The hFOB cells were washed with PBS after RSV treatment and then lysed with 0.6 mL of tris buffer pH 7.5 and 0.1% Triton X-100. The Tris buffer contains 10 mM tris-HCl and 0.5 mM $MgCl_2$, and the pH was adjusted to 7.5. The contents with cell lysate were centrifuged at 2000 rpm and the supernatant was used for the assay of ALP. The soluble fraction of 50 μL each was added to 125-μL glycine buffer (25 mM, pH 9.4), containing 2-mM $MgCl_2$, and 5-mM p-nitrophenyl phosphate (pNPP), and incubated at 37°C for 50 min in a water bath. The enzymatic reaction was stopped by addition of 125-μL 1 M NaOH. The final colored product (p-nitrophenol) was quantified at 405 nm in an ELx808 absorbance microplate reader (BioTek, USA).

Total cellular protein assay

The total protein content was measured using thermo scientific pierce bicinchoninic acid (BCA) protein assay kit. The hFOB cells were seeded in 6-well plates and incubated with and without RSV 500 nM and 1 μM for 7 days at 39.5°C. The wells without RSV serve as a control well. The total protein in cell lysates is estimated using the standard calibration curve of bovine serum albumin. Standard bovine serum albumin was prepared in the range of 3.90–2000 μg/mL. Their absorbance was measured at λ_{max} of 562 nm using an ultraviolet-visible spectrophotometer and a calibration curve was plotted.

Alizarin red staining assay

The Alizarin red staining evaluated the calcium deposition in osteoblast by the procedure described by Gregory *et al.*[17] Mineralization was induced on confluent monolayers in osteogenic media. The cells were seeded in 6-well plates and mixed with 500 nM and 1 μM of RSV. The cultures were incubated at 39.5°C for 7 and 14 days. The medium was replaced after every 3 days. The monolayers of hFOB cells were fixed by 10% (v/v) formaldehyde in 6-well plates (10 cm^2/well) after washing with PBS. The monolayers were washed again with excess distilled water. Alizarin red stain 1 mL (40 mM) was added to each well and incubated for 20 min. The wells were then washed with distilled water and stained monolayers were photographed using inverted microscope attached to the computer.

The acetic acid extraction method was used for quantification of staining. Acetic acid 800 µL 10% (v/v) was added to each well and the monolayer was scraped. The scrapped monolayer then transferred to a 1.5-mL microcentrifuge tube and 500-µL mineral oil was added. It is then heated to 85°C for 10 min cooled in ice for 5 min. The suspension was centrifuged at 12000 rpm for 15 min and 500 µL of the supernatant was removed to a new microcentrifuge tube. The 200 µL of 10% v/v ammonium hydroxide was then added to neutralize the acid and the pH between 4.3 and 4.5 was maintained. The supernatant at 150 µL in triplicate was taken to read the absorbance using ELISA plate reader at 405 nm in 96-well plates.

Statistical analysis

All data were represented as mean ± standard error of the mean (SEM), all data were statistically analyzed by ANOVA and postcomparison was carried out with using Dunnett's multiple comparison test with control group ($P \leq 0.05$).

RESULTS

Effect of resveratrol proliferation and osteogenic differentiation of immortalized human fetal osteoblastic cells 1.19

At the temperature of 33.5°C, the hFOB cells rapidly divide with a cell-doubling time of 36 h whereas at 39.5°C, cell divisions slowed with a doubling time of 96 h and it differentiated into mature osteoblasts as evident from the photomicrograph of cultured cells. Images of undifferentiated hFOB cells, at a temperature 33.5°C [Figure 1a] and differentiated cells at 39.5°C [Figure 1b] were captured.

Determination of EC_{50} of resveratrol in immortalized human fetal osteoblastic cells 1.19 cells

The percentage viability on hFOB was investigated using the MTT assay before the analysis of the anabolic effects of RSV. Figure 2 illustrates the effect of RSV on hFOB cells. RSV showed a dose-dependent manner compared with the control cells. Our results showed that the EC_{50} for RSV was found to be 72.05 µM. EC_{50} values were calculated using dose-response stimulation curve with log (agonist) vs. response-variable slope nonlinear analysis using GraphPad Prism software (version 5.00).

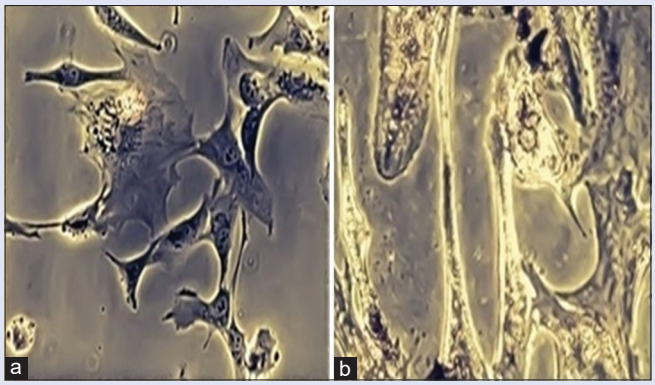

Figure 1: Immortalized human fetal osteoblastic cells 1.19 cells proliferation and differentiation. (a) It was proliferated at the temperature of 33.5°C (undifferentiated) state where rapid cell division occurs. (b) At a temperature above about 37°C little or no cell division occurs, differentiation occurs at elevated temperatures of 39.5°C (differentiated); ×10 images

RSV is found to be safe over wider concentrations in osteoblast. At lowest RSV concentration as 3.9 µM, the percentage mean cell viability was 94.51 ± 2.15 whereas at mid concentration 62.25 µM, it was reduced to 69.37 ± 2.190. At higher concentrations as 125-, 250-, and 500-µM cells detached after they become spherical and significant cell death was observed.

Effect of resveratrol on alkaline phosphatase activity

pNPP, a phosphatase substrate turns yellow when dephosphorylated by ALP and the absorbance can be determined at 405 nm. p-nitrophenol is the hydrolysis product of pNPP and turns yellow under basic conditions and detected using a spectrophotometer at 405 nm. Figure 3 shows the effect of RSV at 500-nM and 1-µM treatment of hFOB cells on ALP activity in 7 days of treatment. The mean absorbance of control group 0.125 ± 0.002 did not show significant change on RSV treatment. On RSV 500-nM and 1-µM treatment, the mean absorbance had little deviation as 0.118 ± 0.0008 and 0.118 ± 0. 001, respectively. It shows that RSV did not affect ALP level significantly. It indicates that RSV had a negative impact on early phase phenotypic osteoblast differentiation.

Effect of resveratrol total protein content

The protein assay using BCA includes the well-known principles of cupric (Cu^{2+}) to cuprous (Cu^{1+}) reduction by protein at higher alkaline pH. In this assay, two molecules of BCA react with one Cu^{1+} and form a bright purple-colored BCA copper complex. This complex is shown stronger linear absorbance at 562 nm with increasing protein concentrations.

Figure 4 refers to the effect of RSV on total protein (in µg/mL, mean ± SEM) on hFOB cells. The total protein content in the control group was found to be 14.51 ± 0.67 µg/mL, which was increased as 18.57 ± 2.06 and 24.68 ± 0.67 at RSV concentrations 500 nM and 1 µM, respectively. These results reveal that the total cellular proteins in the cells are increased with RSV in dose-dependent manner. This indicates that RSV stimulates hFOB cell.

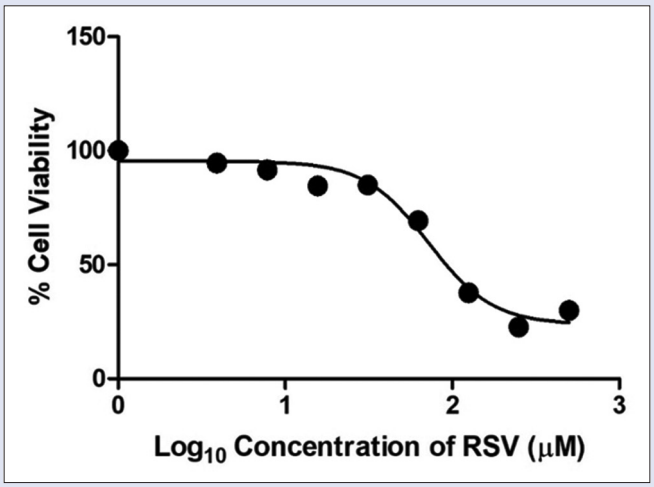

Figure 2: Dose–response curve of resveratrol on immortalized human fetal osteoblastic cells 1.19 cell viability. Graph of percentage of cell viability versus final $\log_{10}$ concentration for resveratrol (3.9–500 µM) incubated for 48 h. All the data were expressed as a mean ± standard error of the mean ($n = 3$). Percentage cell viability was statistically analyzed by calculating the dose-response stimulation curve with log (agonist) versus response variable slope nonlinear analysis using GraphPad Prism software ($P \leq 0.05$)

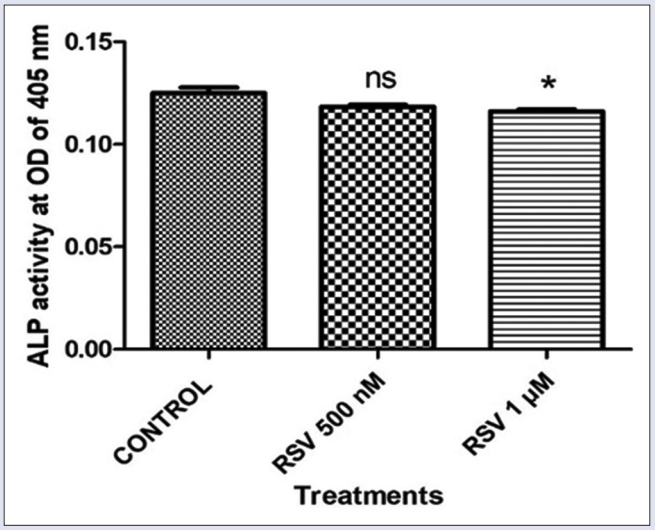

Figure 3: Effect of resveratrol on alkaline phosphatase activity at 500-nM and 1-µM concentration. All the data were expressed as a mean ± standard error of the mean (n = 3). All data were statistically analyzed by ANOVA test and post comparison was carried out with using Dunnett's multiple comparison test with "control group," ($P \leq 0.05$); ALP: Alkaline phosphatase; OD: Optical density; ns: nonsignificant

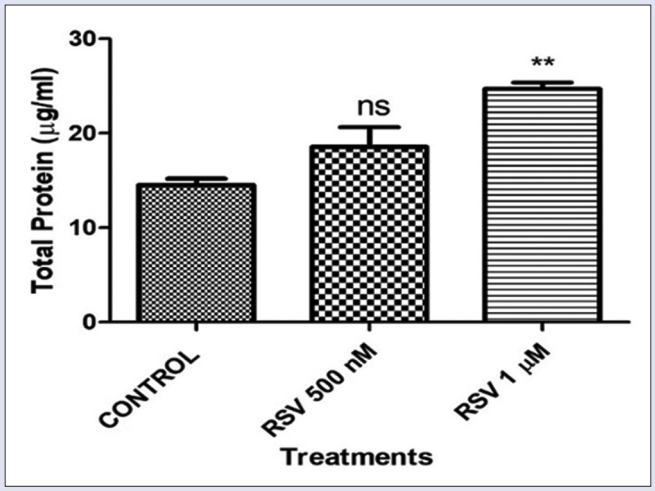

Figure 4: Effect of resveratrol on the total protein content of immortalized human fetal osteoblastic cells 1.19 cell. The assay was done using bovine serum albumin as a standard. All data were represented as mean ± standard error of the mean all data were statistically analyzed by ANOVA test and postcomparison was carried out with using Dunnett's multiple comparison test with "control group," ($P \leq 0.05$). ns: Nonsignificant

Effect of resveratrol on mineralization

The hFOB cells were stained using Alizarin red stain which binds selectively to calcium salts infers the mineralization. Figure 5a-f describes the treatment of hFOB cells with RSV at the concentration of 500 nM and 1 µM showed an increased number of calcified nodules. The mineralization due to the RSV treatment is visualized by bright red calcified nodule. The microscopic appearance of hFOB after mineralization showed increasing amounts of calcium deposition in a time and dose-dependent manner. The marked increase in the mineralization was induced by RSV treatment at 1-µM concentration after 14 days of incubation.

In the quantification of mineralization, RSV at 500-nM and 1-µM concentration showed a significant increase in the mineralization phase after 7- and 14-days treatment is as shown in Figure 6. On 7 days, the control group showed alizarin quantification was 0.399 ± 007 (mean absorbance ± SEM; n = 3) and with RSV at 500-nM and 1-µM concentrations, it was 0.402 ± 0.004 and 0.413 ± 0.004, respectively, indicates slight deviation. However, after 14 days, there is a significant increase in mineralization, from control group 0.950 ± 0.019 to RSV 500-nM and 1-µM treatment as 1.801 ± 0.014 and 2.340 ± 0.041, respectively. The process of bone mineralization is increased over the period and maximum on 14 days incubation. It indicates that RSV is responsible for the late osteoblastic differentiation phase.

DISCUSSION

Osteogenesis is the process of bone formation and synthesis of bone extracellular proteins governed by the osteoblast. In this study, we have investigated the osteogenic potential of RSV on different phases on osteoblast differentiation. Osteoblastic differentiation occurs in the three stages as early proliferative period, matrix maturation period, and late differentiation period. In the early proliferative period, the cells continue to proliferate and express fibronectin, collagen, and osteopontin. In matrix maturation period, the cell cycle stops and osteoblastic differentiation starts. In this phase, maturation of the extracellular matrix starts with an increased expression of alkaline phosphate and collagen level. As the bone consists of 60% inorganic component (hydroxyapatite), 10% water, and 30% organic component (bone matrix proteins), matrix mineralization is the late differentiation phase. In matrix mineralization, deposition of mineral substances takes place with hydroxyapatite synthesis.[18]

The osteogenic potential of RSV was reported in the literature. Many of researcher have used osteogenic and osteoblastic cell lines and the mechanisms are discussed.[12] RSV directly stimulates the proliferation and differentiation osteoblastic MC3T3-E1 cells and has stimulatory effects on DNA synthesis and ALP activity. This effect was blocked in the presence of an antiestrogen tamoxifen. Thus, it is proven that, RSV enhances osteogenesis through estrogen-dependent mechanism.[19] While in another study, RSV stimulated the rat calvarial osteoblast cells independent of their SERM activity by stimulating osteocalcin gene expression.[20] An *in silico* approach by Chakraborty *et al.* 2013, demonstrated partial agonistic characteristic of RSV on estrogen receptor α-isoform.[8,20] hFOB cells express very low levels of the activated estrogen receptor per nucleus. It contains less than about 200 estrogen receptors that are capable of binding 17 β-estradiol and translocating to the cell nucleus.[21,22] Till date, the effect of RSV is not investigated in the hFOB cell line. Thus, the present work demonstrated possible anabolic effect of RSV through estrogen-independent mechanisms.

In our investigation, RSV was found to be safe at the concentration of 62.25 µM and the cell viability was 69.3%. An apoptotic effect was seen only at the higher concentrations as 125, 250, and 500 µM. From the preliminary studies, the therapeutic concentrations as 500 nM and 1 µM were selected for the further assays. The ALP activity was not affected at the therapeutic concentrations as 500 nM and 1 µM. However, total protein content and mineralization were significantly stimulated in a time- and dose-dependent manner. RSV treatment showed a significant increase in calcification within 14 days. Therefore, the present study highlighted the effect of RSV in late mineralization phase of osteoblastic differentiation through estrogen-independent pathway.

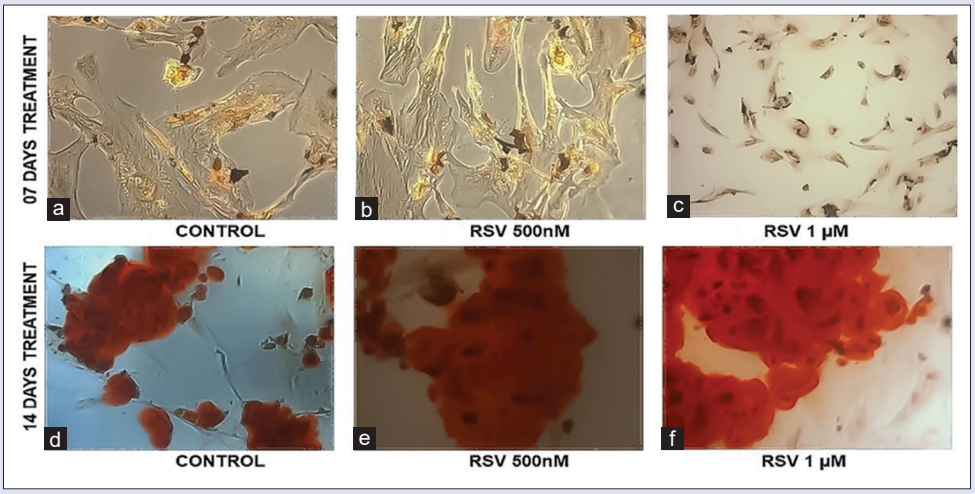

Figure 5: Effect of resveratrol on calcified nodules in immortalized human fetal osteoblastic cells 1.19 on 7- and 14-day treatments by Alizarin red staining. The reaction between calcium ions and Alizarin forms the red-colored nodules visualized under a light microscope. It shows the deposition of bone mineral (calcium phosphate) by osteoblasts. The deposition of calcium crystals observed in (a) Control; (b) RSV 500 nM; (c) RSV 1 µM treatment groups after 07 days and (d) Control; (e) RSV 500 nM; (f) RSV 1 µM observed after 14 days treatment

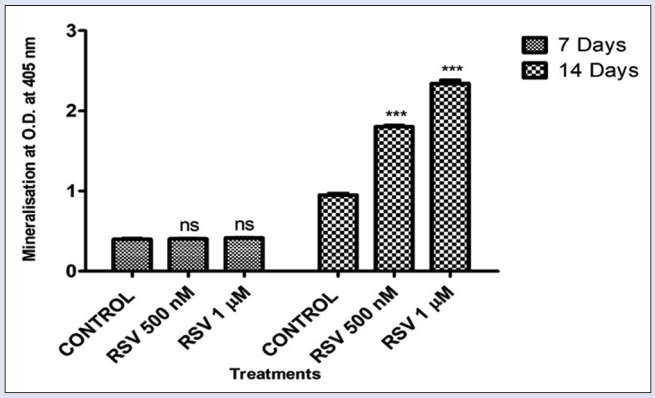

Figure 6: Quantification of mineralization (7 and 14 days treatment) by Alizarin red staining. All the data were expressed as a mean ± standard error of the mean (n = 3). All data were statistically analyzed by ANOVA test and postcomparison were carried out with using Dunnett's multiple comparison test with "control group," $P \leq 0.05$ of each 7 and 14 days, respectively. OD: Optical density; ns: Nonsignificant

CONCLUSION

The RSV stimulated bone formation through activation of late differentiation phase in hFOB cells which strongly suggest its antiosteoporotic activity could also be nonestrogenic signaling mechanism. Further experiments can be carried out to study molecular mechanism to explore the use in clinics.

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Conflicts of interest

There are no conflicts of interest.

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