

Possible Synergistic Role of Cowpea Isoflavones and Vitamin D Combo in the Bone Formation of MG-63 Osteoblasts

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Abstract

Osteoporosis is a major public threat next to diabetes and cardiovascular diseases. In the wake of gravity of osteoporotic morbidity, traditional therapies in the form of diet with fewer side effects are being preferred now days. The present study investigates the efficacy of Cowpea (*Vigna unguiculata*) of Fabaceae family, a source of isoflavones along with Vitamin-D in the bone formation of MG-63 human Osteosarcoma cell lines, a model to study Osteoblasts. The cells are exposed to different dose concentrations of Daidzein (0.01, 0.1, 1, 2.5, 7.5 and 10 μ M), Genestein (0.01, 0.1, 1, 2.5, 7.5 and 10 μ M), Vitamin-D (15 to 50 μ M), Cow Pea extract (having 18 μ M Daidzein: 6 μ M Genestein) and in combinations of Daidzein+Genestein (5 to 30 μ M) and the combination of all at 12, 24, 36, 48 hrs respectively. Biochemical parameters like Osteocalcin activity, Alkaline phosphatase and Acid phosphatase activity assays, Intracellular calcium and reactive oxygen species levels were measured. Antioxidant activity of the enzymes like Catalase, Superoxide dismutase, Malonaldehyde, Vitamin C, and Gluthathione reductase were also tested. The results indicated that the cell proliferation of the exposed groups increased when compared with their respective controls. Similarly, antioxidant activity of the enzymes like Catalase, Superoxide dismutase, Gluthathione reductase, Vitamin C and biochemical parameters like Osteocalcin activity, Alkaline phosphatase and Intracellular calcium levels showed a significant increase in their activities in the exposed groups when compared with their respective controls. In contrast, the exposed groups showed a decrease in Reactive oxygen species, Acid phosphatase and Malonaldehyde activity with respect to their controls. Thus, all different parameters through different approaches showed the stimulating effects of Cowpea isoflavones along with the vitamin D on bone formation.

Keywords: *Vigna unguiculata*; Vitamin D; Antioxidants; Osteoporosis; ROS; Intracellular calcium

Abbreviations

ROS: Reactive oxygen species; Dz-Daidzein; Ge-Genestein; V-D-Vitamin D; CPIF-Cowpea Isoflavones; OC-Osteocalcin; ALP-Alkaline phosphatase; ACP-Acid phosphatase; SOD-Superoxide Dismutase; GSH-Gluthathione reductase; MDA-Malonaldehyde.

Introduction

Osteoporosis (OSP) is most commonly seen in age old people and postmenopausal women. It is a disorder where low bone mass and micro-architectural deterioration of bone tissue are seen as characteristic features [1]. Bone, a rigid organ of the body always undergoes to continuous phases of remodeling throughout entire life. The first phase is an anabolic phase carried by osteoblasts which results in formation of bone cells and the second phase is a catabolic phase carried out by osteoclasts which results in resorption of bone cells. For a proper functioning of the bone a balance should be maintained between these two phases [2]. Traditional therapies such as Hormone replacement therapy [HRT] and the use of certain drugs like bisphosphonates were suggested for the treatment of OSP especially in the postmenopausal women [3]. Even though, therapy reduces OSP but prolonged use results in other disorders like breast cancers and cardiovascular manifestations [4] and usage of drugs further may not increase the incidence of OSP but it won't restore the lost or damaged

bone mass. Therefore, there is a need for the treatment which should be coupled with less undesirable effects and at the same time reduces the usage of drugs. Natural compounds namely Phytoestrogens are gaining importance now a days due to their clinical benefits regarding the bone sparing effects due to their estrogen mimicking activity [5]. Phytoestrogens are classified in to three types namely isoflavones, coumestans, and lignans [6,7]. Daidzein and genestein are the most abundant isoflavones found to have an anabolic effect in stimulating the bone formation and increasing the intracellular alkaline phosphatase activity [8,9]. Both isoflavones have capability to bind to ligand domain of estrogen receptors (ER) more preferably to ER β than ER α and mimic the function of estrogen hormone thus promoting the bone formation process and preventing the bone resorption process [10]. There is one own study of ours where effect of CPIF was tested along with Vitamin D on rat primary osteoblast cultures [11]. Cowpea a Fabaceae family member with voucher specimen number [MPESFPS-02/13] having rich phytoestrogen content was used for our present study where effect of CPIF was tested along with Vitamin D on osteoblast cultures [12].

As studies at cellular level provide insights of the Dz and Ge stimulating effects on bone, with slight modifications our present study was aimed at studying the efficacy of CPIF along with Vitamin D on MG-63 Human osteosarcoma cell lines. The present *in vitro* study involves the effect of Dz, Ge, V-D, CPIF, V-D+CP and combination of all in dose and time dependent manner on MG-63 cells for testing their metabolic activity, ALP, OC, ACP and Intracellular calcium activity and ROS, and Oxidative stress parameters.

Materials and Methods

Reagents

DMEM (#D6171), Pencillin-Streptomycin antibiotics (#PO781), Genestein (#G6649), Daidzein (#O7802), V-D (#C9756), L-Glutamine (#G7513), non-essential aminoacids (#M7145), DMSO (#41640), chemicals were purchased from sigma. Trysin-EDTA (#25200072) and FBS (#1456393) were purchased from Gibco. Remaining all chemicals and solvents used were of high analytical grade.

Extraction and quantification of CPIF using HPLC-DAD analysis

Extraction and quantification of CPIF was done as per the standardized protocol described elsewhere [11].

Cell culture

MG-63 cells were procured from NCCS Pune. The cells were cultured as monolayers in T-75 flasks using DMEM medium supplemented with 10% FBS, 5 ml each of nonessential amino acids, 200 μ M L-Glutamine, 100 units/lit Pen-Strep antibiotics and placed in an incubator at 5% CO₂, 95% humidity and 37°C temperature conditions. By using 0.25% Trypsin-EDTA and 1×PBS the cells were sub cultured for every 2 to 3 days. First the cells were washed by rinsing with PBS, and then 1 ml of Trypsin EDTA was added and kept in an incubator for 3 to 5 min. Then the detached cells were centrifuged and pellet was dissolved in media and further passaged. After 24 hrs the cells were exposed with different concentrations of Dz (0.01, 0.1, 1, 2.5, 7.5 and 10 μ M) Ge (0.01, 0.1, 1, 2.5, 7.5 and 10 μ M), CPIF extract, V-D (15 to 50 μ M), Dz+Ge (5 to 30 μ M), V+C, [V+C+(D+G)], and further cultured for appropriate periods of time (12, 24, 36, 48 HRS). The viability was tested using MTT.

Cell morphology

The cell morphology of the cultured cells was observed using Nikon phase contrast microscopy at 10× magnification and images were saved for further reference.

Cell viability

After 48 hrs of incubation the viability of cells was measured using MTT assay. MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is a pale-yellow substrate which is taken by the live cells that results in cleavage of cells yielding blue formazan product. MTT cell growth assay kit was purchased from Millipore. The assay was conducted as per the user manual provided in the kit (#CT02).

Cell lysates preparation

After 48 hrs of incubation different exposed group of cultured cells were detached using 0.25% Try-EDTA. The detached cells were centrifuged and the pellet collected was washed and dissolved in ice cold PBS. The pellet was dissolved in RIPA buffer with freshly added protease inhibitors for a period of 30 min. Then it was further subjected to ultra-sonication. Finally, cells are further subjected to centrifugation for 15 min at 15000^g rpm. The supernatant was collected and frozen at 8°C for further experiments. Protein concentration in all samples was determined using BCA protein assay

kit (#71285-3). The assay was performed as per the kit manual. The concentration of protein was measured as μ g/ml.

Alkaline and Acid phosphatase (ALP and ACP) activity

The cell lysates of different exposed group of cultured cells were tested for alkaline and acid phosphatase activity. The ACP and ALP activity was measured using TRAP and ALP Assay kit (#MK301). Assay protocol was followed as per the instructions given in the manual. The kit allows the simultaneous detection of the activity of both ACP and ALP enzymes via pNPP substrate. The activity was expressed in terms of percentage.

Osteocalcin (OC) assay

The cell lysates of different exposed groups of cultured cells were tested for Osteocalcin activity. The activity was measured by using ELISA kit for Osteocalcin (#SEA471-Mu) and assay was performed as per the instructions given in the manual. The amount of OC released was expressed as μ g/mol.

Intracellular calcium

After culturing the cells with different exposures treatments for 48 hrs, the cells were detached using a cell scrapper, washed with PBS for three times and 4 μ M fluorescent and cell permeable dye Cal 520 AM (abcam #ab171868) suspended in PBS with equal amounts of pluronic F-127 (Sigma #P2443) suspended in DMSO was added to the pellet and incubated in dark for 30 min at 37°C. Then cells are washed with PBS and were assayed for intracellular calcium levels using flow cytometry. The levels of intracellular calcium was expressed in terms of percentage.

Oxidative stress parameters

Different Cell lysates of the exposed groups were assayed for the antioxidant activity. The antioxidant enzymes like Catalase, SOD, GSH, and antioxidant assays of TBARS, Vitamin C were performed as per the standardized protocols.

GSH assay

Each of the different cell lysates of the exposed groups were taken and OPT(O-phthaldehyde), Sodium phosphate buffer along with EDTA were added together as per the standardized protocol and fluorescence is spectrophotometrically measured at excitation and emission wavelengths of 350 nm and 420 nm respectively. Reduced GSH activity was expressed in terms of μ g/mg protein.

TBARS assay

Each of the different cell lysates of the exposed groups were taken and butylated hydroxy toluene, trichloroacetic acid, thiobarbituric acid, water were added together and boiled for 45 min at 90°C as per the standardized protocol. Centrifugation is done and OD is taken at 532 nm. TBARS activity was expressed in terms of MDA (nmol/ml).

Vitamin C assay

Each of the different cell lysates of the exposed groups were taken and amount of Vitamin C was spectrophotometrically measured according to the standardized method of Zannoni et al. [61] Vitamin C activity was expressed in terms of μ g/mg protein.

Catalase assay

Each of the different cell lysates of the exposed groups was taken and activity of catalase was measured as per the standardized protocol of Deissorth [62]. Catalase activity was expressed in terms of U/mg protein/min.

SOD assay

Each of the different cell lysates of the exposed groups were taken and activity of SOD was measured as per the standardized protocol using TRIS, Pyrogallol, and DTPA (Diethylene triamine penta acetic acid). SOD activity was expressed in terms of U/mg protein.

ROS assay

After culturing the cells with different exposures treatments for 48 hrs, the cells are detached using a cell scraper, washed with PBS for three times and 10 μM fluorescent dye 2171-dihydrofluorescein diacetate (DCFH-DA) (Sigma Cat #35845) suspended in PBS was added to the pellet and incubated in dark for 30 min at 37°C. Then cells are washed with PBS and were assayed for ROS assay using flow cytometry. The ROS activity was expressed in terms of percentage.

Statistical Data

All the values are presented as mean and standard errors of mean (SEM). Data were evaluated by one way ANNOVA. The significance was calculated by using students t-test for non-parametric values. A p value of <0.05 was considered statistically significant.

Results

The effect of influence of Dz and Ge (Figure 1A), CPIF (Figure 1B), V-D (Figure 1C), C+V (Figure 1D) on metabolic activity in MG-63 human osteosarcoma cells assessed by MTT assay showed a significant increase when compared with their respective controls. After treatment, the formation of MTT formazan was increased in both time and dose dependent ways. The cells were cultured for 24 hrs and then were subjected to different exposures, (as shown in figure legends) and allowed to grow for one more day. After 48 hrs of incubation, MTT assay was conducted on cells as per different time periods (12, 24, 36, 48 hrs). The viability of cells was measured and expressed in terms of percentage of viability. The results indicated that the metabolic activity of the MG-63 cells was high in the exposed group of cells when compared to that of control. The effect of Dz on MG-63 cell lines in a time and dose dependent manner at a concentration range of 0.01, 0.1, 1, 2.5, 5, 7.5, 10 μM showed a significant increase in cell number at a percentage (%) increase of 102.6, 106.33, 118.19, 120.23, 123.54, 128.04, 133.07 respectively (Figure 1A). The effect of Ge on MG-63 cell lines in time and dose dependent manner at a concentration range of 0.01, 0.1, 1, 2.5, 5, 7.5, 10 μM showed a significant increase in cell number at a percentage (%) increase of 105.09, 106.72, 109.18, 111.63, 114.03, 118.49, 121.3 respectively (Figure 1A).

The effect of V-D on MG-63 cell lines in time and dose dependent manner at a concentration range of 15, 20, 25, 30, 35, 40, 45, 50 μM showed a significant increase in cell number at a percentage (%) increase of 103.52, 104.72, 106.68, 108.30, 112.18, 115.42, 117.86, 122.23 respectively (Figure 1B). The effect of V-D+CP on MG-63 cell lines in time and dose dependent manner at a concentration range of (as shown in figure legends) showed a significant increase in cell

number at a percentage (%) increase of 105.22, 127.43, 135.24 respectively (Figure 1C).

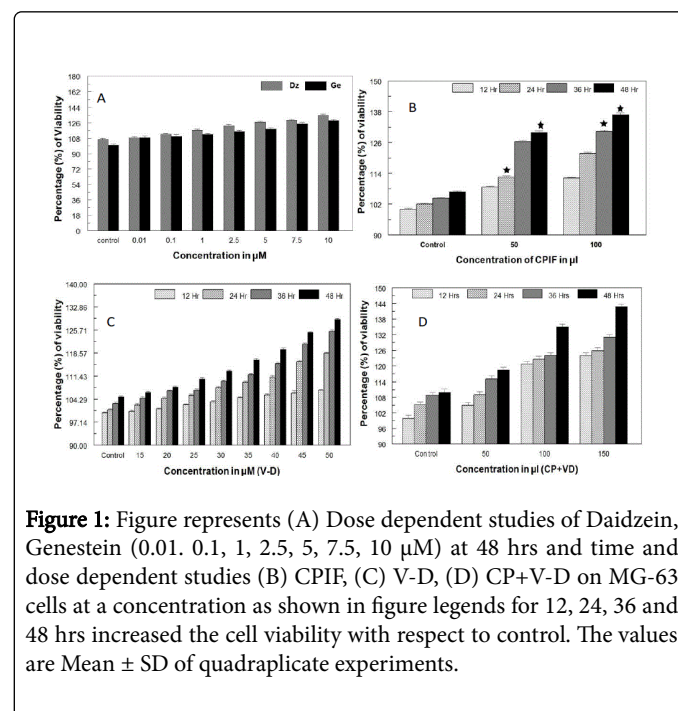


Figure 1: Figure represents (A) Dose dependent studies of Daidzein, Genestein (0.01, 0.1, 1, 2.5, 5, 7.5, 10 μM) at 48 hrs and time and dose dependent studies (B) CPIF, (C) V-D, (D) CP+V-D on MG-63 cells at a concentration as shown in figure legends for 12, 24, 36 and 48 hrs increased the cell viability with respect to control. The values are Mean \pm SD of quadruplicate experiments.

The effect of V+C+D+G on MG-63 cell lines in time and dose dependent manner at a concentration range of (as shown in figure legends) showed a significant increase in cell number at a percentage (%) increase of 111.25, 115.5, 121.25 respectively (Figure 1D). Similarly, different cell lysates of exposed groups at 48 hrs when tested for alkaline phosphatase activity as per the protocol also showed increase in their activity when compared to the group where no exposure is done. The percentage (%) increase in exposed groups like Dz, Ge, Dz+Ge, V-D, CPIF, V+C, V+C+D+G was 127.15, 118.28, 151.61, 344.89, 210.75, 233.87, 263.98 respectively compared to that of the unexposed cells i.e., control where the percentage (%) was 120.42 (Figure 2). But the same when tested for the acid phosphatase activity there was a decrease in levels of ACP in exposed groups when compared with that of the control. The exposed cells showed a significant decrease in percentage (%) in Dz, Ge, Dz+Ge, V-D, CPIF, V+C, V+C+D+G as 13.33, 22.67, 28.67, 23.33, 14.24, 28.83, 20.67 respectively when compared to that of unexposed cells (Figure 2).

Similarly, different exposed groups with different timings when tested for Osteocalcin activity as per the protocol showed increase in their activity when compared to the group where no exposure is done. The exposed cells showed increase in OC activity at a percentage (%) of 11.32, 8.3, 12.2, 10.71, 28.52, 22.75, 53.01, 70 pg/ml for treatments like Dz, Ge, Dz+Ge, CPIF, V-D, V+C, V+C+D+G respectively (Figure 3).

Different treatment groups with different timings when tested for intracellular calcium activity as per the protocol showed increase in their activity when compared to the group where no exposure is done. The levels of intracellular calcium levels in unexposed cells were 5.1% where as in exposed cells like Dz, Ge, Dz+Ge, CP, V-D, V-D+CP, V-D+CP+D+G were 8.5, 10.4, 14.4, 22.6, 25.4, 29.5, and 32.1% respectively (Figure 4). The antioxidant activity of the exposed cells in comparison with that control was also tested. The specific activity of Catalase and SOD enzymes showed an increase in the activity in exposed cells than

control. There was an increase in reduced GSH Activity and vitamin c activity also in exposed cells when compared with that of the control. The TBARS activity in contrast showed decrease in amount of MDA released in exposed groups when compared with that of control.

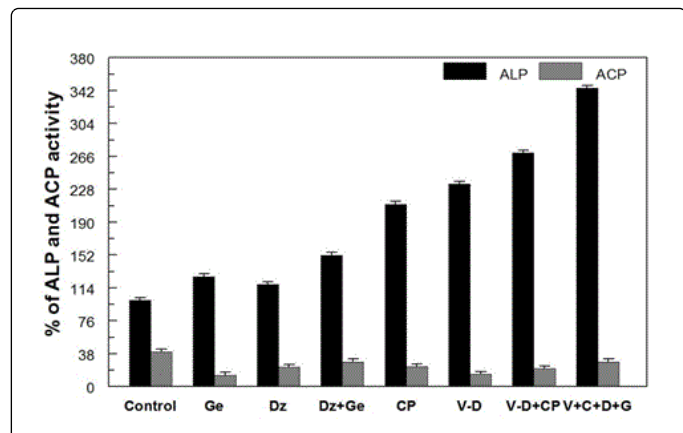


Figure 2: Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP) activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase of ALP activity in exposed cells when compared with that of control and significant decrease of ACP activity in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

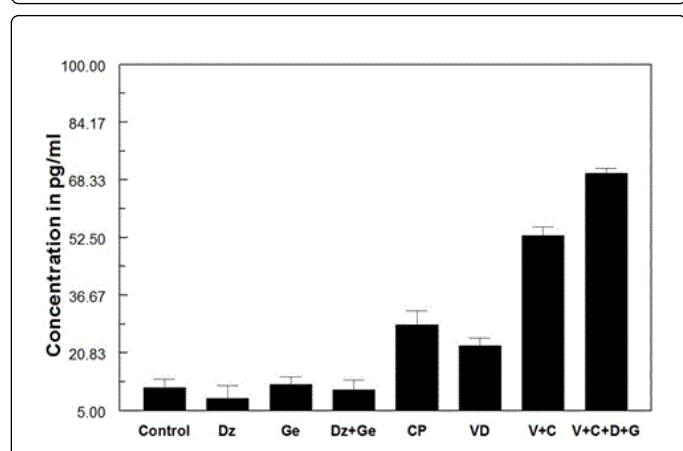


Figure 3: Osteocalcin activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase of OC activity in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

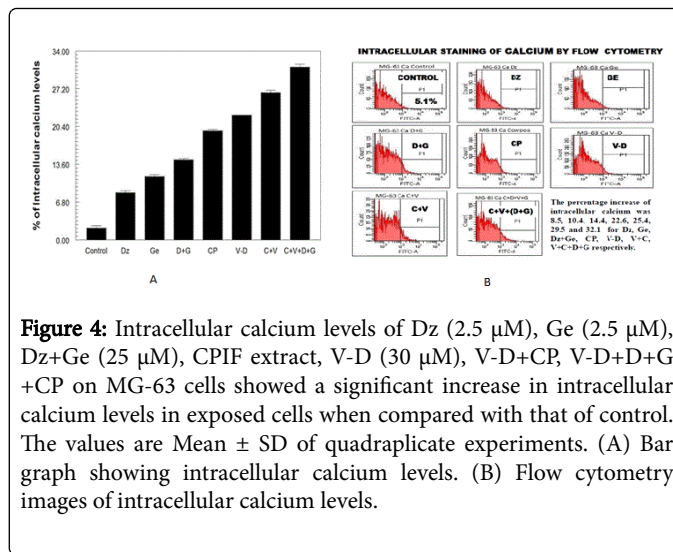


Figure 4: Intracellular calcium levels of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase in intracellular calcium levels in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments. (A) Bar graph showing intracellular calcium levels. (B) Flow cytometry images of intracellular calcium levels.

The levels of glutathione in unexposed cells were 0.285 μ g/mg protein where as in exposed cells like Dz, Ge, Dz+Ge, CP, V-D, V+C, V+C+D+G were 0.6, 0.63, 0.86, 0.68, 1.28, 1.10 μ g/mg protein respectively (Figure 5). The levels of MDA in unexposed cells were 1.24 nm/ml, whereas in the cells exposed to Dz, Ge, Dz+Ge, CP, V-D, V+C, V+C+G+D, were 0.23, 0.193, 0.5, 0.66, 0.54, 1, 1.13 nm/ml respectively (Figure 6).

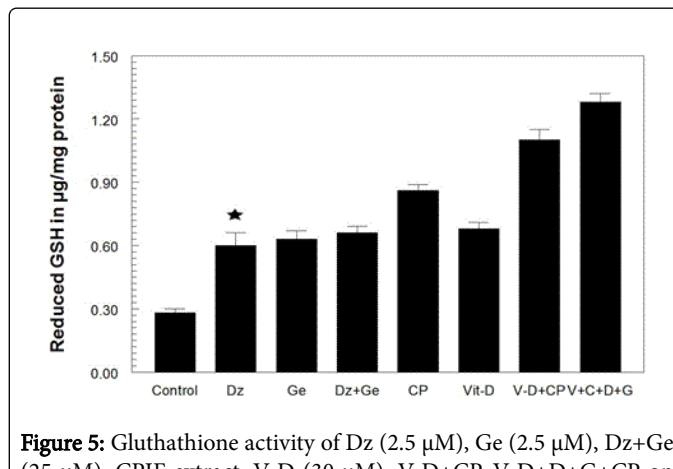


Figure 5: Glutathione activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase a significant increase of GSH activity in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

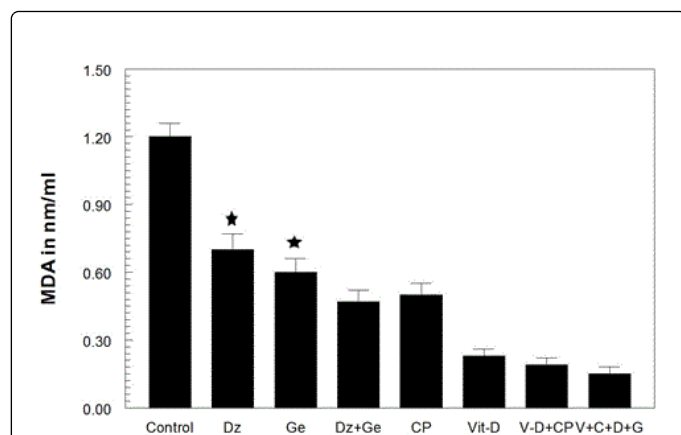


Figure 6: TBARS activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant decrease in levels of released MDA in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

SOD antioxidant showed a significant increase in its activity in exposed cells like Dz, Ge, Dz+Ge, CPIF, V-D, C+V, C+V+D+G 0.56, 0.45, 0.64, 0.78, 0.86, 0.98, 0.76 U/mg protein respectively when compared to that of unexposed cells 0.43 U/mg protein (Figure 7).

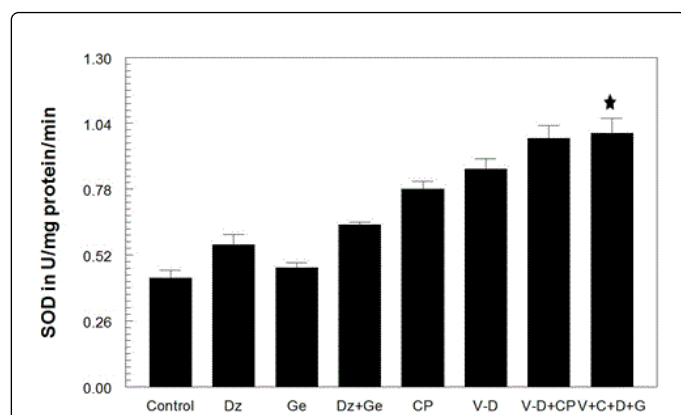


Figure 7: SOD activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase of SOD activity in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

When the specific activity of the catalase levels was observed, a significant increase was observed in exposed cells like Dz, Ge, Dz+Ge, CPIF, V-D, V+C, V+C+G+D at 0.32, 0.24, 0.56, 0.45, 0.75, 0.83, 0.76 U/mg protein/min respectively compared to unexposed cells which showed activity at 0.22 U/mg protein/min (Figure 8).

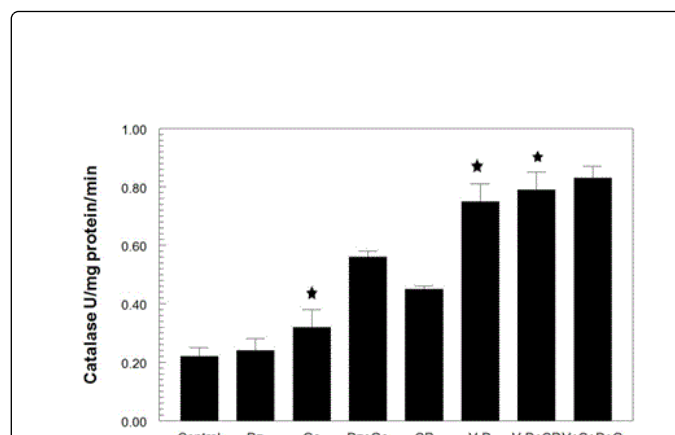


Figure 8: Catalase activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase in levels of Catalase in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.

The levels of vitamin C in exposed cells like Dz, Ge, Dz+Ge, CPIF, V-D, V+C, V+C+G+D were 0.29, 0.42, 0.59, 0.66, 0.57, 0.83, 0.74 μ g/mg protein respectively whereas the levels in unexposed cells were 0.1857 μ g/mg protein (Figure 9). The levels of ROS in unexposed cells was 90.9% whereas in exposed cells like Dz, Ge, Dz+Ge, CP, V-D, V-D+CP, V-D+CP+D+G were 65.5, 61, 60.6, 73.9, 68.7, 54.4 and 51.1 % respectively (Figure 10).

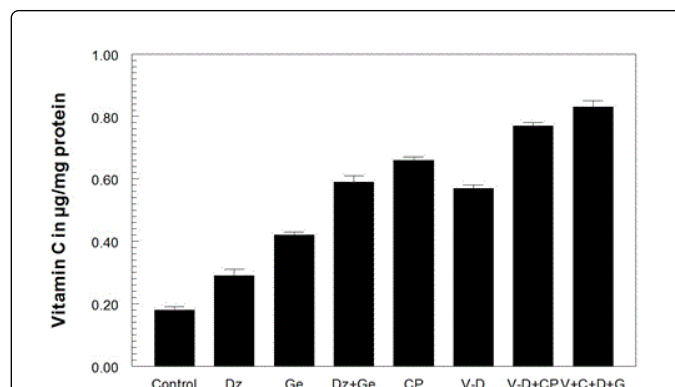
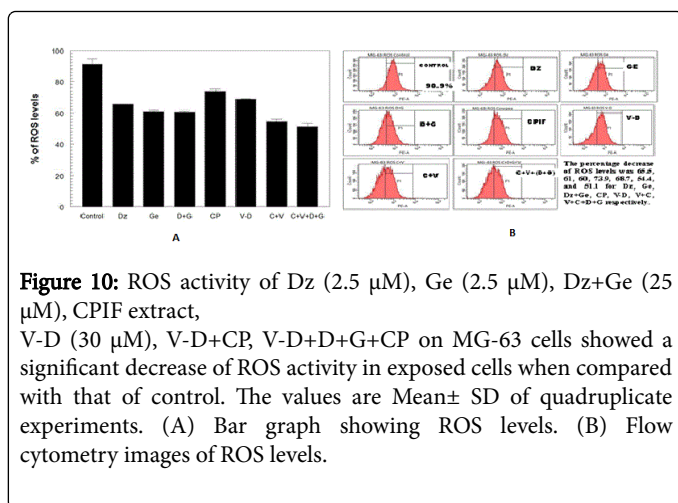
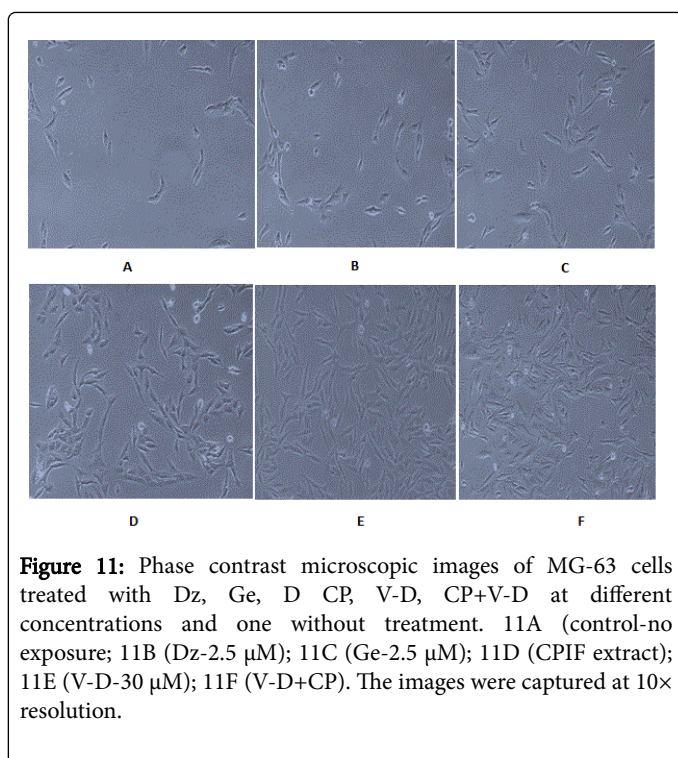


Figure 9: Vitamin C activity of Dz (2.5 μ M), Ge (2.5 μ M), Dz+Ge (25 μ M), CPIF extract, V-D (30 μ M), V-D+CP, V-D+D+G+CP on MG-63 cells showed a significant increase in the levels of vitamin C in exposed cells when compared with that of control. The values are Mean \pm SD of quadruplicate experiments.



When observed, the morphology indicates that, in control (Figure 11A) which is considered to be an osteoporotic group, the cell number was very less and connections between the cells are very far or nil. After treatment, an increase in cell number was observed and connections between the cells also improved and maximum confluence of almost 100% was observed in combination groups after 48 hrs when visualized under phase contrast microscope (Figures 11B-11F).



Discussion

Bone an active organ which maintains a balanced cycle of osteoblastic and osteoclastic activity includes so many factors like growth factors, cytokines and hormones for its balanced activity. Among different hormones, estrogen has a very vital role in bone metabolism especially in women. The effect of estrogen on the bone

cells is mediated by binding to the nuclear receptors ERα and ERβ and forming a complex that undergoes dimerization. The dimers that are formed bind to the estrogen responsive elements in the promoter regions of the target genes and regulates gene expression. Estrogen deficiency in bone has direct effect on osteoclasts which results in up regulation of osteoclastogenesis [13,14] which activates RANKL [15]. Bone formation includes cellular events like chemotaxis, proliferation, and differentiation of osteoblast precursors [16]. These events are probably modulated by local factors or cytokines such as BMP's, Hedgehogs, transcription factors and core binding factors like α-1 (cbfa1) [17-19]. TGF-β/BMP signaling pathways showed to play critical regulatory functions in the formation of bone and differentiation of osteoblasts during mammalian development [20,21]. Disruption in TGF-β/BMP signaling leads to bone related disorders [22]. TGF-β/BMP signal transduction mechanism is specifically through non-canonical smad independent pathway (e.g., p38 MAPK i.e., mitogen activated protein kinase pathway) and canonical smad dependent pathway (TGF-β/BMP ligands receptors and smads). When TGF-β/BMP induction occurs both p38 MAPK and smad pathway converge at Runx-2 gene to control mesenchymal precursor cell differentiation. Coordinated activity of Runx-2 and TGF-β/BMP activated smads is essential for formation of skeleton [23,24]. Present study is performed to find the efficacy of the Cowpea isoflavones along with Vitamin D whether they have any anabolic effect on bone metabolism.

As Isoflavones have antagonistic effects of estrogen hormone, these Phytoestrogens have gained large importance now days. Studies on animals and humans have indicated that bone mass can be initiated or increased by the consumption of IF's. Daidzein and genestein are natural isoflavonoids of the family *leguminosae* and these isoflavones are known to mimick the properties of estrogen hormone. Previous reports have indicated that daidzein stimulates early differentiation of osteoblasts and a significant increase in cell proliferation was observed on MC3T3-E1, the preosteoblastic cells [8]. One more study on wistar rats reports that primary Osteoblastic cultures isolated from newly born rat calvaria showed enhancing effect on viability of cells and also stimulated the differentiation of osteoblasts [25]. Genestein the most abundant isoflavone was shown to have bone sparing effects in ovarioectomized rats [26,27]. Genestein have an inhibitory effect on protein tyrosine kinases by interacting with the plasmalemma tyrosine kinase receptors [28,29] and help in reducing free radical secretion by inflammatory cells receptor. Both Dz and Ge are known to show enhancing effects on cell proliferation, DNA content, ALP activity, OC activity on MC3T3 E1 cell lines. The cells showed significant increase in cell proliferation at a concentration range of 0.01-0.1M [8,9].

In contrast to above studies, in our present study, where MG-63 cell lines were taken as an experimental model, both Dz and Ge at higher concentration ranges with slight variation from above concentrations showed anabolic effects on bone. There was a significant increase in cell proliferation which shows accordance with the above studies. The stimulatory effects of Dz and Ge are found to be equal. When Dz is hydroxylated its structure is similar to that of Ge [30].

The synergistic effects of soy IF on MG-63 cell proliferation could be due to the enhanced estrogenic response of the cells to estrogen receptor and growth signaling pathways which is in support with above results. Our main focus cowpea is found to be very rich in Isoflavones, its absorption and extraction capacity in rats was also found to be high [31,32]. One own study of ours showed enhancing effects of BMD, Ca, and ALP levels in diet induced osteoporotic rat model with the administration of CPIF. The effect was even more

significant and Osteoporotic condition is found to be reversed when CPIF is administered along with Vitamin D [11]. Cowpea a natural isoflavone, which has Dz and Gen in a ratio of 18:6 can be speculated to show results similar more or less as synthetic Dz and Ge. When observed, the cell proliferation of the MG-63 cells upon treatment with Cowpea showed a significant increase in proliferation and the increase was much more significant than proliferation rates of Dz or Ge treated cells [33]. The results are on par with the above studies on rat showing high proliferative rates as it's a cell line. Vitamin D is very essential micronutrient for optimal health. Low levels of Vitamin D lead to multiple illness among them osteoporosis is well known [34]. Vitamin D is very essential for maintaining calcium levels and PHT adds its role for maintaining Ca levels. The effect of vitamin D on MG-63 also showed increased proliferation indicating that Vitamin D may also be having an anabolic effect on bone. When MG-63 cells were exposed to a combination of CP+V-D the proliferation rates were little more significant than V-D alone. The possible mechanism of effect of soyisoflavones and Vitamin D on bone metabolism can be explained in a way that Soyisoflavones and Vitamin D can synergistically stimulate bone formation by inducing $1, 25(\text{OH})_2$ synthesis and Vitamin D Receptor (VDR) expression. $1, 25(\text{OH})_2$ bound to VDR and induces osteoblastic activity and bone formation [35].

Alkaline phosphatase is a phenotypic marker of the early osteoblast differentiation. It is ubiquitous in nature and is a tetrameric enzyme which is bound to membrane. It is attached to Glycosyl-Phosphatidyl-Ionositol moieties located on outer cell surface [36]. Bone specific ALP is preferred for measurement, because of its increased specificity [37-41]. Bone specific ALP is produced by osteoblasts and plays a major role in bone mineralization process. High levels of ALP can be interpreted as a direct indication of more mineralization or bone forming process of osteoblasts. Ca and P together form crystallization product and once enough crystal is formed the ALP is released in to the serum and can be considered as an active indicator of bone turnover [42]. When the ALP levels of the MG-63 cells treated with different exposure treatments were measured the ALP levels were increased. Osteocalcin is another phenotypic marker of osteoblasts but it is produced during the later stages of osteoblast differentiation. ALP and OC can be considered as phenotypic markers of bone turnover [43]. OC is a Bone Gla protein (BGP). It is a hydroxyapatite binding protein exclusively synthesized by osteoblasts [44]. Once OC is released by osteoblasts, the newly synthesized protein is found to be incorporated in to extracellular matrix where it is found to be in form of non-collagenous protein fraction. A small is detected in circulation (15%). When the serum levels of OC were measured, the results correlate with the rate of bone formation [45]. When OC levels of the MG-63 cells treated with different exposure treatments were measured the levels of OC were increased. Thus, CPIF, VD, and isoflavones enhance the ALP levels and OC levels thus showing their anabolic side. The maximum significant increase in ALP and OC levels was observed in CP treated MG-63 cells and C+V+D+G treated MG-63 cells respectively supporting the view of our aimed study that natural isoflavones might be having beneficial effects in treatment of OSP. Tartarate resistant acid phosphate is a marker for bone resorption. Whenever there is an increase in resorption process, more levels of TRAP are observed in serum and plasma [43]. When our cell lines were tested for TRAP activity the levels decreased on all exposure treatments, thus providing an insight for our study.

Bone should contain adequate amounts of nutrients in its matrix for the proper maintaining of bone regulation. Among all nutrients, calcium plays an important role for bone metabolism. In turn,

Vitamin-D is essential for calcium metabolism [46]. One of the studies showed a decrease in the levels of Intracellular calcium where human peripheral blood mononuclear cells (HPBMC) were tested for intracellular calcium using Fluo-3 by flow cytometry by adding Thapsigargin (TG) [47]. In contrast to above when our cell lines were tested for intracellular calcium activity by flow cytometry, the levels of calcium increased significantly in treatment groups compared to that of control. Thus, our compounds Cowpea isoflavones and V-D enhance the levels of intracellular calcium thus providing an insight to our study that these compounds may act synergistically in preventing OSP.

Oxidative stress can cause oxidative damage to the cells mainly due to reactive oxygen species. Oxidative stress plays a crucial role in the pathogenesis of various malignant tumors. Carcinogenesis by oxidant stress appears to be caused mainly by excessive generation of ROS leading to structural changes of DNA. One study exists, where the levels of ROS increased significantly when a flavanoid compound baicalein was exposed on Human Osteosarcoma cell line MG-63 [48]. Our study on MG-63 cell lines decreased the levels of ROS in treatment groups. Our results are in contrary to above results thus indicating that CPIF, V-D and Soy isoflavones decrease Oxidative stress and thus may help in preventing Osteoporotic conditions.

In carcinogenesis, antioxidant enzymes have been suggested to play a functional role. Antioxidants are the substances that may protect cells from damage caused by unstable molecules known as free radicals. All the cells possess elaborate antioxidant defense mechanisms to neutralize the deleterious effects of free radical induced lipid peroxidation [49]. Gluthathione is a tripeptide made up of aminoacids cysteine, glycine and gamma glutamic acid. GSH is a vital antioxidant in cells and it protects tissue against oxidative stress damage, inflammation and injury. GSH acts as a radical scavenging antioxidant. In one previous study, there was a significant decrease in the antioxidant enzymes like GSH, SOD, CAT in the femur of the ovariectomized rats suggesting that deficiency in ovarian hormones failed to combat oxidative stress [50]. Under mild oxidative stress, the cysteine residues of gluthathione undergoes oxidation and causes mixed disulphide formation between low molecular mass thiols and protein thiol groups with the help of GSH. Accumulation of these proteins occurs when there is a balanced redox reaction between oxidized and reduced forms of GSH and this results in disease if balance is not maintained and a decrease is observed [51]. The gluthathione status of a cell (that is excess of reduced over oxidized gluthathione) will perhaps turn out to be the most accurate single indicator of the health of the cell. The reason for decrease in these levels can be attributed to high levels of H_2O_2 production [52].

Catalase is a hemoprotein containing four subunits and it has a capacity to decompose peroxide. The major function of catalase with in the cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a byproduct of metabolic process. The main function is to scavenge for peroxide which is a byproduct of oxidative phosphorylation process. Hydrogen peroxide generated by variety of reactions is converted to water by CAT [53]. In bones, proteins that are involved in cell differentiation are found to be oxidized by Hydrogen peroxide [54]. Thus, hydrogen peroxide increased levels result in ROS in bone tumor.

Superoxide dismutase catalyzes the destruction of oxygen free radical. It protects the oxygen metabolizing cells against harmful effects of superoxide free radicals. SOD is known to have an anti-inflammatory effect. Pyrogallol oxidation inhibition was inhibited by

SOD activity [55]. One more study on rats also showed decrease in GSH, CAT and SOD levels indicating the enhancement of oxidative stress [56].

In contrast to above, our studies showed that levels of GSH, CAT, SOD for all different exposures increased showing that CPIF, VD and Soy IF when treated on cells decreased the oxidative stress [57] thus showing enhancing antioxidant activity hence adding a supportive view for our aimed study.

Vitamin C, is an important enzyme in synthesis of collagen and carnitine and is a water-soluble antioxidant in the human body. Vitamin C promotes osteoblast differentiation and its activity. Even it provides stability of bone matrix. So, its function is found to be protective [58]. The levels of vitamin C were found to be decreased in osteomyelitis (OM) a bone infection in bacteria [59]. The Mechanism is not clearly known. Our studies increased the vitamin C levels indicating that CPIF, VD and Soy IF showed their antioxidant properties on cells and thus may help in improving OSP.

Lipid peroxidation is a free radical related process which occurs under enzymatic control in biological systems. The increase in Hydrogen peroxide levels induced the peroxidation of polyunsaturated fatty acids which further leads to formation of MDA, a byproduct of lipid peroxidation. MDA inhibits synthesis of nucleic acids and proteins and as it has high reactivity towards amino groups, this results in deactivation of enzymes [60]. In this study, there was an increase in MDA levels [50]. Our results are in contrast to the above result, rather, there was a significant decrease in MDA levels when different exposures were done [61].

Overall, the increased levels of GSH, SOD, CAT, Vitamin C, and decreased levels of MDA and ROS activity after treatments on MG-63 cells showed that CPIF and V-D has decreased the oxidative stress on cells which probably may act as an anabolic factor for reducing OSP [62].

Conclusion

To conclude, possible mechanism involving the synergistic effects of Cowpea isoflavones and Vitamin D in improving bone turnover could be due to enhanced osteoblastic activity by promoting osteoblasts to secrete OPG and its binding to RANKL leading to suppression of the preosteoclast and osteoclast differentiation, thereby preventing the binding of RANK and RANKL in RANK-RANKL-OPG pathway which is associated with the increase in the levels of vitamin D metabolites (1, 25(OH)₂D), VDR and ER activity.

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