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# Hydrolyzed collagen improves bone metabolism and biomechanical parameters in ovariectomized mice: An *in vitro* and *in vivo* study

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ABSTRACT

Collagen has an important structural function in several organs of the body, especially in bone and cartilage. The aim of this study was to investigate the effect of hydrolyzed collagen on bone metabolism, especially in the perspective of osteoporosis treatment and understanding of its mechanism of action. An *in vivo* study was carried out in 12-week-old female C3H/HeN mice. These were either ovariectomized (OVX) or sham-operated (SHAM) and fed for 12 weeks with a diet containing 10 or 25 g/kg of hydrolyzed collagen. We measured bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA). C-terminal telopeptide of type I collagen (CTX), marker of bone resorption, and alkaline phosphatase (ALP), marker of bone formation, were assayed after 4 and 12 weeks. Femur biomechanical properties were studied by a 3-point bending test and bone architecture by microtomography. The BMD for OVX mice fed the diet including 25 g/kg of hydrolyzed collagen was significantly higher as compared to OVX mice. The blood CTX level significantly decreased when mice were fed with either of the diets containing hydrolyzed collagen. Finally, we have shown a significant increase in bone strength correlated to geometrical changes for the OVX mice fed the 25 g/kg hydrolyzed collagen diet. Primary cultures of murine bone cells were established from the tibia and femur marrow of BALB/c mice. The growth and differentiation of osteoclasts and osteoblasts cultured with different concentrations (from 0.2 to 1.0 mg/mL) of bovine, porcine or fish hydrolyzed collagens (2 or 5 kDa) were measured. Hydrolyzed collagens (2 or 5 kDa) in the tissue culture medium did not have any significant effects on cell growth as compared to controls. However, there was a significant and dose-dependent increase in ALP activity, a well-known marker of osteogenesis, and a decrease in octeoclast activity in primary culture of bone cells cultured with hydrolyzed collagens (2 kDa only) as compared to the control.

It is concluded that dietary hydrolyzed collagen increases osteoblast activity (as measured in primary tissue culture), which acts on bone remodeling and increases the external diameter of cortical areas of the femurs.



## Introduction

Osteoporosis is a chronic disorder occurring mainly in postmenopausal women, characterized by reduced BMD and an increased risk of fracture. Bone loss originates from an imbalance between bone formation and bone resorption, especially after the menopause, which induces an increase of bone turnover by excess osteoclast activity. Nutritional components with potential anti-resorptive activity generally include calcium and cholecalciferol, but other components such as hydrolyzed type I collagen are also presumed to have an impact on bone metabolism [1].

Type I collagen is the major structural protein distributed throughout the whole body accounting for 25% of total body protein and for 80% of total conjunctive tissue in humans. It is an important component of bone, being the main extra cellular matrix protein for calcification, which also plays a role in osteoblast differentiation [2,3]. Some studies suggest that hydrolyzed collagen-enriched diet improves bone collagen metabolism and BMD. Oral administration of hydrolyzed collagen increased bone mass content and density in rats and mice fed a calcium- or protein-deficient diet [4,5]. Oral administration of hydrolyzed collagen was also demonstrated to increase the quantity of type I collagen and proteoglycans in the bone matrix of ovariectomized rats [1]. Moreover, in patients with osteoporosis, oral intake of hydrolyzed collagen with calcitonin had a stronger inhibitory effect on bone resorption than calcitonin alone [6]. Proteins represent a key nutriment for bone health and

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thereby in the prevention of osteoporosis [7]. Protein undernutrition contributes to the occurrence of osteoporotic fracture in elderly. This may be associated with an uncoupling between increased bone formation and bone resorption, due to a decreased plasma insulin-like growth factor-I (IGF-I) levels [8]. Previous works showed that protein repletion after hip fracture in elderly patients was associated with increased serum levels of IGF-1 and attenuation of proximal femur bone loss [9].

The mechanisms by which ingestion of hydrolyzed collagen could improve bone formation remain unclear but have been suspected to be associated with the release and absorption of collagen-derived peptides acting on bone metabolism. Since Oesser et al. [10] demonstrated the intestinal absorption and the cartilage accumulation of collagen-derived peptides, it has been generally assumed that collagen-rich diets interact with the bone matrix. Indeed, collagen-derived di- and tripeptides rich in hydroxyproline such as Hyp, Pro-Hyp, Pro-Hyp-Gly or Gly-Pro-Val have been detected in human blood following the ingestion of hydrolyzed collagen [11]. Negligible amounts of the peptide form of hydroxyproline (Hyp) were observed in human blood before collagen ingestion. After its oral ingestion, the peptide form of Hyp significantly increased and reached a maximum level (20−60 nmol/mL of plasma) after 1−2 h and then decreased to half of the maximum level 4 h after ingestion. Moreover, some studies demonstrated the time-dependent degradation of Gly-Pro-Hyp, which is frequently found in collagen sequences, into the free-form Gly and a dipeptide, Pro-Hyp. The PEPT1 proton-dependent transporter assures the transport of Pro-Hyp across the intestinal barrier [12]. Among the collagen-derived peptides, Pro-Hyp-Gly, Pro-Hyp and analogs display chemotactic activity to fibroblasts, peripheral blood neutrophils [13,14] and monocytes [15], Asp-Gly-Glu-Ala stimulates osteoblast-related gene expression of bone marrow cells [16], Ala-Hyp and Gly-Pro-Val are potential inhibitors of angiotensin-converting enzyme [17,18], and Gly-Pro-Hyp could be involved in platelet aggregation [19].

In such a context, the aim of this study was to investigate both *in vivo* and *in vitro* the effects of hydrolyzed collagen used as a nutritional component on bone metabolism. For such a purpose, we used an ovariectomized C3H/HeN mouse model of postmenopausal osteoporosis and a primary culture of murine bone cells.

## Materials and methods

### *Hydrolyzed collagens*

Enzymatic hydrolyzed collagens were provided by Rousselot SAS, a Vion Company, (Puteaux, France) from the Rousselot® Peptan™ range. The hydrolyzed collagens were from bovine (Peptan™ B coded RDH), porcine (Peptan™ P coded PCH) and fish (Peptan™ F coded FGH) origins with molecular weights of 2 kDa (RDH-N, PCH-N, FGH-N) and 5 kDa (RDH, PCH, FGH). All preparations were food grade and can be obtained commercially. The hydrolyzed collagens were derived from the enzymatic hydrolysis of animal skins which are predominantly type I collagen. Bovine serum albumin (BSA; Euromedex; fraction V) was used as control protein for the *in vitro* experiments.

### *Ovariectomized mouse model*

Thirty-two 6-week-old female C3H/HeN mice (Harlan) were housed in a room controlled for temperature (22 ± 1 °C) and a 12:12 light–dark cycle with free access to food and water for a 6-week adaptation period prior to the study. All experimental procedures used during the experiments complied with institutional policies and guidelines designed to prevent pain and distress. At 12 weeks of age, 24 mice were ovariectomized (OVX) and 8 sham-operated (SHAM). For the surgery, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Morphine was used as analgesic. One week after the surgery, the OVX mice were divided into three groups of eight mice each. They were fed *ad libitum* for 12 weeks with a diet (AIN-93M with 240 g/kg soy protein as a protein source) containing 0 (OVX and SHAM), 10 (OVX10) or 25 (OVX25) g/kg hydrolyzed collagen PCH (Table 1). The monitoring of diet consumption showed an intake of about 3.5 g/day. These concentrations correspond to approximately 1 g/kg body weight of hydrolyzed collagen for OVX10 and 2.5 g/kg for OVX25. The mice had free access to water throughout the experiment. Twelve weeks after surgery mice were anesthetized and whole blood collected by cardiac puncture. Body composition was determined by dissection. Four white adipose tissue (WAT) pads (periovarian, retroperitoneal, mesenteric and total subcutaneous) were removed and weighed. The liver, intestines, uterus, brown adipose tissue and carcass (muscles and skeleton) were also weighed. Femurs and tibias were collected.

### *BMD quantification*

Two, 4, 8, and 12 weeks after the surgery, bone mineral density (BMD) and bone mineral content (BMC) were measured by dual-energy X-ray absorptiometry (DXA) with a Lumar Piximus densitometer (GE Medical Systems, software version 1.4 lunar). The Piximus allows automated, accurate and precise measurement of bone density for small animals (10–50 g). The bone measurements exhibit excellent correlation between their ashes or chemical extraction weights ($r = 0.99$). Blood samples were also collected (150 µl). The mice were weighed, anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed on the belly under the X-ray of the Lumar Piximus. BMD and BMC were measured for the whole body (excluding head and tail to improve sensitivity as suggested by the manufacturer), lumbar spines and the right femoral bone of each mouse [20].

### *Bone composition, geometric and biomechanical parameters*

Both femurs and tibias were collected and preserved in dry conditions at -80 °C. One tibia from each mouse was dried overnight at 100 °C and ashed at 550 °C for 48 h. The ashes were extracted with 1 mL of 1 M HCl in order to quantify bone calcium content by atomic absorption spectrophotometry with a Zeeman polarized spectrophotometer. Non-mineral content was determined as the difference between the weight of the dried bones at 100 °C and the weight of the ashes. The mechanical properties of the mouse femurs were assessed by 3-point bending test using a Universal Testing Machine (Instron 4501, Instron, Canton, MA). Each femur was centrally loaded at the

**Table 1**
Composition of the diets (g/kg diet).

| Ingredient (g/ kg diet) | Control | OVX10 | OVX25 |
|---|---|---|---|
| Soy protein[a] | 200.2 | 190.2 | 175.2 |
| PCH[b] | 0 | 10 | 25 |
| Corn starch[c] | 569.2 | 569.2 | 569.2 |
| Saccharose[d] | 91.7 | 91.7 | 91.7 |
| Soybean oil[e] | 40 | 40 | 40 |
| AIN 93 M mineral mixture[f] | 35 | 35 | 35 |
| AIN 93 M vitamin mixture[f] | 10 | 10 | 10 |
| Alpha-cellulose[g] | 50.1 | 50.1 | 50.1 |
| Choline[g] | 2.3 | 2.3 | 2.3 |
| Methionine[g] | 1 | 1 | 1 |
| Cysteine[g] | 0.4 | 0.4 | 0.4 |

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[d] Eurosucre, Paris, France.
[e] Bailly SA, Aulnay-sous-Bois, France.
[f] ICN Pharmaceuticals, Orsay, France.
[g] Medias Filtrants Durieux, Marne-la-Vallée. France (Alphacel, ICN Pharmaceuticals, Orsay, France).

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mid-diaphysis at a speed of 1 mm/s. The extrinsic biomechanical parameters of stiffness ($S$, N/mm) and ultimate strength ($F_U$, N) were determined from load-displacement curves. The intrinsic biomechanical parameters Young's modulus ($E$, MPa) and ultimate stress ($\sigma_U$, MPa) were calculated from the load displacement curves and geometric properties as described previously [21]. The cross-sectional geometry of the diaphysis of each mouse femur was imaged by microcomputed tomography (μCT, Skyscan 1072, Skyscan, Kontich, Belgium). Both medio-lateral and antero-posterior external and internal diameters of the cortical bone were measured at the mid-diaphysis. Cross-sectional cortical area (CSA, $mm^2$) and moment of inertia ($I$, $mm^4$) in relation to the horizontal axis were calculated as previously described [22].

### *CTX, glucose and bone ALP concentrations in blood*

C-terminal telopeptide of type I collagen (CTX) concentration was measured in blood using an ELISA RatLaps™ kit (Osteomedical). Serum glucose was evaluated using a bioMerieux glucose RTU™ kit. Bone-specific alkaline phosphatase (ALP) activity was measured using *p*-nitrophenylphosphate in presence or absence of L-phenylalanine 0.1 M for the inhibition of the intestinal ALP as described by Dimai et al. [23]. Cell ALP activity was evaluated without L-phenylalanine and calculated as milliunits per mL of serum or milliunits per $1 \times 10^5$ cells. One unit is defined as the activity which hydrolyzes 1 μmol of substrate to product per minute at 37 °C.

### *Primary tissue culture*

Primary tissue co-culture of murine bone osteoblast and osteoclast cells was established from tibia and femur bone marrow obtained from 4-week-old BALB/c female mice (Harlan). Mice were killed by decapitation; tibias and femurs were removed aseptically then crushed with a scalpel on petri dishes containing α-MEM (Invitrogen) with 10% fetal bovine serum (FBS; Hyclone). Bone pieces and the medium with the cells were collected in a tube and vigorously shaken. The solution was filtered through a 70-μm cell strainer. Cells were collected by centrifugation for 5 min at 800 *g* and resuspended in α-MEM with 10% FBS and $10^{-8}$ M of 1α, 25-dihydroxyvitamin $D_3$ (Sigma). Cells obtained from four to five tibias were plated in one 75-$cm^2$ flask and cultured at 37 °C under a 5% $CO_2$ 95% air atmosphere. After 6 days, cells were scraped off, collected and seeded in petri dishes at a density of $2.5 \times 10^4$ cells/$cm^2$. Twenty-four hours after seeding, proteins were added to the culture medium. Cells were grown in the presence of hydrolyzed collagens or BSA at a concentration of 0.2, 0.5 or 1.0 mg/mL. After 14 days, cell growth was measured using a FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes) as previously described [24]. The fluorometric method we used has been developed to count adherent cells in the range of 1000 to 100,000 cells per well for proliferation studies. In a first experiment, cells were counted and compared to DNA quantification. The standard curve obtained was used to correlate DNA quantification to number of cells per well. Morphological observation was performed by light microscopy at 100 × magnification. Osteoclasts, which are large plurinucleate cells, were counted using Giemsa staining (Sigma).

The BD BioCoat™ Osteologic™ bone cell culture system (BD Bioscience) was used to characterize and measure osteoclast-mediated bone resorption. The preculture cell suspension was seeded at density of $4 \times 10^4$ cells/$cm^2$. Twenty-four hours after seeding, cells were incubated in the presence of 1 mg/mL BSA or PCH-N for 14 days. The digestion of sub-micron synthetic calcium phosphate thin films was observed using a light microscope with 10 × magnification to evaluate resorption activity of the cells.

### *Statistical analysis*

Statistical analyses were carried out using SAS version 9.1 with the data expressed as means ± S.D. One-way ANOVA was performed using the GLM procedure of SAS with a Duncan multiple comparison test and a Tukey test *post hoc*. The MIXED procedure was used to perform repeated-measures ANOVA.

## Results

### *Body composition of ovariectomized mice fed hydrolyzed collagen supplemented diet*

Body weight change as function of time in SHAM, OVX, OVX10 and OVX25 mice is reported on Fig. 1. The OVX procedure induces a higher weight gain as compare to SHAM. However, no difference in body weight could be observed between OVX, OVX10 and OVX25 mice. Analysis of body composition as determined by dissection showed that after 12 weeks, OVX, OVX10 and OVX25 mice had significantly higher subcutaneous, perirenal and periovarian adipose tissue pad masses and a significantly lower uterine weight than the SHAM mice (Table 2). These results clearly show the effectiveness of ovariectomy. They also show that a hydrolyzed collagen enriched diet has no effect not only on body weight but also on adipose tissue or uterine weight of OVX mice. Moreover, no difference was observed between SHAM and OVX, OVX10 or OVX25 for carcass and kidney weight. Serum glucose determination after 12 weeks of diet supplementation showed that SHAM mice had a blood glucose level of 1.46 ± 0.14 g/L. This value is smaller than the values reported for the OVX, OVX10 or OVX25 mice which are similar with an average value of 1.67 ± 0.16 g/L. However, no significant differences between the groups are reported.

### *BMD in ovariectomized C3H/HeN mice fed hydrolyzed collagen supplemented diet*

The change of total body BMD is shown in Fig. 2. Before surgery, the mean BMD for the mice was 0.041 g/cm. Surgery was performed

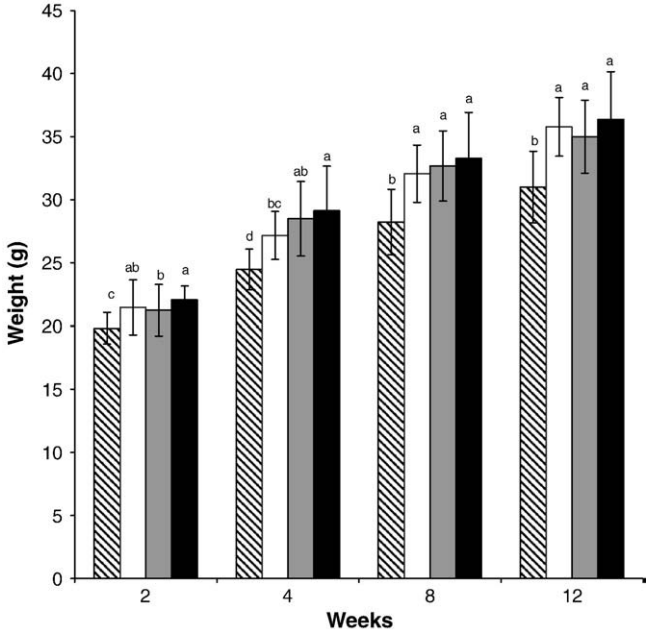


**Fig. 1.** Change of body weight 12 weeks after ovariectomy. Body weight was evaluated for SHAM (hatched), OVX (white), OVX10 (grey) and OVX25 (black) mice. Values are means ± SD, $n = 8$. Groups with different letters are significantly different ($p < 0.05$).

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**Table 2**
Body composition of SHAM, OVX, OVX10 and OVX25 mice 12 weeks after surgery.

| Dietetary groups | Initial body weight (g) | Final body weight (g) | Periovarian fat mass (g) | Mesenteric fat mass (g) | Perirenal fat mass (g) | Subcutuneous fat mass in the down part of the body (g) | Uterus (mg) | Kidneys (mg/g BW) | Carcass (g) |
|---|---|---|---|---|---|---|---|---|---|
| Sham | 19.83 ± 1.24 | 32.6 ± 2.9 [b] | 2.19 ± 0.83 | 0.83 ± 0.2 | 0.73 ± 0.19 [b] | 1.54 ± 0.39 [b] | 2.60 ± 0.65 [a] | 10.9 ± 0.8 | 11.03 ± 0.63 |
| OVX | 21.47 ± 2.17 | 37.1 ± 4.1 [a] | 1.89 ± 0.47 | 0.91 ± 0.29 | 0.86 ± 0.15 [ab] | 1.89 ± 0.6 [ab] | 1.03 ± 0.33 [b] | 11.5 ± 0.9 | 12.85 ± 0.66 |
| OVX10 | 21.25 ± 2.04 | 36.7 ± 3.9 [a] | 2.19 ± 0.58 | 0.82 ± 0.37 | 1.07 ± 0.29 [a] | 2.03 ± 0.56 [a] | 0.92 ± 0.54 [b] | 10.1 ± 0.8 | 11.82 ± 0.82 |
| OVX25 | 22.06 ± 1.09 | 39.05 ± 4.6 [a] | 2.10 ± 0.57 | 1.07 ± 0.36 | 1.09 ± 0.47 [a] | 1.77 ± 0.64 [a] | 1.29 ± 0.35 [b] | 10.7 ± 0.9 | 12.58 ± 1.67 |

Values are means ± SD, $n = 8$. Values in a column with different letters are significantly different ($p < 0.05$).

at 12 weeks when BMD was still increasing. As reported previously [20], BMD continues to increase until 6 months; however the OVX procedure slows down BMD gain of OVX mice as compared to SHAM mice. As expected, the OVX mice had a significantly lower increase in BMD than the SHAM group from at least 8 weeks post-surgery until the end of the experiment (0.0049 ± 0.0018 vs. 0.0074 ± 0.0018 g/cm$^2$ at week 4 and 0.021 ± 0.002 vs. 0.017 ± 0.0019 g/cm$^2$ at week 12, respectively). This result shows the effectiveness of ovariectomy to increase bone resorption.

In a first experiment we tested a wide range of hydrolyzed collagen on bone metabolism from 0.2 to 10 g/kg of body weight per day. At 0.2 g/kg of body weight per day we did not observe any effect on the Bone Mineral Density (BMD) but at 1 and 2.5 g/kg of body weight per day we observed an increase of the BMD. Higher concentrations than 2.5 g/kg of body weight per day did not further improve BMD. Four and 8 weeks after the surgery the BMD of the OVX25 group was not significantly different from OVX and SHAM. However, after 12 weeks, OVX25 had a significantly higher increase in BMD compared to OVX (0.02 ± 0.0023 g/cm$^2$ vs. 0.017 ± 0.0019 g/cm$^2$, respectively, $p < 0.05$). Fig. 2 shows that OVX10 BMD was intermediate between OVX and OVX25 8 weeks after surgery, and had a significant higher increase in BMD at 12 weeks after the OVX procedure compared to OVX(0.0018 ± 0.0022 g/cm$^2$ vs. 0.017 ± 0.0019 g/cm$^2$ respectively, $p < 0.05$). Femoral BMD was also evaluated. Values reported for OVX25 were higher compared to OVX, but the difference was not significant (data not shown).

*Bone composition, mechanical properties and microarchitecture of ovariectomized mice fed a hydrolyzed collagen supplemented diet*

12 weeks after surgery, the tibia non-mineral contents were not significantly different between OVX and SHAM, but were higher for OVX10 and OVX25 (321 ± 35 and 370 ± 44 mg/g bone vs. 396 ± 40 and 388 ± 63 mg/g bone respectively, $p < 0.05$; Table 3). Evaluation of femur mechanical properties showed no significant differences between groups for ultimate stress, Young's modulus and stiffness, whereas the moments of inertia were significantly different between OVX and OVX25 groups. Significant differences between OVX and OVX25 groups were also observed for some biomechanical parameters (Fig. 3): femur microarchitecture analysis showed that both the cortical area at mid-diaphysis and the ultimate strength were significantly greater in OVX25 than in OVX mice (1.25 ± 0.11 vs. 1.14 ± 0.07 mm$^2$ and 30.80 ± 2.88 vs. 28.67 ± 2.07 N respectively, $p < 0.05$). The increased thickness of the cortical area was related to a significant increase in the external mid-diaphysis diameters without change in the medullar area (data not shown).

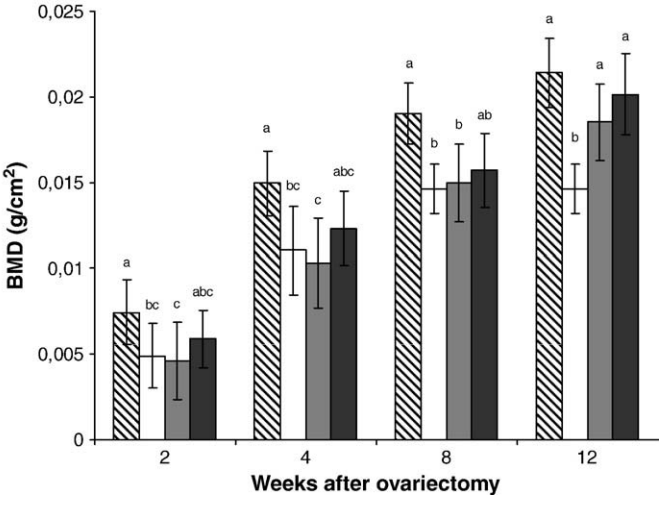


**Fig. 2.** Increase of bone mass density (BMD) of whole body 12 weeks after ovariectomy. BMD was evaluated for SHAM (hatched), OVX (white), OVX10 (grey) and OVX25 (black) mice. Values are means ± SD, $n = 8$. Groups with different letters are significantly different ($p < 0.05$).

*Ingestion of a hydrolyzed collagen supplemented diet decreases bone resorption in ovariectomized mice*

The bone turnover markers CTX and bone ALP were assayed in mouse plasma (Fig. 4). As expected, 12 weeks after surgery, we reported a significantly higher concentration of CTX in the plasma of OVX mice than in SHAM (12.42 ± 1.70 vs. 8.97 ± 2.43 µg/mL, respectively, $p < 0.05$) confirming the effectiveness of ovariectomy to increase osteoclast activity. Moreover, CTX plasma concentration was significantly lower for OVX10 and OVX25 (9.62 ± 2.02 and 9.45 ± 1.33 respectively) as compared to OVX, and did not differ from SHAM (Fig. 4A). Bone ALP was assayed 4 and 12 weeks after surgery (Fig. 4B). As expected this showed that its activity is always higher for SHAM than for OVX (27.68 ± 4.89 vs. 18.57 ± 4.67 mg/mL/min and 17.43 ± 2.93 vs. 12.76 ± 3.01 mg/mL/min for 4 and 12 weeks after surgery, respectively). This confirms the effectiveness of ovariectomy to decrease osteoblast activity (Fig. 4B). Four weeks after surgery, bone ALP for OVX 25 was higher than for OVX. However no difference could be seen after 12 weeks.

*Hydrolyzed collagen increases osteoblast activity in co-culture of murine bone cells*

We used primary culture of murine bone cells established from femur bone marrow obtained from Balb/c mice. The primary co-culture of osteoblasts and osteoclasts was grown in 48-well microplates in the presence of 1, 0.5 or 0.2 mg/mL BSA, PCH-N, FGH-N, RDH-N, PCH, RDH or FGH for 14 days. For all the hydrolyzed collagens tested, there was no effect on cell growth in comparison to the BSA control (Table 4). The results were expressed as a stimulation index (SI), calculated as the ratio of DNA content in the presence of hydrolyzed collagen as compared to a BSA control. ALP activity, a marker of the osteogenic activity of osteoblasts, was significantly increased in the presence of 1 mg/mL PCH-N, RDH-N and FGH-N but was not modified in the presence of 1 mg/mL PCH, RDH and FGH as compared to BSA. Addition of 0.5 mg/mL PCH-N and RDH-N also induced an increase in ALP activity whereas FGH-N, PCH, RDH and FGH had no effect when compared to BSA. However, no effect on ALP activity was reported after addition of 0.2 mg/mL of any of the

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**Table 3**
Composition of tibias and biomechanical parameters of femurs 12 weeks after surgery.

| Dietetary groups | Tibia composition | | | | Femur mechanical properties | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Weight of dried bones (mg) | Mineral content (mg/g bone) | Calcium content (mg/g mineral content) | Non-mineral content (mg/g bone) | Moment of inertia, $I$ ($mm^4$) | Ultimate stress, $\sigma_U$ (MPa) | Young's modulus, $E$ (MPa) | Stiffness, $S$ (N/mm) | Energy (N mm) |
| SHAM | 555 ± 69 | 254 ± 4 | 325 ± 47 | 370 ± 44 $^{ab}$ | 0.124 ± 0.019 $^{ab}$ | 280 ± 24 | 13106 ± 2462 | 151 ± 22 | 8.73 ± 1.58 |
| OVX | 523 ± 65 | 262 ± 2 | 289 ± 14 | 321 ± 35 $^{b}$ | 0.124 ± 0.016 $^{a}$ | 275 ± 14 | 13366 ± 1193 | 155 ± 11 | 8.16 ± 0.99 |
| OVX10 | 557 ± 53 | 241 ± 2 | 337 ± 29 | 396 ± 40 $^{a}$ | 0.114 ± 0.015 $^{b}$ | 285 ± 20 | 14276 ± 1555 | 152 ± 21 | 8.40 ± 1.31 |
| OVX25 | 554 ± 83 | 238 ± 5 | 358 ± 64 | 388 ± 63 $^{a}$ | 0.143 ± 0.011 $^{a}$ | 267 ± 27 | 12570 ± 1739 | 167 ± 20 | 9.18 ± 1.99 |

Values are means ± SD, $n = 8$. Values in a column with different letters are significantly different ($p < 0.05$).

hydrolyzed collagens tested (Table 4). Moreover, as reported in Figs. 5A and B, the presence of 1 mg/mL PCH-N in the culture medium allowed the osteoblasts to acquire a star shape whereas osteoclast numbers were not modified, the control experiments being performed with BSA dissolved in the culture medium. To better characterize the effects of PCH-N on osteoclasts, murine bone primary tissue culture was performed for 14 days using the BD BioCoat™ Osteologic™ bone cell culture system which allows measurement of osteoclast-mediated bone resorption. Osteoclast activity was measured by digestion of a sub-micron synthetic calcium phosphate film. As indicated in Figs. 5C and D, presence of PCH-N (1 mg/mL) in the culture medium reduced resorption area as compared to the BSA control.

## Discussion

The present study shows that hydrolyzed collagens are able to increase *in vivo* and *in vitro* bone metabolism. To evaluate the efficiency of hydrolyzed collagen to modify bone turnover, we used an ovariectomized mice model. Our results show that feeding a diet enriched with 25 g/kg hydrolyzed collagen is able to significantly increase bone metabolism as well as the biomechanical properties (such as BMD) of OVX animals. Moreover, an assay of the bone turnover marker CTX reinforced the view that a diet enriched with hydrolyzed collagen is able to decrease bone resorption in OVX mice. Taking into account that osteoporosis can be defined as the capacity of

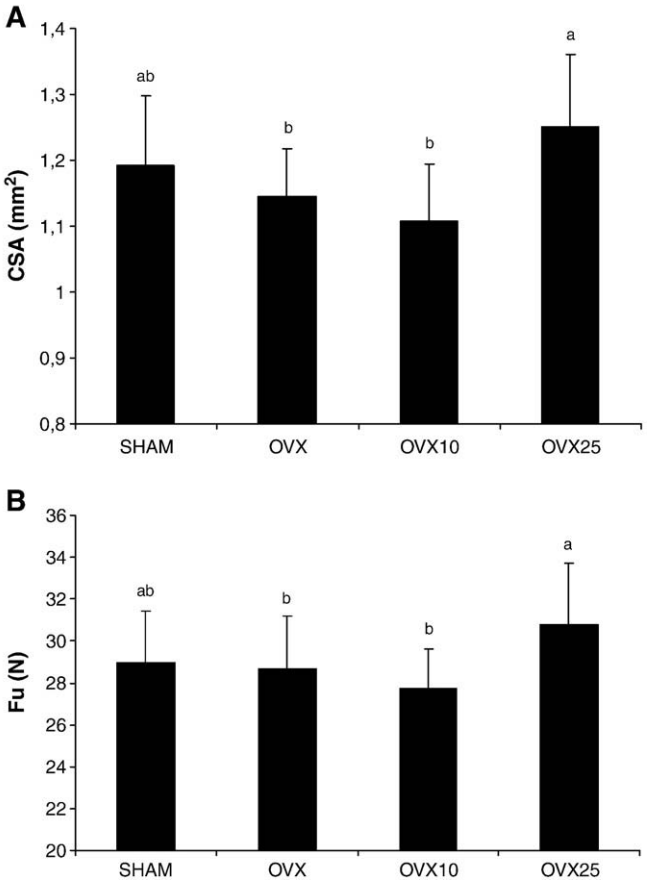


**Fig. 3.** Cross sectional area and ultimate strength of femurs after 12 weeks ingestion of the experimental diets. Ovariectomized mice were fed for 12 weeks with control diet (OVX), or the diet supplemented with 10 (OVX10) or 25 (OVX25) g/kg of PCH. The sham-operated mice were fed for 12 weeks with the control diet (SHAM). (A) Cross-sectional area 12 weeks after ovariectomy. (B) Ultimate strength 12 weeks after ovariectomy. Groups with different letters are significantly different ($p < 0.05$).

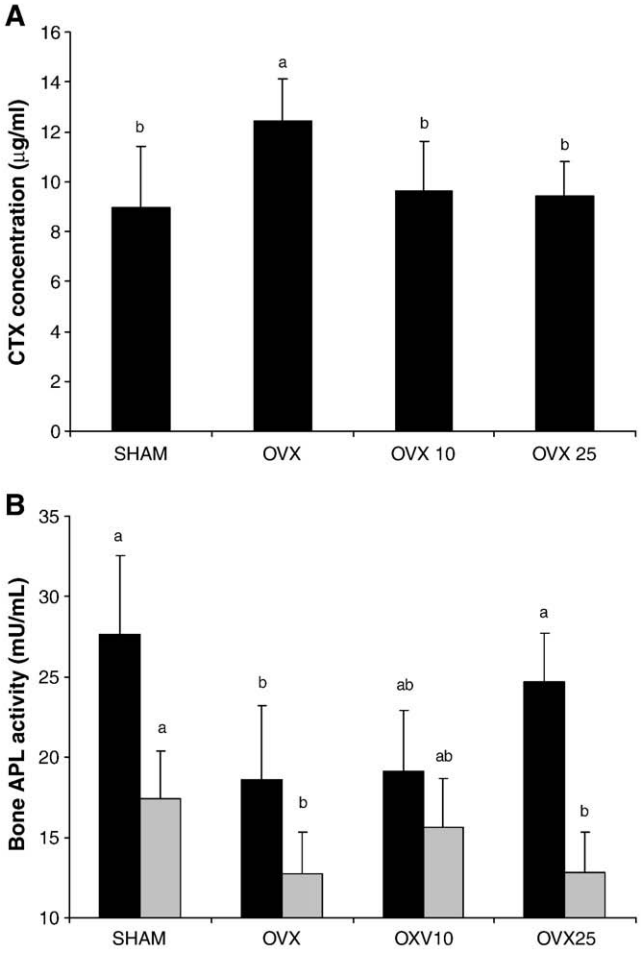


**Fig. 4.** CTX and ALP quantification at 12 weeks after surgery. Ovariectomized mice were fed for 12 weeks with the control diet (OVX), or the diet supplemented with 10 (OVX10) or 25 (OVX25) g/kg of PCH. The sham-operated mice were fed for 12 weeks with the control diet (SHAM). (A) CTX quantification 12 weeks after ovariectomy. (B) ALP quantification 4 (black) and 12 (grey) weeks after ovariectomy. Groups with different letters are significantly different ($p < 0.05$).

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**Table 4**
Effect of hydrolyzed collagens on cell growth and ALP activity of osteoblasts in primary culture of murine bone cells obtained from 4-week-old Balb/c female mice.

| Protein | Cell growth (SI) | ALP (mU/1 × $10^5$ cells) | Cell growth (SI) | ALP ( mU/1 × $10^5$ cells ) | Cell growth (SI) | ALP ( mU/1 × $10^5$ cells) |
|---|---|---|---|---|---|---|
| | 1 mg/mL | | 0.5 mg/mL | | 0.2 mg/mL | |
| BSA | 1.00 ± 0.18 | 0.42 ± 0.17 [b] | 1.00 ± 0.16 | 0.45 ± 0,068 [b] | 1.00 ± 0.06 | 0.51 ± 0.17 |
| RDH-N | 0.95 ± 0.03 | 2.13 ± 0.76 [a] | 1.04 ± 0.22 | 1.45 ± 0.089 [a] | 1.01 ± 0.15 | 0.64 ± 0.10 |
| PCH-N | 1.08 ± 0.16 | 1.71 ± 0.85 [a] | 1.18 ± 0.20 | 1.81 ± 0.84 [ab] | 1.09 ± 0.05 | 0.87 ± 0.48 |
| FGH-N | 0.99 ± 0.08 | 2.13 ± 0.76 [a] | 1.11 ± 0.19 | 1.80 ± 0.3 [a] | 0.92 ± 0.12 | 0.80 ± 0.047 |
| RDH | 0.75 ± 0.07 | 0.77 ± 0.53 [b] | 0.80 ± 0.18 | 0.78 ± 0.26 [b] | 0.91 ± 0.18 | 1.01 ± 0.47 |
| PCH | 0.87 ± 0.11 | 0.77 ± 0.37 [b] | 1.03 ± 0.09 | 0.65 ± 0.44 [b] | 1.10 ± 0.20 | 0.61 ± 0.21 |
| FGH | 0.75 ± 0.07 | 0.68 ± 0.41 [b] | 0.84 ± 0.19 | 0.34 ± 0.11 [b] | 0.88 ± 0.11 | 0.72 ± 0.43 |

Alkaline phosphatase (ALP) was evaluated as function of cell density in the presence of hydrolyzed collagens for 15 days. Values are means ± SD, of three different cell cultures done in triplicate. Values in a column with different letters are significantly different ($p<0.05$).

the bone to increase bone resorption, the understanding of how osteoporosis can be reduced requires the study of the mechanical behavior of the bone tissue. Biomechanical properties of mouse femurs were then assessed by a three-point bending test which is one of the most common test used with small animals such as mice [25]. A model of low bone mass phenotype such as C57BL/6 and high mass such as C3H/HeN [29] may be useful for further studies. In the present study, C3H/HeN ovariectomized mice or sham-operated mice were used as an *in vivo* animal model of postmenopausal osteoporosis. The OVX procedure was performed at 12 weeks when BMD was still increasing. Using this model, we observed a reduced rate of bone mass gain and 12 weeks later a lower BMD for OVX was observed as compare to SHAM, but no significant difference in biomechanical properties between OVX and SHAM mice was measured. Interestingly, previous studies show a genetic regulation of bone loss induced by estrogen deficiency [30-32].

The present study indicates that ingestion of hydrolyzed collagen diet induced the growth of the external diameter of the bone cortical zone in OVX mice. The increased cortical area of the OVX25 mice was correlated with a significant increase in the femur external diameter, without modification of the size of the medullar area. Therefore, the increased size of the cortical area was induced by a periosteal apposition of bone on the mouse femur. Due to this increase in bone size, the ultimate strength of OVX10 and OVX25 mice femurs was significantly greater than OVX. These results are in agreement with previous studies showing a correlation between bone strength and external diameter [26]. The increase of the external diameter of the femur for OVX25 as compared to OVX also suggests a higher level of bone formation for mice eating the hydrolyzed collagen-rich diets. However, 12 weeks after surgery, OVX25 bone ALP was similar to OVX. This result suggests that hydrolyzed collagen intake had a rapid and maybe transient effect on bone formation. However the effect allowed an increase of bone cortical area which increased femur strength. There was no significant difference between OVX25 and OVX for the ultimate stress. Therefore, no significant change was shown for the intrinsic properties of bone. Consequently, the intrinsic material properties of the femurs remained unchanged after treatment. There was no significant modification of stiffness or Young's

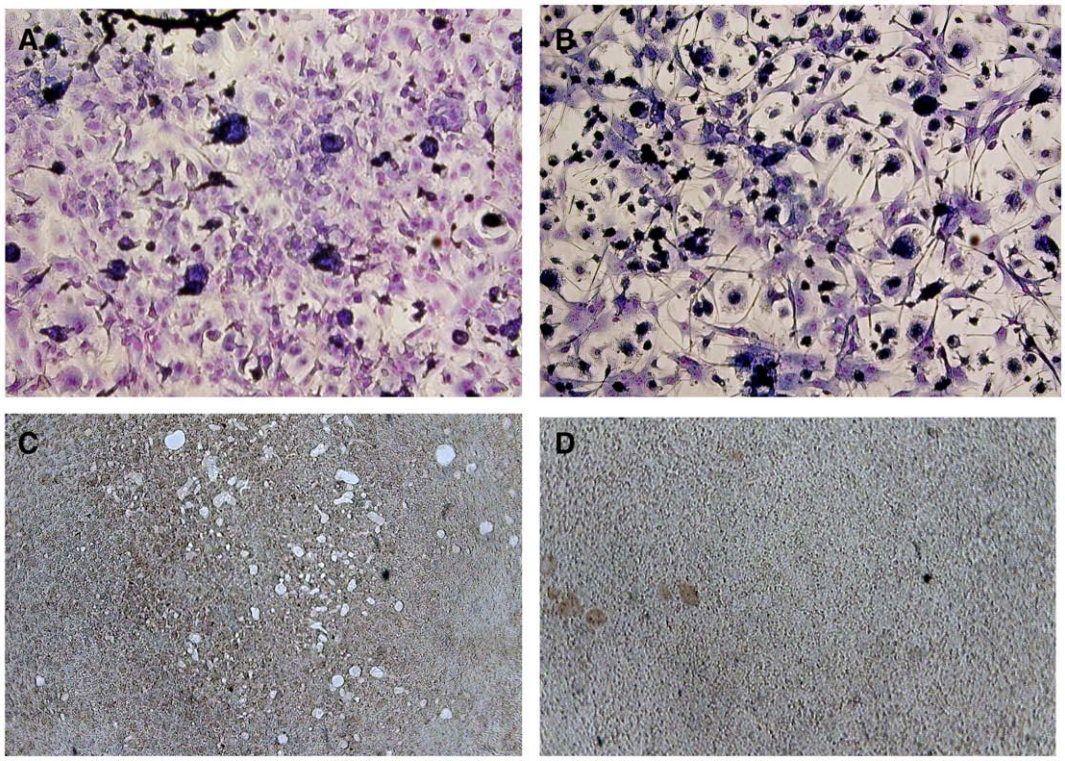


**Fig. 5.** Effect of hydrolyzed collagen on primary culture of murine bone cells in the presence of hydrolyzed collagens for 15 days. (A) Observation of bone cells with optical microscope (100) for cells cultured with 1 mg/mL of BSA added in the culture medium. (B) Observation of bone cells with optical microscope (100) for cells cultured with 1 mg/mL of PCH-N added in the culture medium. (C) Observation of pit assays with optical microscope (10) for cells culture with 1 mg/mL of BSA added in the culture medium. (D) Observation of pit assays with optical microscope (10) for cells culture with 1 mg/mL of PCH-N added in the culture medium.

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modulus between groups. However there was a trend towards greater bone stiffness in OVX25 than for other groups. Assuming that the stiffness of bone is correlated to the amount of type I collagen present [27,28], and since some previous studies showed an increase of type I collagen and proteoglycan excretion for mice fed hydrolyzed collagen [5], further studies are needed to quantify the new formation of type I collagen in the bone of these mice.

The *in vitro* results obtained with primary tissue culture of murine bone cells demonstrated that some of the tested hydrolyzed collagens were able to stimulate cell growth and ALP activity, a marker of osteogenesis. A study of Ohara et al. [33] compared quantity and structures of food-derived gelatin hydrolysates in human blood from fish scale, fish skin and porcin skin type I collagen in a single blind crossover study. Amounts of free Hyp and Hyp-containing peptide were measured over a 24-h period. Hyp-containing peptides comprised approximately 30% of all detected Hyp. The total area under the concentration−time curve of the fish scale group was significantly higher than that of the porcine skin group. Pro-Hyp was a major constituent of Hyp-containing peptides. Ala-Hyp, Leu-Hyp, Ile-Hyp, Phe-Hyp, and Pro-Hyp-Gly were detected only with fish scale or fish skin gelatin hydrolysates. Ala-Hyp-Gly and Ser-Hyp-Gly were detected only with fish scale gelatin hydrolysate. The quantity and structure of Hyp-containing peptides in human blood after oral administration of hydrolyzed collagen depends on the collagen source. But this effect depends not only on collagen origin but also on the molecular size of the hydrolyzed collagens, proving that small peptides interact with bone cells, and not all the collagen molecule. Collagen needs to be hydrolysate to be able to interact with bone metabolism. In our studies, all the tested collagens were able to increase osteoblast activity but PCH-N was the most efficient *in vitro*. These results are in line with previous observations showing that type I collagen was able to increase MG-63 osteoblastic cell line differentiation [34]. Similar observations were also reported with osteoblasts grown on collagen type I films compared to a plastic support with an improvement in various bone markers including increased ALP activity and an accelerated and uniform mineralization of the bone matrix [35]. Moreover, the present results showed that PCH-N hydrolyzed collagen did not modify osteoclast growth but reduced osteoclast differentiation. As observed using the *in vitro* BD BioCoat™ Osteologic™ bone cell culture system, this combination of a stimulation of osteoblast activity with a reduced osteoclast differentiation led to reduced bone resorption in the presence of PCH-N hydrolyzed collagen.

The present study furthermore showed that hydrolyzed collagen has an effect on bone resorption factors and supports the idea that it stimulates osteoblast activity. Osteoclast growth was not modified. However, we reported a lower differentiation and maturation of osteoclasts. This effect, combined with increased osteoblast activity, is likely to modulate bone turnover leading to the growth of the external diameter of cortical bone. Several potential mechanisms can be proposed to explain the influence of hydrolyzed collagen-derived peptides on bone metabolism. Some results have suggested that ingestion of type I hydrolyzed collagen leads to the production and absorption of collagen-derived peptides similar to peptides released from type I collagen *in situ* during bone resorption which act on bone cell metabolism [6]. Osteoblast activity involves three steps including proliferation, matrix protein synthesis (type I collagen and proteoglycans) and mineralization of the bone matrix (hydroxyapatite) [36-38]. Several hormones and cytokines can modulate osteoblast and osteoclast differentiation and activity. The cytokine TGF-β which is stored in a latent form in the bone matrix, and secreted during the bone resorption phase is believed to exert such an effect [39]. TGF-β stimulates type I collagen and proteoglycan production while inhibiting that of hydroxyapatite. Interestingly, the type I collagen-derived peptide DGEA (asparagine, glycine, glutamine and alanine) was shown to interact with α2β1 integrin located on the osteoblast cell membrane. This interaction leads to inhibition of TGF-β and consequently bone matrix protein synthesis [2,3,10,40]. Other results showed that hydrolyzed collagen-derived peptides increase type I collagen and proteoglycan production but in this case the active peptides remains to be identified [1].

Taken together, the results of the present study indicate that hydrolyzed collagen modulates bone formation and mineralization of the bone matrix by stimulating osteoblast growth and differentiation while reducing osteoclast differentiation. These effects led to growth of the external diameter of the cortical zone. These results could be of potential interest for nutritional intervention in the prevention of bone loss.

## References


[1] Nomura Y, et al. Increase in bone mineral density through oral administration of shark gelatin to ovariectomized rats. Nutrition 2005;21(11-12):1120–6.
[2] Takeuchi Y, Nakayama K, Matsumoto T. Differentiation and cell surface expression of transforming growth factor-beta receptors are regulated by interaction with matrix collagen in murine osteoblastic cells. J Biol Chem 1996;271(7):3938–44.
[3] Takeuchi Y, et al. Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem 1997;272(46):29309–16.
[4] Wu J, et al. Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. J Bone Miner Metab 2004;22(6):547–53.
[5] Koyama Y, et al. Ingestion of gelatin has differential effect on bone mineral density and body weight in protein undernutrition. J Nutr Sci Vitaminol (Tokyo) 2001;47(1):84–6.
[6] Adam M, et al. Postmenopausal osteoporosis. Treatment with calcitonin and a diet rich in collagen proteins. Cas Lek Cesk 1996;135(3):74–8.
[7] Bonjour JP. Dietary protein: an essential nutrient for bone health. J Am Coll Nutr 2005;24(6 Suppl):526S–36S.
[8] Thissen JP, Ketelslegers JM, Underwood LE. Nutritional regulation of the insulin-like growth factors. Endocr Rev 1994;15(1):80–101.
[9] Schurch MA, et al. Protein supplements increase serum insulin-like growth factor-I levels and attenuate proximal femur bone loss in patients with recent hip fracture. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 1998;128(10):801–9.
[10] Oesser S, et al. Oral administration of (14)C labeled gelatin hydrolysate leads to an accumulation of radioactivity in cartilage of mice (C57/BL). J Nutr 1999;129(10):1891–5.
[11] Iwai K, et al. Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. J Agric Food Chem 2005;53(16):6531–6.
[12] Aito-Inoue M, et al. Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane. J Pept Sci 2007;13(7):468–74.
[13] Postlethwaite AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. Proc Natl Acad Sci U S A 1978;75(2):871–5.
[14] Laskin DL, et al. Chemotactic activity of collagen-like polypeptides for human peripheral blood neutrophils. J Leukoc Biol 1986;39(3):255–66.
[15] Postlethwaite AE, Kang AH. Collagen-and collagen peptide-induced chemotaxis of human blood monocytes. J Exp Med 1976;143(6):1299–307.
[16] Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J Biochem 2001;129(1):133–8.
[17] Kim SK, et al. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. J Agric Food Chem 2001;49(6):2992–7.
[18] Oshima G, Shimabukuro H, Nagasawa K. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. Biochim Biophys Acta 1979;566(1):128–37.
[19] Knight CG, et al. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. Cardiovasc Res 1999;41(2):450–7.
[20] Nagy TR, Clair AL. Precision and accuracy of dual-energy X-ray absorptiometry for determining in vivo body composition of mice. Obes Res 2000;8(5):392–8.
[21] Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone 1993;14(4):595–608.
[22] Di Masso RJ, et al. Long-bone biomechanics in mice selected for body conformation. Bone 1997;20(6):539–45.
[23] Dimai HP, et al. Alkaline phosphatase levels and osteoprogenitor cell numbers suggest bone formation may contribute to peak bone density differences between two inbred strains of mice. Bone 1998;22(3):211–6.
[24] Blais A, et al. Oral bovine lactoferrin improves bone status of ovariectomized mice. Am J Physiol Endocrinol Metab 2009.
[25] Beaupied H, Lespessailles E, Benhamou CL. Evaluation of macrostructural bone biomechanics. Joint Bone Spine 2007;74(3):233–9.
[26] Currey JD. Bone strength: what are we trying to measure? Calcif Tissue Int 2001;68(4):205–10.
[27] Burr DB. The contribution of the organic matrix to bone's material properties. Bone 2002;31(1):8–11.


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[28] Mann V, et al. A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. J Clin Invest 2001; 107(7):899–907.
[29] Voide R, van Lenthe GH, Muller R. Differential effects of bone structural and material properties on bone competence in C57BL/6 and C3H/He inbred strains of mice. Calcif Tissue Int 2008;83(1):61–9.
[30] Bouxsein ML, et al. Ovariectomy-induced bone loss varies among inbred strains of mice. J Bone Miner Res 2005;20(7):1085–92.
[31] Li CY, et al. Genetic background influences cortical bone response to ovariectomy. J Bone Miner Res 2005;20(12):2150–8.
[32] Ward WE, et al. Serum equol, bone mineral density and biomechanical bone strength differ among four mouse strains. J Nutr Biochem 2005;16(12):743–9.
[33] Ohara H, Matsumoto H, Ito K, Iwai K, Sato K. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. J Agric Food Chem 2007;55(4):1532–5.
[34] Andrianarivo AG, et al. Growth on type I collagen promotes expression of the osteoblastic phenotype in human osteosarcoma MG-63 cells. J Cell Physiol 1992;153(2):256–65.
[35] Lynch MP, et al. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res 1995;216(1):35–45.
[36] Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 1993;14(4):424–42.
[37] Owen TA, et al. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 1991;128(3): 1496–504.
[38] Quarles LD, et al. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 1992;7(6):683–92.
[39] Oreffo RO, et al. Activation of the bone-derived latent TGF beta complex by isolated osteoclasts. Biochem Biophys Res Commun 1989;158(3):817–23.
[40] Xiao G, et al. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 1998;273(49):32988–94.

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