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Effect of calcium phosphate and vitamin D₃ supplementation on bone remodelling and metabolism of calcium, phosphorus, magnesium and iron

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Abstract

Background: The aim of the present study was to determine the effect of calcium phosphate and/or vitamin D₃ on bone and mineral metabolism.

Methods: Sixty omnivorous healthy subjects participated in the double-blind, placebo-controlled parallel designed study. Supplements were tricalcium phosphate (CaP) and cholecalciferol (vitamin D₃). At the beginning of the study (baseline), all subjects documented their normal nutritional habits in a dietary record for three successive days. After baseline, subjects were allocated to three intervention groups: CaP (additional 1 g calcium/d), vitamin D₃ (additional 10 µg/d) and CaP + vitamin D₃. In the first two weeks, all groups consumed placebo bread, and afterwards, for eight weeks, the test bread according to the intervention group. In the last week of each study period (baseline, placebo, after four and eight weeks of intervention), a faecal (three days) and a urine (24 h) collection and a fasting blood sampling took place. Calcium, phosphorus, magnesium and iron were determined in faeces, urine and blood. Bone formation and resorption markers were analysed in blood and urine.

Results: After four and eight weeks, CaP and CaP + vitamin D₃ supplementations increased faecal excretion of calcium and phosphorus significantly compared to placebo. Due to the vitamin D₃ supplementations (vitamin D₃, CaP + vitamin D₃), the plasma 25-(OH)D concentration significantly increased after eight weeks compared to placebo. The additional application of CaP led to a significant increase of the 25-(OH)D concentration already after four weeks. Bone resorption and bone formation markers were not influenced by any intervention.

Conclusions: Supplementation with daily 10 µg vitamin D₃ significantly increases plasma 25-(OH)D concentration. The combination with daily 1 g calcium (as CaP) has a further increasing effect on the 25-(OH)D concentration. Both CaP alone and in combination with vitamin D₃ have no beneficial effect on bone remodelling markers and on the metabolism of calcium, phosphorus, magnesium and iron.

Trial registration: NCT01297023

Keywords: Vitamin D, Calcium, Phosphorus, Magnesium, Iron, Human study, Bone remodelling

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Background

Vitamin D is of large importance for homeostasis of calcium and bone metabolism. For humans, there are two ways for an adequate supply with vitamin D: Firstly, via cutaneous synthesis under UVB exposure, and secondly, via intake with the diet [1]. International scientific literature indicated a worldwide problem of vitamin D deficiency [2]. According to Hintzpeter *et al.*, 57% of men and 58% of women in Germany had 25-hydroxyvitamin D (25-(OH)D) concentrations below the recommended concentration of 50 nmol/l [3]. Furthermore, the optimal concentration is defined as a 25-(OH)D > 75 nmol/l [4]. In the first place, vitamin D is responsible for maintaining the extracellular calcium concentrations by controlling absorption of calcium and by direct effects on bone and on parathormone (PTH) secretion [2]. In recent years, vitamin D gained increasing attention because of its association with the risk of overall mortality, diabetes, cancer, musculoskeletal disorders, mental and physical performance, hypertension and cardiovascular diseases [5,6].

The beneficial effects of calcium phosphate mainly focus on the intestinal metabolism, e.g., bile acid metabolism, fatty acid excretion, and modulation of the gut microbiota [7-10]. Calcium from tricalcium phosphate (CaP, a water-insoluble compound at neutral pH value), is partly absorbed in the human gut; but the main part of the calcium and phosphorus is precipitated to amorphous calcium phosphate in the gut, and thus, not absorbed [11]. Nevertheless, CaP showed in combination with vitamin D₃ beneficial modulation on bone metabolism in elderly women [12]. The aim of the present study was to determine the impact of daily 1 g calcium (as CaP) with or without 10 µg vitamin D₃ on bone formation and resorption markers as well as on calcium, phosphorus, magnesium and iron metabolism in healthy middle-aged men and women.

Methods

Supplements

Two supplements were used in this study: CaP (Ca₅(PO₄)₃OH; cfb, Budenheim, Germany) and cholecalciferol (vitamin D₃; Brenntag, Mülheim an der Ruhr, Germany). In order to achieve a calcium supplementation of additional 1 g/d and a vitamin D₃ supplementation of 10 µg/d, CaP and/or vitamin D₃ were incorporated in wholewheat bread. Participants consumed approximately 135 g of this bread daily. Placebo bread was prepared in the same manner as the test bread, but without CaP and vitamin D₃. Taste and visual properties of placebo and test breads were comparable.

Subjects and study design

The study was conducted at the Friedrich Schiller University Jena, Department of Nutritional Physiology, between January and April 2011.

Sixty omnivorous healthy subjects (men, n = 24; women n = 36) participated in this double-blind, placebo-controlled parallel designed study. Eligibility criteria for participants were age between 20 and 70 years and physical health. All volunteers were informed of the purpose, the course and the possible risks of the study and gave their written consent. The study protocol was approved by the Ethical Committee of the Friedrich Schiller University Jena (No.: 2959-11/10). Four participants dropped out because of pregnancy, illness and personal reasons (Figure 1). The remaining 56 volunteers aged 42 ± 12 y had a BMI of 25 ± 4 kg/m².

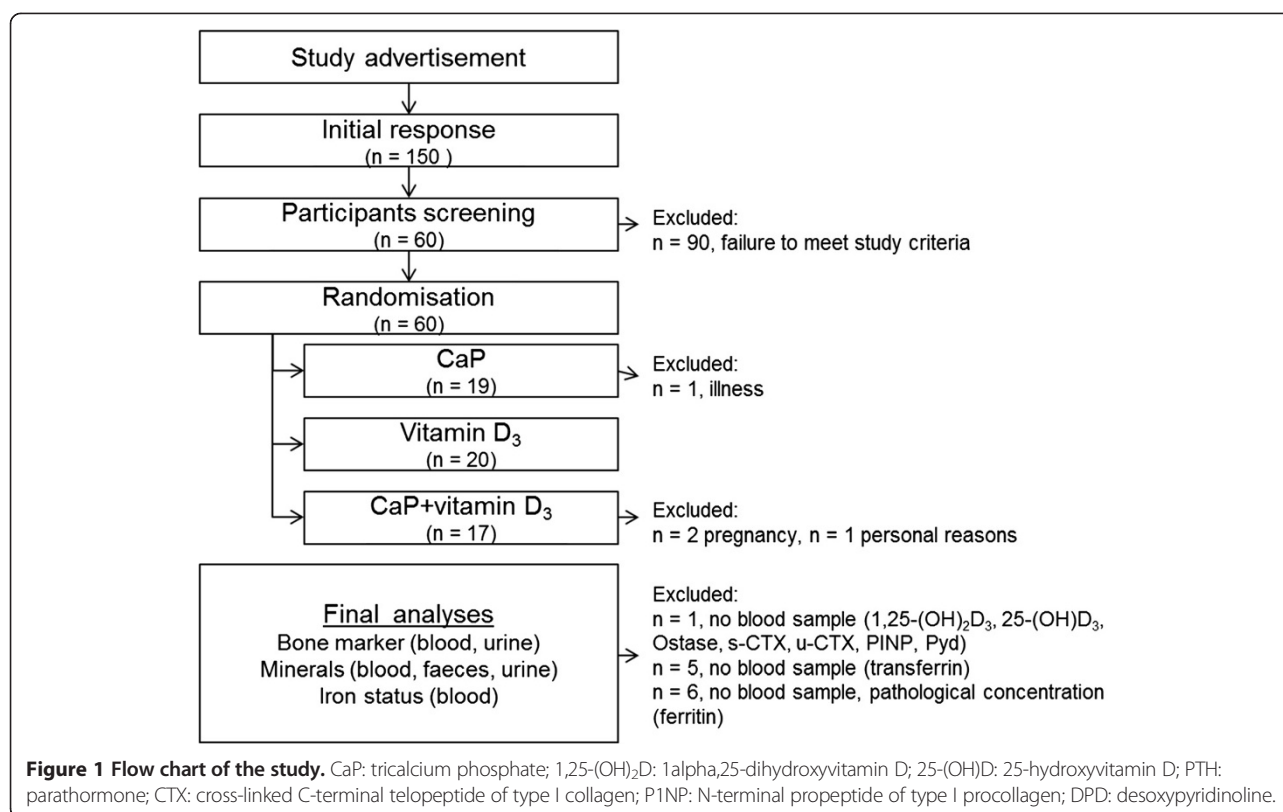
At the beginning of the study (baseline), all subjects documented their normal nutritional habits in a dietary record for three successive days. The participants were encouraged to weigh all eaten foods with proved scales. One day later, the subjects collected quantitatively their faeces for three days. From day two to day three of the faeces collection, the subjects collected their urine for 24 h, too. At the last day of the faeces collection, a fasting blood sampling took place. This procedure aimed to familiarise the subjects to the sample collection and to establish a baseline profile of each subject. Afterwards, subjects were allocated to the three intervention groups, so that there were no significant changes in age, BMI and 25-(OH) D concentration between the groups.

After allocation, subjects consumed for two weeks the placebo bread. Then they consumed for eight weeks either the CaP, vitamin D₃ or the CaP + vitamin D₃ bread. In the last week of the placebo and after four and eight weeks of intervention, subjects consumed a defined diet for three days. This diet contained all the foods required per subject over the three days and was prepared and pre-weighed in the study centre. The subjects were instructed to consume no other foods than provided. Food intake was calculated by weighing food residues. Samples from each food component were frozen and stored at -20°C until analysis. Furthermore, subjects collected faeces quantitatively for three days, beginning at the second day of the defined diet. From day two to three of the defined diet, the subjects collected their urine for 24 h. On the third day of the defined diet, a fasting venous blood sample was taken.

Sample preparation

Blood samples were drawn by venipuncture and collected in lithium heparin and serum tubes. Lithium heparin tubes were centrifuged (2500 × g, 15 min, 20°C) and the plasma supernatants were stored at -20°C until analysis. Serum tubes were centrifuged (2500 × g, 10 min, 20°C) and the serum was stored at -20°C until analysis.

The faecal samples were transported to the study centre without delay. Each specimen was weighed, frozen and stored at -20°C. At the end of the study, faeces samples were homogenised, portioned, and the pH-value was



determined. The complete 24 h urine was transported to the study centre at the day of the blood sampling. The urine volume from every participant was measured, and aliquots were frozen at -20°C until analysis.

Food analysis

The intake of calcium, phosphorus, magnesium, and iron was analysed via ICP-OES as described previously [10]. The intake of energy, fat, proteins, carbohydrates and vitamin D was verified using the Prodi[®] 5.4 software (Nutri-Science GmbH, Freiburg, Germany). Retained samples of placebo and test breads were homogenised, freeze-dried and analysed for vitamin D₃ concentration by certified methods (GC-MS) according to the Food GmbH Jena, Analytic and Consulting.

Blood analysis

Concentrations of calcium, phosphate, magnesium, iron, ferritin and transferrin in plasma and osteocalcin, alkaline phosphatase, 1α,25-dihydroxyvitamin D (1,25-(OH)₂D), 25-(OH)D, PTH and calcitonin in serum were ascertained according to certified methods of the Institute of Clinical Chemistry and Laboratory Medicine, Jena University Hospital.

Serum cross-linked C-terminal telopeptide of type I collagen (CTX) and N-terminal propeptide of type I procollagen (P1NP) were determined using methods according to the

Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostic, University Leipzig.

Faeces and urine analyses

Mineral concentrations in faeces were analysed via ICP-OES as described previously [10].

The concentrations of calcium, phosphorus, magnesium and iron in urine were measured according to certified methods of the Institute of Clinical Chemistry and Laboratory Medicine, Jena University Hospital.

The concentrations of CTX and desoxypyridinoline (DPD) were determined using methods according to the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostic, University Leipzig.

Statistics

Samples from each participant were coded to protect volunteer identity and to mask treatment groups during the analysis. All values in the text and tables are means with standard deviations. Data analysis was performed using the statistical software package IBM SPSS Statistics 19 (SPSS Inc. IBM Company, Chicago, USA). Variance homogeneity was tested using the Levene test. In case of variance heterogeneity data were \log_{10} transformed (signed with ^t in text, tables and figures). The effect of time in each intervention group was tested using general linear model with repeated measurements (with pairwise

comparisons based on Fishers-LSD test). The effect of supplementation between groups was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test. Differences were considered significant at $p \leq 0.05$.

Results

The baseline characteristics of participants of the three intervention groups are presented in Table 1. Due to randomisation, there were no significant differences in age, BMI and 25-(OH)D between intervention groups.

Nutrient intake

There were no differences in the intake of energy, fat, protein, and carbohydrates between study periods (placebo, four and eight weeks) and between intervention groups. The mean intakes of energy, fat, protein, and carbohydrates were 8.7 ± 1.8 MJ/d, 78.6 ± 22.5 g/d, 73.6 ± 12.5 g/d, and 260.2 ± 47.4 g/d, respectively. Due to the CaP and vitamin D₃ supplementations, the intake of calcium, phosphorus and vitamin D₃ increased significantly (Table 2). The intake of magnesium and iron did not change between the three study periods and between the interventions. The mean magnesium and iron intakes were 277.8 ± 42.2 mg/d and 7.8 ± 1.1 mg/d, respectively.

Minerals

The plasma calcium concentration decreased significantly in all intervention groups after four and eight weeks of intervention compared to placebo (Table 3; $p \leq 0.05$). In all interventions, no effect of time on the renal calcium excretion was observed. After four weeks of intervention, the CaP group had a significantly higher renal calcium excretion compared to vitamin D₃ ($p \leq 0.05$). Due to CaP supplementations (CaP, CaP + vitamin D₃), faecal excretion of calcium increased significantly after four and eight weeks compared to placebo. The faecal calcium excretion was significantly lower in the vitamin D₃ group compared to the CaP and CaP + vitamin D₃ groups at four and eight weeks of intervention ($p \leq 0.05$).

There were no changes in plasma phosphate concentration in all intervention groups. After vitamin D₃ supplementation, the renal phosphorus excretion significantly increased after eight weeks compared to placebo

($p \leq 0.05$). Due to CaP supplementations (CaP and CaP + vitamin D₃), the faecal excretion of phosphorus increased significantly after four and eight weeks compared to placebo. The faecal phosphorus excretion was significantly lower in the vitamin D₃ group compared to the CaP and the CaP + vitamin D₃ groups after eight weeks of intervention ($p \leq 0.05$).

Both the plasma magnesium concentration and the faecal magnesium excretion did not change by any intervention. After vitamin D₃ supplementation, the renal magnesium excretion significantly increased after eight weeks compared to four weeks and to placebo ($p \leq 0.05$).

Plasma iron, ferritin, transferrin and transferrin saturation did not change due to any intervention (Table 4). After placebo and four weeks of intervention, the CaP + vitamin D₃ group had significantly higher ferritin concentrations compared to the CaP group ($p \leq 0.05$). The renal iron concentration was in most cases (67%) below the limit of detection ($0.75 \mu\text{mol/l}$) and was not subjected to statistical analysis.

Bone metabolism markers

Due to the vitamin D₃ supplementation, the 25-(OH)D concentration in plasma significantly increased in the vitamin D₃ group after eight weeks compared to placebo (Figure 2; $p \leq 0.05$). The combined supplementation of CaP + vitamin D₃ led to a significant increase after four and eight weeks of intervention compared to placebo ($p \leq 0.05$). After CaP intervention, the plasma concentration of 25-(OH)D significantly decreased after four weeks compared to placebo ($p \leq 0.05$). After four and eight weeks of intervention, the 25-(OH)D concentration was significantly higher in the vitamin D₃ and CaP + vitamin D₃ groups compared to the CaP group ($p \leq 0.05$).

After four and eight weeks of CaP supplementation, 1,25-(OH)₂D concentration in plasma decreased significantly compared to placebo (Table 5; $p \leq 0.05$). After four weeks of CaP + vitamin D₃ intervention, 1,25-(OH)₂D concentration was significantly higher compared to placebo and eight weeks of CaP + vitamin D₃ intervention ($p \leq 0.05$). There were no changes in plasma PTH and calcitonin concentrations.

Bone resorption markers, serum and urinary CTX and urinary DPD did not change due to the three interventions (Table 5). The bone formation markers plasma osteocalcin and serum P1NP did not change after any intervention. All supplementations led to a significant increase of plasma alkaline phosphatase concentration after four weeks compared to placebo and to eight weeks (Table 5, $p \leq 0.05$).

Discussion

The additional intake of 1 g calcium (via CaP) per day was used in other studies due to beneficial effects on intestinal

Table 1 Baseline characteristics of participants who completed the study

	CaP	Vitamin D ₃	CaP+vitamin D ₃
N	19	20	17
Age [y]	42 (12)	45 (13)	41 (12)
BMI [kg/m ²]	24 (3)	25 (5)	25 (4)
25-(OH)D [nmol/l]	59 (30)	46 (20)	50 (16)

n = 56; data are expressed as mean (standard deviation); CaP: tricalcium phosphate; 25-(OH)D: 25-hydroxyvitamin D.

Table 2 Mean intake of calcium, phosphorus, and vitamin D during defined diet in all study periods

Parameter	Supplements	Placebo	Interventions	
			4 weeks	8 weeks
Calcium [mg/d]	CaP	938 ^b (197)	1998 ^a (192) ¹	2014 ^a (194) ¹
	Vitamin D ₃	905 (187)	897 (219) ²	916 (203) ²
	CaP+vitamin D ₃	889 ^b (154)	1913 ^a (151) ¹	1872 ^a (164) ¹
Phosphorous [mg/d]	CaP	1337 ^b (250)	1803 ^a (247) ¹	1833 ^a (242) ¹
	Vitamin D ₃	1311 (236)	1303 (247) ²	1324 (226) ²
	CaP+vitamin D ₃	1286 ^b (172)	1733 ^a (180) ¹	1705 ^a (187) ²
Vitamin D ₃ [µg/d]	CaP	6.5 (0.6)	6.6 (0.6) ¹	6.6 (0.5) ¹
	Vitamin D ₃	6.2 ^b (1.3)	15.2 ^a (1.0) ²	15.2 ^a (1.0) ²
	CaP+vitamin D ₃	6.5 ^b (0.4)	15.1 ^a (0.4) ²	15.1 ^a (0.3) ²

n = 56; data are expressed as mean (standard deviation); CaP: tricalcium phosphate; ^{a, b} mean values within a row with dissimilar superscript letters are significantly different (p ≤ 0.05); ^{1, 2} mean values within a column with dissimilar superscript numbers are significantly different (p ≤ 0.05); results without superscripts in a row/column have no significant differences; effect of time was tested using general linear model with repeated measurements (with pairwise comparisons based on Fishers-LSD test); effect of supplementation was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test; [†] for statistical analysis the data were log₁₀ transformed, because of variance heterogeneity.

metabolism [10,13]. The additional daily intake of 10 µg vitamin D₃ was chosen, because 5 µg/d was the previously recommended vitamin D intake in Germany [14]. In 2012, the German Nutrition Society set up this value to 20 µg/d for adolescents and adults under 65 years [15], because the UV index in Germany is lower than 3 for about six months of the year [16]. A rule of thumb says, that indexes lower than 3 are insufficient to produce vitamin D in the skin [16]. In the present study, the total intake of vitamin D (supplement, defined diet and bread) was approximately 15 µg/d in the vitamin D₃-supplemented groups, and therefore below the actual recommendation of the German Nutrition Society [15].

However, after vitamin D₃ supplementation, the 25-(OH)D concentration significantly increased after eight weeks (Figure 2). The mean increases in the vitamin D₃ and in the CaP + vitamin D₃ groups were 22.5 nmol/l and 19.8 nmol/l, respectively (Figure 2). The present results indicate, that the supplementation with 10 µg/d was enough to increase 25-(OH)D significantly. Only 5% of participants treated with vitamin D₃ had 25-(OH)D concentrations below 50 nmol/l after eight weeks of intervention. Furthermore, the mean increase is in accordance with other vitamin D₃ supplementation studies [17,18].

Interestingly, in the CaP + vitamin D₃ group the increase in 25-(OH)D was significant after four and eight weeks, but in the vitamin D₃ group only after eight weeks (Figure 2). It seems that the combination with CaP leads to a faster increase in 25-(OH)D concentration. A low calcium intake could lead to a higher turnover of vitamin D metabolites (higher production of 1,25-(OH)₂D, due to PTH increase), while a high calcium intake could be 25-(OH)D sparing [19]. These findings were based on rat experiments, in which the half-life of 25-(OH)D was longer when calcium intake was high. Furthermore, in a rat experiment

conducted by Anderson *et al.*, animals fed high calcium combined with vitamin D₃ had significantly higher serum 25-(OH)D concentrations compared to animals fed low calcium combined with vitamin D₃ [20]. In the present study, plasma calcium and PTH were not significantly affected by the interventions. Only the 1,25-(OH)₂D concentration decreased compared to placebo after eight weeks of CaP (CaP alone and CaP + vitamin D₃) supplementations. From the increase of 25-(OH)D concentration after four weeks of CaP + vitamin D₃ supplementation it can be concluded, that the combination of vitamin D₃ and calcium is more effective than vitamin D₃ alone.

In the present study, we determined different biomarkers of bone metabolism, like urinary CTX, serum CTX and urinary DPD for bone resorption and plasma alkaline phosphatase, serum P1NP and plasma osteocalcin for bone formation. None of the bone metabolism markers changed related to the CaP, vitamin D₃ or CaP + vitamin D₃ supplementations. Literature indicates that a modulation of bone metabolism through calcium and/or vitamin D supplementations occurred especially in older subjects with a vitamin D deficiency [12,21]. In studies involving subjects with no or only marginal vitamin D deficiency, no beneficial effects on bone metabolism were observed [22,23].

Due to CaP supplementation, the faecal excretion of calcium and phosphorus significantly increased. This is based on the formation of amorphous calcium phosphate, a well-known process in the intestine [8,24]. Thus, only a minor part of the calcium can be absorbed [11]. The unchanged excretion of calcium and phosphorus in urine and the comparable plasma concentration confirm this suggestion. Vitamin D is known to increase the absorption of calcium and phosphate in the human gut, in order to maintain the calcium homeostasis. After supplementation

Table 3 Concentrations of plasma and excretion of renal and faecal minerals after intervention with either tricalcium phosphate, vitamin D₃ or both

Parameter	Supplements	Placebo	Interventions	
			4 weeks	8 weeks
Plasma calcium [mmol/l]	CaP	2.4 ^a (0.1)	2.3 ^b (0.1)	2.3 ^b (0.1)
	Vitamin D ₃	2.4 ^a (0.1)	2.3 ^b (0.1)	2.3 ^b (0.1)
	CaP+vitamin D ₃	2.4 ^a (0.1)	2.3 ^b (0.1)	2.4 ^b (0.1)
Renal calcium excretion [mg/d]	CaP	158 (84)	187 (98) ¹	171 (79)
	Vitamin D ₃	133 (53)	141 (64) ²	152 (58)
	CaP+vitamin D ₃	117 (60)	143 (63) ¹²	130 (57)
Faecal calcium excretion [mg/d]	CaP	763 ^a (434)	1214 ^b (386) ¹	1324 ^b (465) ¹
	Vitamin D ₃	810 (328)	723 (366) ²	708 (423) ²
	CaP+vitamin D ₃	657 ^a (318)	1149 ^b (426) ¹	1254 ^b (449) ¹
Plasma phosphate concentration [mmol/l]	CaP	1.1 (0.1)	1.2 (0.2)	1.1 (0.1)
	Vitamin D ₃	1.1 (0.1)	1.1 (0.2)	1.1 (0.1)
	CaP+vitamin D ₃	1.2 (0.1)	1.2 (0.1)	1.1 (0.2)
Renal phosphorus excretion [mg/d]	CaP	917 (354)	881 (289)	816 (289)
	Vitamin D ₃	857 ^a (236)	841 ^{ab} (276)	963 ^b (300)
	CaP+vitamin D ₃	807 (276)	818 (290)	894 (288)
Faecal phosphorus excretion [mg/d]	CaP	554 ^a (382)	716 ^b (331)	754 ^b (331) ¹
	Vitamin D ₃	584 (218)	544 (309)	450 (275) ²
	CaP+vitamin D ₃	496 ^a (229)	696 ^b (270)	740 ^b (260) ¹
Plasma magnesium concentration [mmol/l]	CaP	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)
	Vitamin D ₃	1.0 (0.1)	0.9 (0.0)	0.9 (0.0)
	CaP+vitamin D ₃	0.9 (0.1)	0.9 (0.1)	0.9 (0.0)
Renal magnesium excretion [mg/d]	CaP	93 (41)	90 (45)	86 (31)
	Vitamin D ₃	79 ^a (23)	84 ^a (30)	98 ^b (31)
	CaP+vitamin D ₃	75 (31)	76 (25)	73 (26)
Faecal magnesium excretion [mg/d]	CaP	207 (136)	191 (103)	208 (119)
	Vitamin D ₃	234 (85)	242 (135)	205 (97)
	CaP+vitamin D ₃	191 (74)	178 (56)	180 (54)

n = 56; data are expressed as mean (standard deviation); CaP: tricalcium phosphate; ^a ^b mean values within a row with dissimilar superscript letters are significantly different (p ≤ 0.05); ¹ ² mean values within a column with dissimilar superscript numbers are significantly different (p ≤ 0.05); results without superscripts in a row/column have no significant differences; effect of time was tested using general linear model with repeated measurements (with pairwise comparisons based on Fishers-LSD test); effect of supplementation was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test.

of vitamin D₃ alone, only the renal excretion of phosphorus significantly increased after eight weeks compared to placebo. In phosphorus equilibrium, the amounts of intestinally absorbed phosphorus is equal to the excreted phosphorus by the kidney [25]. Thus, the increased excretion indicates higher phosphorus absorption due to vitamin D₃ supplementation.

In a rat experiment, it has been shown that magnesium is also able to form an insoluble complex with calcium and phosphate [26]. When this formation occurs in the human gut, the absorption of magnesium would be inhibited. After supplementation with high amounts of phosphorus, Greger *et al.* showed a significant increase

in faecal excretion of magnesium, but simultaneously a decrease of magnesium in urinary excretion [27]. The authors concluded that magnesium metabolism was not negatively influenced by high phosphorus doses [27]. In the present study, CaP supplementation had no harmful effects on magnesium metabolism. After eight weeks of vitamin D₃ supplementation, the renal excretion of magnesium increased compared to placebo. Hardwick *et al.* reviewed the literature concerning magnesium absorption and suggested that a significant amount of magnesium absorption is vitamin D-dependent [28].

Literature indicates, that calcium supplementation can decrease iron absorption, both from haem and non-haem

Table 4 Parameter of iron status after intervention with either tricalcium phosphate, vitamin D₃ or both

Parameter	Supplements	Placebo	Interventions	
			4 weeks	8 weeks
Plasma iron concentration [$\mu\text{mol/l}$]	CaP	18.8 (5.4)	18.3 (7.3)	19.5 (6.5)
	Vitamin D ₃	22.3 (5.2)	20.8 (6.8)	20.5 (5.1)
	CaP+vitamin D ₃	18.8 (6.7)	21.5 (5.2)	20.5 (5.5)
Plasma transferrin concentration [g/l]	CaP	2.5 (0.4)	2.4 (0.5)	2.5 (0.4)
	Vitamin D ₃	2.7 (0.5)	2.6 (0.5)	2.6 (0.6)
	CaP+vitamin D ₃	2.7 (0.4)	2.7 (0.4)	2.8 (0.6)
Transferrin saturation [%]	CaP	31.1 (10.1)	32.3 (16.8)	32.9 (10.9)
	Vitamin D ₃	35.0 (11.2)	24.1 (14.4)	33.6 (9.2)
	CaP+vitamin D ₃	29.1 (13.2)	32.0 (8.7)	31.3 (12.3)
Plasma ferritin [$\mu\text{g/l}$]	CaP	54.1 (28.4) ¹	47.5 (21.7) ¹	45.4 (22.6)
	Vitamin D ₃	88.3 (65.1) ¹²	81.0 (60.1) ¹²	72.6 (43.4)
	CaP+vitamin D ₃	134.6 (138.5) ²	139.3 (151.3) ²	105.0 (99.6)
Faecal iron excretion [mg/d]	CaP	8.7 (6.4)	7.7 (4.5)	7.7 (4.0)
	Vitamin D ₃	9.1 (4.0)	9.5 (5.0)	8.3 (3.7)
	CaP+vitamin D ₃	7.4 (2.9)	6.7 (2.4)	7.3 (2.9)

n = 51, except plasma transferrin n = 50; data are expressed as mean (standard deviation); CaP: tricalcium phosphate; ^{1 2} mean values within a column with dissimilar superscript numbers are significantly different ($p \leq 0.05$); results without superscripts in a row/column have no significant differences; effect of time was tested using general linear model with repeated measurements (with pairwise comparisons based on Fishers-LSD test); effect of supplementation was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test.

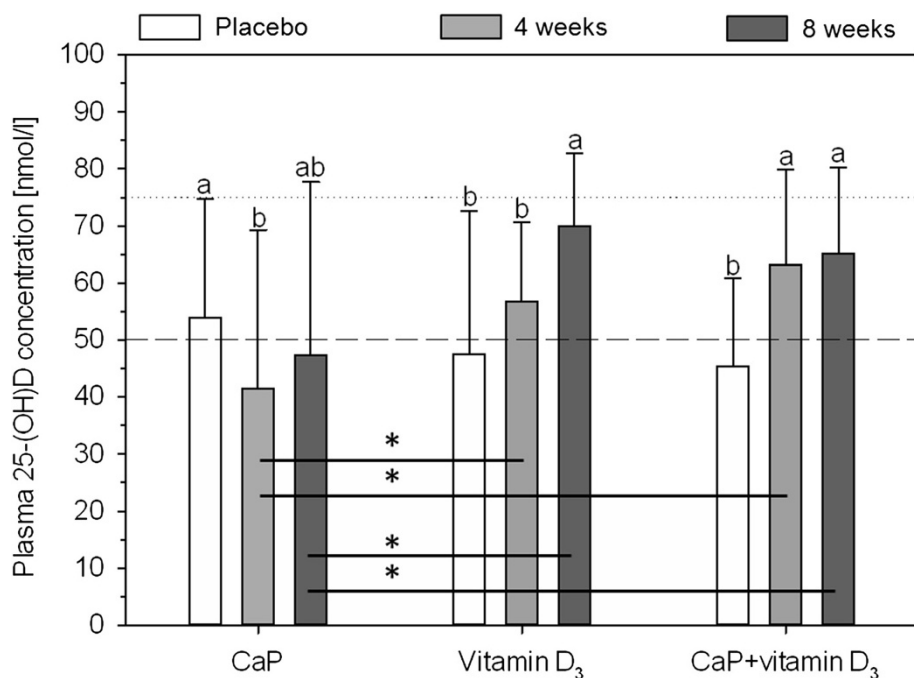


Figure 2 Concentration of plasma 25-(OH)D after intervention with either tricalcium phosphate, vitamin D₃ or both. n = 55; CaP: tricalcium phosphate; 25-(OH)D: 25-hydroxyvitamin D; broken line: recommended 25-(OH)D concentration; dotted line: optimal 25-(OH)D concentration; ^{a b} data are expressed as mean and standard deviation; mean values within a row with dissimilar superscript letters are significantly different ($p \leq 0.05$); * significant difference between groups; effect of time was tested using general linear model with repeated measurements (with pairwise comparisons based on Fishers-LSD test); effect of supplementation was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test.

Table 5 Bone metabolism markers and bone-related hormones in blood and urine after intervention with either tricalcium phosphate, vitamin D₃ or both

Parameter	Supplements	Placebo	Interventions	
			4 weeks	8 weeks
Plasma 1,25-(OH) ₂ D [pmol/l]	CaP ^t	102.9 ^a (24.7)	93.3 ^b (24.7)	76.3 ^c (24.5)
	Vitamin D ₃	97.9 (27.5)	108.3 (23.9)	99.2 (38.1)
	CaP+vitamin D ₃	92.2 ^b (31.0)	100.4 ^a (31.1)	86.0 ^b (23.1)
Plasma osteocalcin [ng/ml]	CaP	20.3 (6.5)	20.4 (6.5)	20.9 (7.2)
	Vitamin D ₃	21.8 (7.2)	23.4 (8.2)	20.7 (8.8)
	CaP+vitamin D ₃	20.1 (7.0)	20.2 (6.4)	19.9 (7.6)
Plasma alkaline phosphatase	CaP	9.6 ^a (3.9)	10.7 ^b (3.9)	9.3 ^a (3.7)
	Vitamin D ₃	9.1 ^a (3.5)	10.3 ^b (3.6)	9.0 ^a (3.6)
	CaP+vitamin D ₃	9.8 ^a (3.4)	11.3 ^b (3.9)	9.5 ^a (3.1)
Serum PTH [µg/l]	CaP	56.6 (12.2)	74.4 (35.8)	71.9 (25.1)
	Vitamin D ₃	65.0 (24.7)	71.2 (29.2)	66.5 (21.0)
	CaP+vitamin D ₃	62.0 (20.1)	63.3 (17.8)	60.6 (19.8)
Serum CTX [ng/ml]	CaP	0.3 (0.1)	0.2 (0.1)	0.3 (0.2)
	Vitamin D ₃	0.3 (0.2)	0.3 (0.2)	0.3 (0.1)
	CaP+vitamin D ₃	0.3 (0.1)	0.2 (0.1)	0.2 (0.1)
Serum P1NP [ng/ml]	CaP	44.5 (19.1)	42.5 (16.5)	44.2 (19.7)
	Vitamin D ₃	48.8 (18.9)	44.4 (20.7)	50.9 (24.7)
	CaP+vitamin D ₃	41.4 (17.6)	36.3 (19.7)	40.4 (15.9)
Urine DPD [nmol/l]	CaP	29.2 (14.9)	31.6 (20.0)	27.0 (14.5)
	Vitamin D ₃	25.3 (13.7)	22.8 (11.5)	25.5 (9.8)
	CaP+vitamin D ₃	23.7 (9.0)	25.8 (16.0)	28.1 (15.2)
Urine CTX [mg/l]	CaP	1.0 (0.6)	1.1 (1.1)	0.7 (0.5)
	Vitamin D ₃	0.9 (0.7)	0.8 (0.6)	1.1 (0.6)
	CaP+vitamin D ₃	0.9 (0.7)	0.9 (0.7)	1.2 (1.1)

n = 55; data are expressed as mean (standard deviation); CaP: tricalcium phosphate; 1,25-(OH)₂D: 1alpha,25-dihydroxyvitamin D; 25-(OH)D: 25-hydroxyvitamin D; PTH: parathormone; CTX: cross-linked C-terminal telopeptide of type I collagen; P1NP: N-terminal propeptide of type I procollagen; DPD: desoxypyridinoline; ^{a b} mean values within a row with dissimilar superscript letters are significantly different (p ≤ 0.05); results without superscripts in a row/column have no significant differences; effect of time was tested using general linear model with repeated measurements (with pairwise comparisons based on Fishers-LSD test); effect of supplementation was tested using univariate analysis of variance followed by Student-Newman-Keuls *post hoc* test; ^t for statistical analysis the data were log₁₀ transformed, because of variance heterogeneity.

iron [29]. This decrease in iron absorption has been shown mostly in short-term studies [30-32]. In contrast, Minihane and Fairweather-Tait supplemented calcium carbonate in a short-term (1200 mg calcium carbonate for one day) and in a long-term study (1200 mg calcium carbonate over six months) and showed that the non-haem iron absorption decreased only in the short-term supplementation [33]. By supplementing 1200 mg calcium as calcium carbonate over six months, they found no changes in any of the haematologic indexes (i.e. haemoglobin, plasma ferritin). The mechanism proposed by the authors is an adaptive response in the intestinal mucosa cell: the lower supply of iron to plasma after calcium intake may modify the developing enterocytes in order to stimulate specific proteins [33]. Thus, the dietary iron is used more efficiently, when developing cells are mature [33]. The unchanged results

of faecal iron and of iron status on the present study indexes (transferrin, transferrin saturation, ferritin) confirm the results of Minihane and Fairweather-Tait, that long-term CaP supplementation does not negatively influence the parameters of iron metabolism. The significantly higher ferritin concentration in the CaP + vitamin D₃ is due to two participants with concentrations in the upper limits of normal. In all, the presented results underline that supplementation with CaP and/or vitamin D₃ did not negatively influence the metabolism of magnesium and iron.

Conclusion

The results of the present human study shows that supplementation with 10 µg vitamin D₃ significantly increases 25-(OH)D. Furthermore, a combination with 1 g calcium (as CaP) beneficially influences the increasing effect on

the 25-(OH)D concentration. Both CaP alone or in combination with vitamin D₃ has no beneficial or harmful effects on bone metabolism markers and on the metabolism of calcium, phosphorus, magnesium and iron in middle-aged healthy persons.

Abbreviations

CaP: Tricalcium phosphate; CTX: Cross-linked C-terminal telopeptide of type I collagen; DPD: Desoxypyridinoline; PTH: Parathormone; P1NP: N-terminal propeptide of type I procollagen; 1,25-(OH)₂D: 1alpha,25-dihydroxyvitamin D; 25-(OH)D: 25-hydroxyvitamin D.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UT conducted research, wrote the manuscript and performed statistical analysis; UT, NN, ML and JK were involved in analyses; UT and GJ designed research and had primary responsibility for final content. All authors read and approved the final manuscript.

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