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Effect of the supplementation of dietary rich phytoestrogens in altering the vitamin D levels in diet induced osteoporotic rat model☆

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ABSTRACT

Plant-derived estrogen-like compounds such as isoflavones (IF) especially daidzein and genistein are said to be preserving the bone in the osteoporotic conditions. However, it is not known whether a combination of IF and calcium (Ca) supplementation attenuates losses in bone mass and prevents the loss of vitamin D (VD). The present study addresses the role of phytoestrogens (PE) and Ca supplementation in low Ca and low VD diet induced osteoporosis (OSP). Cowpea (CP) which has high amount of the IF was selected to study its effect on diet induced osteoporotic conditions. Female weanling WNIN rats (total of 68) were divided into five groups and fed for five weeks on semisynthetic diet with low Ca (0.15%) and low VD (0.1 IU/day/rat) in combination with low (10 mg/kg) or high (25 mg/kg) concentrations of PEs derived from CPIF. The study groups are: (I) normal Ca(0.47%) and normal VD (25 IU/day/rat), (II) low Ca + low VD, (III) low Ca + low VD + low CPIF (10 mg/kg diet), (IV) low Ca + low VD + high CPIF (25 mg/kg diet) and (V) low Ca + low VD + 17-(-estradiol (3.2 mg/kg diet). After the development of OSP the group II was subgrouped into: (SG I) continued on low Ca + VD, (SG II) low CPIF, (SG III) high CPIF, (SG IV) 17-β-estradiol and (SG V) normal Ca and VD. Serum 25-VD levels were in the range of 14–38 ng/ml in groups I, III, IV and V, where as the values were very low in the group II (5.8 ng/ml). These were partially reversed upon supplementation of CPIF. The results correlated with altered Ca levels, body weight, bone mineral density and content and other related biochemical parameters. The paper further explains the possibility of protective and therapeutic role of VD in the presence of CPIF in osteoporotic health manifestations.

1. Introduction

Osteoporosis (OSP) with its accompanying reduction in bone mass is universally recognized as a major public health problem. OSP is a metabolic condition characterized by low bone mass, deterioration of bone tissues and increased risk of fracture [1–3]. OSP creates significant economic burden on society as well as the families of patients who are suffering from related fractures [3–5]. In women, hormone replacement therapy (HRT) used to be the major regimen for prevention and treatment of OSP. However, with the recent discovery that HRT is associated with an increased risk in developing breast, endometrial and ovarian cancers also [6,7], there is a strong demand for developing alternative approaches for the management of OSP.

Nutritional and pharmacological strategies are need of the hour to prevent age related bone loss. Traditional therapies for the OSP have emphasized the use of antiresorptive agents such as estrogen, calcitonin and bisphosphonates. Although these agents may prevent further bone loss in established OSP, they may not restore bone mass that has been lost already. Thus, it is necessary to develop alternative therapy in the form of naturally occurring compounds with less desirable side effects that can immensely reduce the need for drugs usage. Polyphenolic non-steroidal plant compounds namely phytoestrogens (PE) are naturally available biological compounds found in a wide variety of sources such as plant foods and are said to exhibit estrogen-like activity because of its structural similarities as that of estrogens [8,9]. Isoflavones (IF) are one of the classes of PE and are abundant in plants and have received increasing attention as dietary components that can affect several aspects of human health IF like genistein (Ge) and daidzein (Dz) bind to the ligand binding domain of both Estrogen receptor (ER) isoforms with moderate affinity, but preferentially to ERβ, in a manner similar to E2 [10,11].

The reports indicate that use of soy isoflavones for protection against or reduction of bone loss is inconsistent [12–14]. Further, VD and its derivatives have an important role in OSP [15], and the active forms of VD can significantly improve bone mass and reduce vertebral fracture rates in osteoporotic conditions [16,17]. There is

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no data available till now in the situations where OSP is induced by the low Ca and low VD diet and the effects of PE against the induced OSP derived from foods. This study was primarily designed to elucidate whether supplementation of PE rich CP is capable of preventing the rapid bone loss occurring after diet induced OSP in the rats.

2. Materials and methods

2.1. Animals and diet

2.1.1. Animals

Female weanling WNIN rats (30–35 g) were maintained under controlled conditions of temperature (20 ± 2 °C, relative humidity 50–80%) and illumination (12 h light, 12 h dark). All animal experiments were duly approved by the institute's animal ethical committee and complied with accepted veterinary medical practice.

2.1.2. Study design and animal experimentation

The animals a total of 68 were fed a semisynthetic diet as described by Suda et al. [18] with low Ca (0.15%) and low VD (0.1 IU/day/rat). The CPIF mixed diet was fed the animal (Table 1). Group I of six rats included the Control diet (with normal Ca) (0.47%) and normal VD (25 IU/day/rat), Group II containing 38 rats fed with low Ca (0.15%) and 0.1 IU VD, Group III having eight rats were fed with low Ca (0.15%) and 0.1 IU VD and supplemented with low concentrations of CPIF (10 mg/kg diet), Group IV included eight rats and fed with low calcium (0.15%), 0.1 IU VD and supplemented with high concentrations of CPIF (25 mg/kg diet) and Group V contains eight rats fed with low Ca (0.15%) and 0.1 IU VD supplemented with 17-β-estradiol (3.2 mg/kg diet). After the development of OSP (approximately six weeks) as indicated by BMD, BMC and biochemical analysis, the group II was sub-divided in to five subgroups (SG) and supplemented CPIF. Number of rats in all the SGs was maintained with eight except group V which was having only six animals. In the Group II, SG I was continued with low Ca (0.15%) and 0.1 IU VD, SG II was fed with low concentrations CPIF (10 mg/kg), SG III was fed with high concentrations of CPIF (25 mg/kg diet), SG IV was fed with 17-β-estradiol (3.2 mg/kg diet) and SG V was replenished with normal Ca and VD. Rats had free access to deionized water and food intake was recorded every alternative, and body weight was measured once in four days. After a period of 90 days the animals were sacrificed and the investigations were carried out to find the protective and therapeutic effects of the CP.

2.1.3. Preparation of vitamin D_3 (VD3) drops

Crystalline VD3 is procured from Sigma chemical company. A pinch of crystalline VD3 was dissolved in 3 ml of 95% ethanol and OD is measured at 265 nm. The exact concentration of VD3 is calculated based on its molar extinction coefficient and OD at 265 nm. For the preparation of VD3 drops, an aliquot containing a known amount of VD3 is evaporated to dryness under a stream of nitrogen and resuspened in refined cotton seed oil, so as to get 50 IU of VD3 in 0.1 ml aliquot. This is further diluted to get different concentrations of VD3.

2.2. Extraction of CPIF

2.2.1. Extraction

Five grams of each powdered sample was defatted in duplicates and extracted twice into 70 ml of 70% methanol. The extract was hydrolyzed with 2.0 M HCl and then concentrated in a flash evaporator, and made up to known volume and then filtered through 0.45 μ filters and used for HPLC analysis. C18 Sep-Pack cartridges were preconditioned with methanol followed by water for Solid-phase extraction within 24 h. One ml of concentrated extract was loaded on the column and washed with water to remove sugars. Retained IF were eluted with ethyl acetate and than analyzed by HPLC [19].

2.2.2. HPLC-DAD analysis

Lichrosphere- ODS 2, C18 column (250 mm × 4.6 mm), was used for chromatographic analysis on Shimadzu LC- 2010A equipped with auto injector, binary pump and diode array detector (Shimadzu SPDM10A). Linear gradient of mobile phase 0.1% phosphoric acid (A) and Acetonitrile (B) with a flow of 0.8 ml/min. The linear gradient was started at 10% acetonitrile and increased up to 100% and brought back to 10% in 35 min of run time. A wavelength range of 200–800 nm was used in the DAD detector [20].

2.3. Bone mineral density (BMD) and mineral content (BMC)

BMD (g/cm^2) and BMC (g) of the whole body, other regions of the bone arrangement were assessed by dual-energy X-ray absorptiometry (DXA; QDR-2000, Hologic Inc., Waltham, MA) equipped with appropriate software for use with small laboratory animals. The regions analyzed were divided in to four parts such as right hind limb (R1), left hind limb (R2), thoracolumbosacral spine (R3) and both fore limbs, a part of basal skull and cervical spine (R4). The results are expressed as the net values for both BMD and BMC.

2.4. Quantification of serum vitamin D

Serum 25-hydroxy vitamin D (25-VD) was quantified by the RIA method and procured from DiaSorin (Stillwater, MN 55082-0285, USA). Briefly the assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-VD and other hydroxylated metabolites from serum with acetonitrile. Following extraction, the treated sample was then assayed using an equilibrium RIA procedure. The RIA method is based on an antibody with specificity to 25-VD. The sample, antibody and tracer are incubated for 90 min at 20–25 °C. Phase separation is accomplished after a 20 min incubation at 20–25 °C with a second antibody precipitating complex. A NSB/Addition buffer is added after this incubation prior to centrifugation to aid in reducing non-specific binding in terms of average CPM of NSB Tube/Average CPM of Total Count Tubes. The values of 25-VD are expressed in ng/ml of the sample.

2.5. Determination of serum alkaline phosphatase (ALP)

Serum ALP was determined by the method of Walter and Schutt [21]. Enzyme activity was expressed as mmol of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al. [22].

2.6. Determination of serum calcium (Ca) and phosphorus (P)

Serum Ca was measured using atomic absorption spectrophotometry by appropriately diluting with 0.1% lanthanum chloride solution as per the modified method of Zettner and Seligson [23]. Serum P was determined by the modified method of Taussky and Shorr [24].

2.7. Statistical analysis

Data were expressed as mean ± standard error (SE). The comparison between the groups was done by Student's two-tailed *t* test. Comparisons among treated groups were statistically processed by one-way analysis of variance (ANOVA) with Tukey's post hoc

Table 1
Effects of supplementation of CPIF on food intake, body weight and total body fat percentage.

Parameter	G I	G II					G III	G IV	G V
		SG I	SG II	SG III	SG IV	SG V			
Food intake (g/day)									
Initial	15.4 ± 0.2	12.2 ± 0.2	12.3 ± 0.21	11.9 ± 0.3	12.9 ± 0.3	12.3 ± 0.3	13.7 ± 0.4	13.4 ± 0.3	14.7 ± 0.3
Final	14.9+0.2	12.6 ± 0.3	12.9 ± 0.3	11.4 ± 0.2	12.7 ± 0.2	14.6 ± 0.2*	13.9 ± 0.3	13.7 ± 0.2	14.2 ± 0.3
Body weights (g)									
Initial	92.3 ± 0.9	64.2 ± 0.8	73.2 ± 0.7	68.2 ± 0.8	67.2 ± 0.9	65.2 ± 0.9	74.2 ± 0.7	71.3 ± 0.8	70.2 ± 0.9
Final	99.3 ± 0.9	62.3 ± 0.7	69.2 ± 0.6	69.3 ± 0.8	66.2 ± 0.72	88.3 ± 0.8*	72.2 ± 0.76	73.1 ± 0.8	71.8 ± 0.7
Fat (%)									
Initial	26.9 ± 0.1	13.4 ± 0.2	12.6 ± 0.2	11.2 ± 0.1	12.7 ± 0.2	12.2 ± 0.2	11.7 ± 0.3	13.3 ± 0.2	14.3 ± 0.3
Final	28.9 ± 0.3	14.3 ± 0.2	14.3 ± 0.2*	9.8 ± 0.2*	11.2 ± 0.2	23.4 ± 0.2*	12.4 ± 0.3	12.8 ± 0.2	13.7 ± 0.2

Values are expressed as mean ± SE.
* The significant differences are expressed with respective initial values when compared with their respective final values at $p < 0.05$

analysis by use of a SAS software package (SAS Institute Japan Ltd., Tokyo). p values of less than 0.05 were considered significant.

3. Results

Animal diet was designed to develop OSP with Ca and P free salt mixture and incorporating calcium carbonate with variation in the composition in the control (1.17 g/100 g diet) and experimental osteoporotic diet (0.37 g/100 g diet) with low VD (0.1 U/rat/day) levels. The important identified constituents of the CPIF are Dz (18.07 mg/100 g dry weight) and Ge (6.6 mg/100 g dry weight) and were utilized to formulate the experimental diet with low concentration (10 mg/kg diet) and high concentration (25 mg/kg diet) and incorporated in the diet appropriately. In the data, Initial always indicates the point where the completion of feeding of the osteoporotic diet and initiation of the CPIF diet for the G II animals and the Final always indicates the point where the study was completed unless mentioned specifically.

The results indicate that food intake of the animals are more or less the same through out the experiment and there were no significant differences between the initial and final groups and the average consumption was found to be in the range of 12–14 g/day (Table 1). Body weights were significantly high in the G I animals (Control diet) when compared with other groups. The weight of the SG V in the G II was significantly increased when compared to the diet given to develop OSP in the same group, except this there is no visible change in the body weights in the groups. G II maintained low body weights till the end of the experimentation (Table 1).

The fat deposition percentage is also found to be the similar as that of body weights, except in the SG V of G II, where the animals are replenished with the control diet with normal Ca and VD (Table 2). BMD and BMC were found to be significantly increased in the SG II, III, IV and V of the G II ($p < 0.05$). There is no significant difference observed in the other groups when compared to the initial and final status of the supplementation of CPIF (Figs. 1 and 2). The DXA report indicates that the hind limbs of the rat had low BMD and BMC in the osteoporotic group and was partially but significantly reversed with the supplementation of CPIF (data not reported). The status of Ca and P levels in the final point significantly altered in the SG II, III and IV of the G II when compared with the initial point. Inspite of the levels altered in the G III, but there were no significant changes when compared with their corresponding comparative group. The changes in the ALP levels were significant particularly in the SG II, III, IV and V ($p < 0.05$) and difference in the range of 65–80 U/l was observed. Similarly the 25-hydroxy VD (25-VD) levels were significantly altered in all the SG of G II ($p < 0.05$) and other groups almost remains the same with their respective initial and final points of the experimentation (Fig. 3).

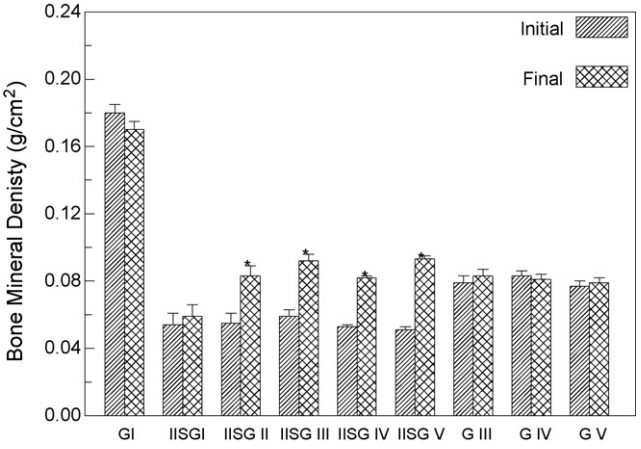

Fig. 1. Effects of dietary supplementation of CPIF on bone mineral density.

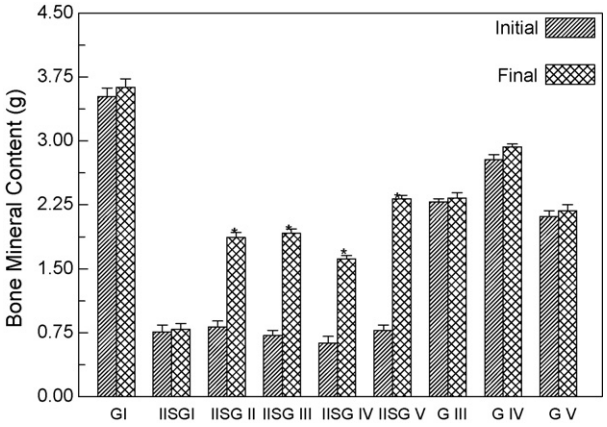

Fig. 2. Effects of dietary supplementation of CPIF on bone mineral content.

4. Discussion

Inhibition of the bone loss and reduction in the risk of fractures are the hallmarks for an effective antiosteoporotic treatment. There are a number of conventional medicines available for preventing and/or treating OSP, these medicines are mostly having the side effects and costly to procure. The purpose of this study is to find the action of naturally available foods that can act as the agents in preventing the bone loss or promoting bone restoration.

Study was designed to determine whether CPIF can be efficient enough to restore the bone loss as a result of diet induced

Table 2
Effects of supplementation of CPIF on the serum levels of calcium, phosphorus and alkaline phosphatase.

Parameter	G I	G II					G III	G IV	G V
		SG I	SG II	SG III	SG IV	SG V			
Calcium (mg/dl)									
Initial	10.4 ± 0.27	5.75 ± 0.4	5.83 ± 0.38	5.89 ± 0.29	5.36 ± 0.33	5.67 ± 0.32	6.99 ± 0.28	7.34 ± 0.3	6.78 ± 0.3
Final	10.5 ± 0.21	5.82 ± 0.32	7.33 ± 0.32*	7.89 ± 0.34*	6.33 ± 0.52*	8.32 ± 0.3*	7.34 ± 0.36	7.56 ± 0.31	6.97 ± 0.18
Phosphorous (mg/dl)									
Initial	6.97 ± 0.33	9.76 ± 0.29	9.66 ± 0.31	9.87 ± 0.3	9.56 ± 0.3	9.67 ± 0.2	7.33 ± 0.3	7.53 ± 0.26	8.01 ± 0.2
Final	7.23 ± 0.33	9.87 ± 0.23	7.83 ± 0.23*	7.89 ± 0.31*	8.3 ± 0.3*	7.66 ± 0.24*	7.42 ± 0.3	7.49 ± 0.21	7.99 ± 0.11
Alkaline Phosphatase (U/l)									
Initial	115.2 ± 3.2	216.2 ± 6.2	221.3 ± 3.2	216.3 ± 4.2	224.7 ± 3.8	216.2 ± 3.2	142.4 ± 2.8	136.3 ± 2.7	163.2 ± 2.4
Final	112.2 ± 2.9	221.3 ± 5.3	152.6 ± 3.8*	138.3 ± 3.2*	183.2 ± 4.2*	128.1 ± 4.2*	137.3 ± 2.6	138 ± 2.6	158.3 ± 2.9

Values are expressed mean ± SE.
* The significant differences are expressed with respective initial values when compared with their respective final values at $p < 0.05$.

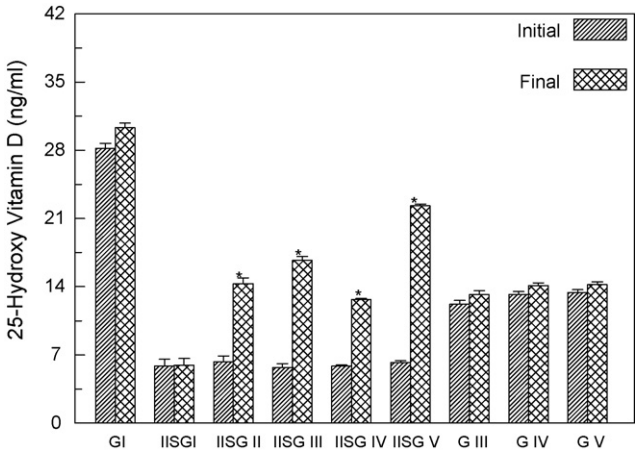

Fig. 3. Effects of dietary supplementation of CPIF on 25-hydroxy vitamin D levels.

OSP. Upon screening the different pulses and legumes, Cowpea was found to have rich IF content. In rats, these IF are highly bioavailable in nature, they not only well absorbed from the intestine, but also extracted efficiently from the portal blood into the liver and ultimately excreted into bile [25,26]. Among all the IF, Dz was considered to be more bioavailable than Ge in rats and also in humans [27–31]. In the present study, the OSP was generated by alterations in the composition of the diet in the levels of Ca and VD. The results obtained are on par with the earlier reports in improving the BMD, BMC of the whole body, Ca, P and ALP levels, in addition, 25-VD also found to play a role in partially in reversing the clinical manifestation of the osteoporotic situation in the rats.

Further, the findings indicates that, the CPIF diet alone was not sufficient to reverse the loss in whole-body BMD due to the diet induced OSP but can provide additive effects in terms of bone density. BMD was maintained normally in the control diet with normal Ca and VD, however, the low BMD levels were significantly improved in the SG II, III and V of G II, where, after the development of OSP the CPIF were supplemented. The similar kind of situation was observed for BMC also. The results obtained were in contrast to the observations reported previously was specifically in cortical bone [32,33], where, Dz and Ge improved BMD at both the cancellous and cortical sites by suppressing the bone turnover increase [34]. In a report, orally administered genistein induced a bone tissue retention at a low dose whereas a slight improvement in the effects at a higher dose [35]. CPIF might suppressed the osteoclastic activity through tyrosine-kinase inhibition [36] and the bone loss preventive effects of IF resulted from suppression in bone turnover increase and could be due to a mechanism similar to that of estrogen [37,38]. Exhibition of different effects of CPIF on the improvement of both the BMD and BMC at different levels when compared with the other groups needs to be investigated.

In the present report, the rats receiving the very low levels of Ca and VD failed to gain weight and also were found to be severely hypocalcemic. The serum P levels were elevated initially in the all the SGs of G II animals and tetany or paralysis however, was not observed in the low Ca diet fed rats. In these G II animals, where the low Ca and along with the low levels of VD might have helped in the maintenance of the serum Ca levels by increasing the intestinal Ca absorption and conserving it by decreasing the urinary calcium excretion and resorption of the bone. The significant reversal in the Ca levels upon the CPIF supplementation in the SG II, III and IV of G II animals reflects the close association of the IF mechanism of bone calcification. The enhanced intestinal absorption of Ca along with modulation of parathyroid hormone (PTH) and renal function may provide a partial explanation for the beneficial effects of CPIF on bone health, as has been suggested by earlier reports [39]. In addition, these IF diet was linked to increased urinary Ca excretion [40], might be due to the oxidation of sulfur-containing amino acids. In fact, improvement in Ca signalling pathways might also in part mediated by the IF inhibitory effects on osteoclasts, as inhibitors of the calcium-dependent signaling molecules, calmodulin and protein kinase C, which antagonizes the reduction in osteoclast number induced by these compounds [41,42]. Increases in intracellular Ca levels induction could be mediated by direct inhibition K^+ channels independent of IF activity on tyrosine kinases [43].

Bone-specific ALP is produced by osteoblasts and is essential for proper mineralization of the bone. Bone mineralization is the result of osteoblasts forming matrix vesicles. ALP in the vesicle membrane transports phosphate into the vesicle. Ca diffuses passively through the membrane, and, together with phosphate, forms the crystallization product. When enough mineral has been crystallized, the vesicle ruptures and ALP is released. Therefore serum ALP could be regarded as an indicator of the active crystallization process and reflects bone turnover. In the present investigation, increased serum ALP levels were observed initially in all the SGs of G II animals which were significantly reduced upon the supplementation of CPIF and could by preventing the leakage of the enzyme from bone.

The 25-VD levels were found to be altered in a significant way in the SG II, III and V of G II indicating the fact that CPIF has a role in the Ca, P and VD metabolism which has mutual interlinking of different pathways of bone mineralization. In fact, 25-VD plays a important role in the development, growth and mineralization of the overall morphology of the bone. VD insufficiency or VD deficiency has a more subtle effect on the skeleton. As the body becomes VD insufficient, the efficiency of intestinal Ca absorption decreases

and this results in a decrease in the ionized Ca concentration in the blood, which signals the calcium sensor in the parathyroid glands resulting in an increase in the synthesis and secretion of PTH. The mechanism underlying the improvement in the levels of VD especially in the osteoportic rats by CPIF needs be further investigated.

In conclusion CPIF had a convincing effect in protecting the bone mineralization in the critical situations like OSP in terms of improving the BMD, BMC, Ca, ALP and 25-VD levels, it is inconclusive whether these IF had a therapeutic effect and needs to be further investigated

Conflict of interest

The authors declared that they have no conflict of interest.

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