

Synergistic Effect of Genistein and Casein Phosphopeptides on Bone Components in Young and Elderly Female Rats

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The effect of genistein and casein phosphopeptides (CPP) on bone components in the femoral tissue of young and elderly rats was investigated. Genistein has been shown to directly stimulate bone formation, and CPP has been reported to increase intestinal calcium absorption. Genistein (10 or 50 $\mu\text{g}/100\text{ g}$ body weight) or CPP (40 mg/100 g) was orally administered to young (5 weeks old) or elderly (50 weeks old) rats for 14 d. The administration of genistein (50 $\mu\text{g}/100\text{ g}$) resulted in a significant increase in the femoral dry weight, calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the femoral-diaphyseal and metaphyseal tissue of young and elderly rats. The administration of CPP (40 mg/100 g) caused a significant increase in the femoral dry weight of young and elderly rats. CPP administration increased significantly the diaphyseal calcium content in young rats, but it did not have an effect on the diaphyseal and metaphyseal alkaline phosphatase activity and DNA content. The genistein (50 $\mu\text{g}/100\text{ g}$)-increased femoral dry weight, calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissue of young rats was significantly enhanced by the simultaneous administration of CPP (40 mg/100 g). In elderly rats, this enhancement was resulted in the metaphyseal tissue. This study demonstrates that the combination of genistein and CPP administration has a synergistic-anabolic effect on bone components in rats with increasing age, suggesting that it may have a role in the prevention of osteoporosis.

Key words — isoflavone, genistein, casein phosphopeptides, bone metabolism, osteoporosis

INTRODUCTION

Bone mass decreases with increasing age and this decrease is due to increased bone resorption and reduced bone formation. The decrease in bone mass induces osteoporosis, which is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur.^{1,2)} Nutritional factors can help in preventing bone loss with increasing age but these factors are poorly understood.³⁾

Recent studies have shown that daidzein and genistein, natural isoflavonoid phytoestrogens found in *Leguminosae*, have an anabolic effect on bone metabolism in rats.⁴⁻⁹⁾ Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybean at a comparatively higher concentration.

Daidzin and genistin are hydrolyzed to daidzein and genistein by β -glucuronidase in gastric juice, respectively. Daidzein and genistein have been shown to stimulate osteoblastic bone formation,^{10,11)} and to inhibit osteoclastic bone resorption,¹²⁻¹⁵⁾ although genistein has more potent effect than daidzein. Isoflavone can prevent bone loss in ovariectomized (OVX) rats,^{16,17)} and it may be an important nutritional factor in preventing osteoporosis.

Casein phosphopeptides (CPP), which is a product of tryptic casein digestion, have been shown to enhance paracellular transport of calcium in the distal small intestine.¹⁸⁾ CPP has been reported to prevent the precipitation of insoluble calcium phosphate salts by forming soluble complexes with ionized calcium, *in vitro* and *in vivo*, in intestinal luminal contents, thereby promoting the passive absorption of calcium in the ileum.^{19,20)} CPP-containing diets have been shown to have a preventive effect on OVX-induced bone loss in rats.²¹⁾

Whether the combination of nutritional factors exhibits an additive or synergistic effect on bone

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components has not been fully clarified. This information may be important in preventing bone loss with increasing age. This study therefore was undertaken to determine the effect of the combination of genistein and CPP on bone components in young and elderly rats. We found that the anabolic effect of genistein on bone components is enhanced by the combination of CPP.

MATERIALS AND METHODS

Animals — Female Wistar rats (conventional) weighing 100–110 g (5 weeks old) or 210–250 g (50 weeks old) were obtained from Japan SLC (Hamamatsu). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and phosphorus at a room temperature of 25°C, with free access to distilled water.

Administration Procedures — Casein phosphopeptides-III (CPP) (Meiji Seika Kaisha, Ltd., Tokyo) was dissolved in distilled water to give a concentration of 80 mg per milliliter (ml). Genistein (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in 10% ethanol solution to give a concentration of 20 or 100 microgram (μg) per ml. These solutions were orally given 0.5 ml per 100 g body weight of rats with a stomach tube. Young (5 weeks old) rats were administered either vehicle, CPP (40 mg/100 g body weight), genistein (10 or 50 $\mu\text{g}/100$ g) or CPP (40 mg/100 g) plus genistein (10 or 50 $\mu\text{g}/100$ g) for 14 d. Elderly (50 weeks old) rats were administered either vehicle, CPP (40 mg/100 g) plus genistein (50 $\mu\text{g}/100$ g) for 14 d. These animals were sacrificed 24 h after the last administration.

Analytical Procedures — Rats were sacrificed by cardiac puncture under light ether anesthesia, and blood samples and femur were removed immediately. Blood samples were centrifuged for 30 min after collection, and the serum was separated. The serum calcium concentration was determined by the method of Willis,²²⁾ serum inorganic phosphorus was measured by the method of Taussky and Shon.²³⁾

The femur was removed after bleeding and soaked in ice-cold 0.25 M sucrose solution. It was cleaned of soft tissue, and then dried for 16 h at 100°C to measure dry bone weight. After this measurement, the femurs were separated into diaphysis and metaphysis (not containing epiphyseal tissues) and weighed to determine the calcium content. The femoral–metaphyseal and diaphyseal tissues were

digested with nitric acid, and calcium was determined by atomic absorption spectrophotometry.⁴⁾

To assay bone alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a Potter–Elvehjem homogenizer, and disrupted for 60 s using an ultrasonic device. The supernatant centrifuged at $600 \times g$ for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.²⁴⁾ Enzyme activity was expressed as (μmol of *p*-nitrophenol liberated per minutes (min) per mg protein. Protein concentration was determined by the method of Lowry *et al.*²⁵⁾

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after the homogenization of the bone tissues.²⁶⁾ After alkali extraction, the samples were centrifuged at $1000 \times g$ for 5 min, and supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti²⁷⁾ and expressed as the amount of DNA (mg)/g wet weight of bone tissues.

Statistical Analysis — The significance of the difference between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of CPP and Genistein on Bone Components in Young Rats

Rats (5 weeks old) were orally administered either vehicle (distilled water), CPP (40 mg/100 g body weight), genistein (10 or 50 $\mu\text{g}/100$ g) or CPP (40 mg/100 g) plus genistein (10 or 50 $\mu\text{g}/100$ g) with a stomach tube for 14 d. Femoral dry weight was significantly increased by the administration of CPP, genistein (10 or 50 $\mu\text{g}/100$ g) or CPP plus genistein (10 or 50 $\mu\text{g}/100$ g) (Fig. 1). The genistein (50 $\mu\text{g}/100$ g)-increased femoral dry weight was additionally enhanced by the simultaneous administration of CPP.

The change in calcium content in the femoral–diaphyseal and metaphyseal tissue of young rats administered CPP and genistein is shown in Fig. 2. The administration of CPP (40 mg/100 g) caused a significant increase in calcium content in the dia-

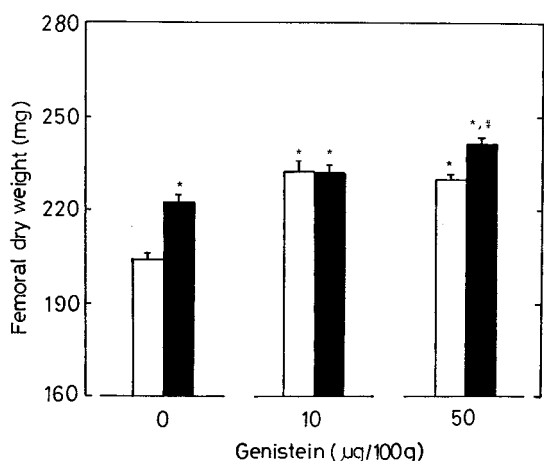


Fig. 1. Changes in the Femoral Dry Weight of Young Rats Administered CPP and Genistein

Rats were orally administered either vehicle (distilled water), CPP (40 mg/100 g body weight), genistein (10 or 50 µg/100 g) or CPP (40 mg/100 g) plus genistein (10 or 50 µg/100 g) with a stomach tube for 14 d. Animals were sacrificed 24 h after the last administration. Each value is the mean \pm S.E.M. of five animals. * $p < 0.01$, compared with the control value. [#] $p < 0.01$, compared with the value for CPP or genistein (50 µg/100 g) alone. □, Control; ■, CPP.

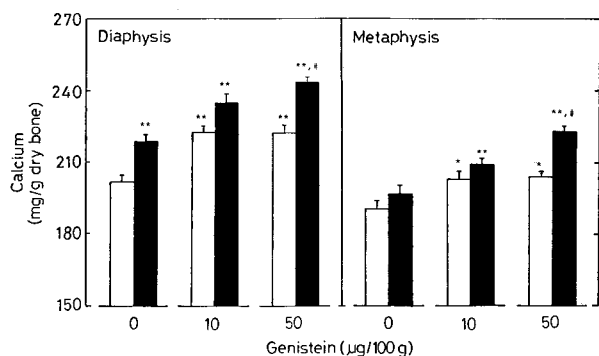


Fig. 2. Changes in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissue of Young Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 1. Each value is the mean \pm S.E.M. of five animals. * $p < 0.05$ and ** $p < 0.01$, compared with the control value. [#] $p < 0.01$, compared with the value for CPP or genistein (50 µg/100 g) alone. □, Control; ■, CPP.

physeal tissues, but this increase was not seen in the metaphyseal tissues. The administration of genistein (10 or 50 µg/100 g) resulted in a significant increase in calcium content in the diaphyseal and metaphyseal tissues. The genistein (50 µg/100 g)-increased calcium content in the diaphyseal and metaphyseal tissues was significantly enhanced by the simultaneous administration of CPP. This enhancement was synergistic.

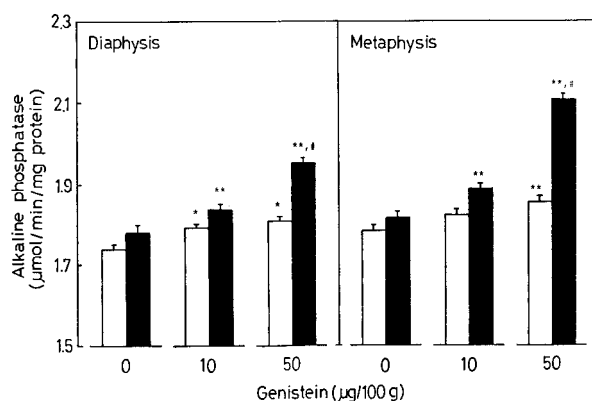


Fig. 3. Changes in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissue of Young Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 1. Each value is the mean \pm S.E.M. of five animals. * $p < 0.05$ and ** $p < 0.01$, compared with the control value. [#] $p < 0.01$, compared with the value for CPP or genistein (50 µg/100 g) alone. □, Control; ■, CPP.

The change in alkaline phosphatase activity in the femoral-diaphyseal and metaphyseal tissue of young rats administered CPP and genistein is shown in Fig. 3. CPP (40 mg/100 g) administration did not have an appreciable effect in the enzyme activity. Genistein (10 µg/100 g) increased significantly the enzyme activity in the diaphyseal tissues, although the effect was not seen in the metaphyseal tissues. However, the metaphyseal alkaline phosphatase activity was significantly increased by the simultaneous administration of CPP and genistein (10 µg/100 g). The enzyme activity in both tissues was significantly increased by the administration of genistein (50 µg/100 g). The simultaneous administration of CPP (40 mg/100 g) and genistein (50 µg/100 g) resulted in a synergistic effect on alkaline phosphatase activity in the diaphyseal and metaphyseal tissues.

The change in DNA content in the femoral-diaphyseal and metaphyseal tissue of young rats administered CPP and genistein is shown in Fig. 4. CPP (40 mg/100 g) did not have an effect on the femoral DNA content. The diaphyseal and metaphyseal DNA content was significantly increased by the administration of genistein (50 µg/100 g). This increase was significantly enhanced by the simultaneous administration of CPP (40 mg/100 g). The enhancing effect in the metaphyseal tissues was synergistic.

Effect of CPP and Genistein on Bone Components in Elderly Rats

The serum calcium and inorganic phosphorus

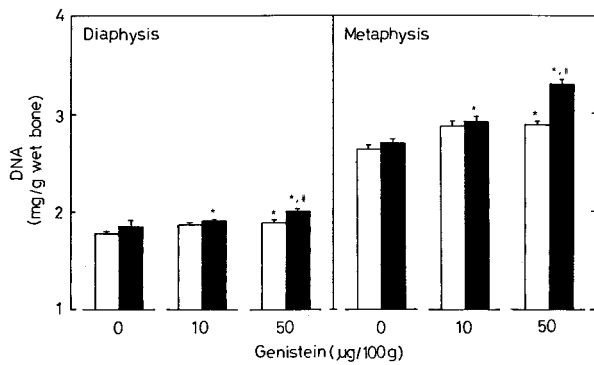


Fig. 4. Changes in DNA Content in the Femoral-Diaphyseal and Metaphyseal Tissue of Young Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 1. Each value is the mean \pm S.E.M. of five animals. * $p < 0.01$, compared with the control value. # $p < 0.01$, compared with the value for CPP or genistein (50 $\mu\text{g}/100\text{ g}$) alone. , Control; , CPP.

Table 1. Changes in Body Weight and Serum Components with Increasing Age

Age	Body weight (g)	Serum components (mg/dl)	
		Calcium	Inorganic phosphorus
5 weeks	111.4 \pm 2.7	10.66 \pm 0.27	7.62 \pm 0.18
50 weeks	228.8 \pm 5.9*	9.25 \pm 0.12*	4.55 \pm 0.19*

Each value is mean \pm S.E.M. of five animals. * $p < 0.01$, compared with the value for 5 weeks old rats.

concentrations in young (5 weeks old) and elderly (50 weeks old) rats are shown in Table 1. These concentrations were significantly decreased with increasing age. The serum components in young and elderly rats were not significantly changed by the administration of CPP (40 mg/100 g), genistein (50 $\mu\text{g}/100\text{ g}$), and CPP (40 mg/100 g) plus genistein (50 $\mu\text{g}/100\text{ g}$) for 14 d (data not shown).

When elderly rats were orally administered either vehicle, CPP (40 mg/100 g), genistein (50 $\mu\text{g}/100\text{ g}$) or CPP (40 mg/100 g) plus genistein (50 $\mu\text{g}/100\text{ g}$) for 14 d, the femoral dry weight was significantly increased (Fig. 5). The genistein-increased femoral dry weight was significantly enhanced by the simultaneous administration of CPP. This effect was additive.

The change in bone components in the femoral-diaphyseal and metaphyseal tissue of elderly rats administered CPP and genistein for 14 d was determined. The administration of CPP (40 mg/100 g) produced a significant increase in calcium content (Fig. 6) and alkaline phosphatase activity (Fig. 7) in the metaphyseal tissues. Such increase was not seen

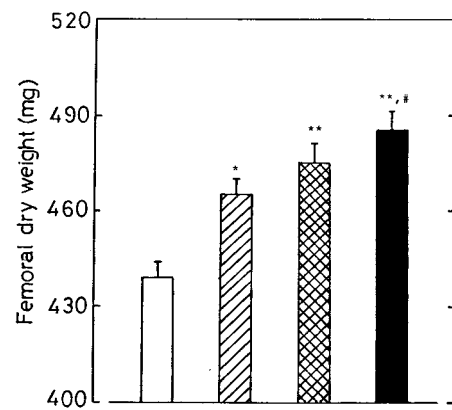


Fig. 5. Changes in the Femoral Dry Weight of Elderly Rats Administered CPP and Genistein

Rats were orally administered either vehicle (distilled water), CPP (40 mg/100 g body weight), genistein (50 $\mu\text{g}/100\text{ g}$) or CPP (40 mg/100 g) plus genistein (50 $\mu\text{g}/100\text{ g}$) with a stomach tube for 14 d. Animals were sacrificed 24 h after the last administration. Each value is the mean \pm S.E.M. of five animals. * $p < 0.025$ and ** $p < 0.01$, compared with the control value. # $p < 0.01$, compared with the value for CPP alone. , Control; ▨, CPP; ▩, genistein; , CPP plus genistein.

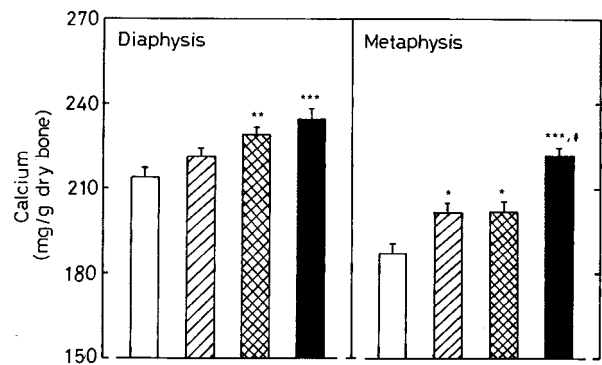


Fig. 6. Changes in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissue of Elderly Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 5. Each value is the mean \pm S.E.M. of five animals. * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.01$, compared with the control value. # $p < 0.01$, compared with the value for CPP or genistein alone. , Control; ▨, CPP; ▩, genistein; , CPP plus genistein.

in the diaphyseal tissues. CPP administration caused a significant increase in DNA content in the diaphyseal tissues, although this increase was not seen in the metaphyseal tissues (Fig. 8). The administration of genistein (50 $\mu\text{g}/100\text{ g}$) resulted in a significant increase in calcium content (Fig. 6), alkaline phosphatase activity (Fig. 7), and DNA content (Fig. 8) in the diaphyseal and metaphyseal tissues. These increases in the metaphyseal tissues were significantly enhanced by the simultaneous administration

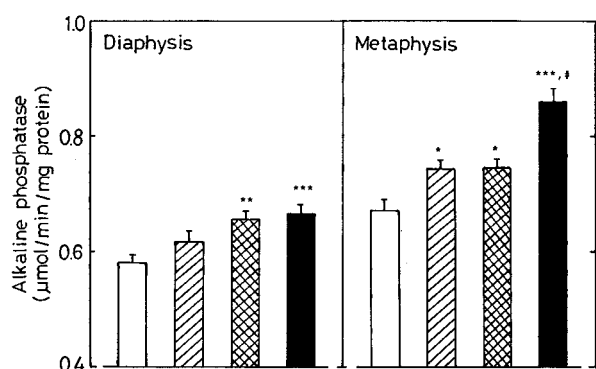


Fig. 7. Changes in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissue of Elderly Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 5. Each value is the mean \pm S.E.M. of five animals. * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.01$, compared with the control value. # $p < 0.01$, compared with the value for CPP or genistein alone. , Control; /, CPP; , genistein; , CPP plus genistein.

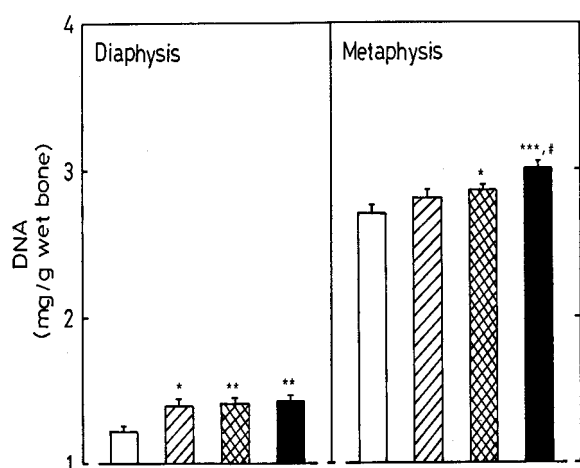


Fig. 8. Changes in DNA Content in the Femoral-Diaphyseal and Metaphyseal Tissue of Elderly Rats Administered CPP and Genistein

Rats were administered CPP and genistein as described in the legend for Fig. 5. Each value is the mean \pm S.E.M. of five animals. * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.01$, compared with the control value. # $p < 0.01$, compared with the value for CPP or genistein alone. , Control; /, CPP; , genistein; , CPP plus genistein.

of CPP (50 mg/100 g) (Figs. 6, 7, and 8). These enhancing effects were synergistic. This enhancement, however, was not seen in the diaphyseal tissues.

DISCUSSION

The deterioration of bone metabolism is induced with increasing age.^{28,29} We used young (5 weeks old) and elderly (50 weeks old) female rats in this

study. Increasing age was shown to have reduced serum calcium and inorganic phosphorus concentrations, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and metaphyseal tissue, although it did not result in the reduction of the diaphyseal and metaphyseal calcium content. The oral administration of genistein (50 μ g/100 g) to young and elderly rats for 14 d caused a significant increase in the femoral dry weight, calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissue, indicating that the isoflavone has an anabolic effect on bone components in aged rats.

Alkaline phosphatase is a marker enzyme of osteoblasts, and the enzyme participates in bone mineralization.³⁰ DNA content is an index of bone growth and the number of bone cells including osteocytes, osteoblasts, and osteoclasts in bone tissues.³¹ Genistein has been shown to stimulate a cell proliferation due to increasing protein synthesis in osteoblastic cells.¹⁰ Genistein may stimulate osteoblastic bone formation and mineralization in the femoral-diaphyseal and metaphyseal tissues of young and elderly rats. Genistein has also been shown to inhibit osteoclastic bone resorption.¹²⁻¹⁴ Bone resorption in aged rats may be inhibited by the administration of genistein, thereby increasing bone calcium content.

The oral administration of CPP to young and elderly rats for 14 d produced a significant increase in the femoral dry weight, and it exhibited a partial-anabolic effect on calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal or metaphyseal tissues. CPP has been shown to stimulate an absorption of dietary calcium in rat intestine.¹⁸⁻²⁰ The absorbed calcium may be accumulated to bone tissues, and it may have an influence on osteoblastic cell function.

The simultaneous administration of genistein and CPP to young and elderly rats for 14 d resulted in a synergistic enhancing effect on the femoral dry weight and the diaphyseal and metaphyseal components. This effect may be from the stimulatory action of genistein on osteoblastic cells, which are related to bone formation and mineralization, and the enhancing effect of CPP on intestinal calcium absorption, due to exhibiting different mechanisms.

The present finding, that the anabolic effect of genistein on bone components in aged rats is enhanced by CPP administration, suggests that a combination of food factors has a potent effect in the prevention of osteoporosis with increasing age. The

synergistic effect of genistein and CPP on bone components was pronounced in the femoral–metaphyseal tissues of aged rats. The metaphyseal tissue is trabecular bone and the diaphyseal tissue is cortical bone. Trabecular bone is secondary spongiosum, and bone mass in this tissue is the first to decrease with increasing age. The combination of genistein and CPP in dietary supplementation may be a good tool in the prevention of trabecular bone loss with ageing.

In conclusion, it has been demonstrated that the simultaneous administration of genistein and CPP has a synergistic–anabolic effect on bone components in the femoral tissue of young and elderly rats.

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