

BIOLOGICAL EFFECT OF HYDROLYZED COLLAGEN ON BONE METABOLISM

Journal:	<i>Critical Reviews in Food Science and Nutrition</i>
Manuscript ID:	BFSN-2015-1585.R1
Manuscript Type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Daneault, Audrey; INRA, Coxam, Véronique; INRA, Wittrant, Yohann; INRA, Centre de Recherche Clermont-Ferrand/Theix
Keywords:	Osteoporosis, Collagen, Hydrolyzed collagen, Bone

SCHOLARONE™
Manuscripts

BIOLOGICAL EFFECT OF HYDROLYZED COLLAGEN ON BONE METABOLISM

Abstract

Osteoporosis is a chronic and asymptomatic disease characterized by low bone mass and skeletal microarchitectural deterioration, increased risk of fracture and associated co-morbidities most prevalent in the elderly. Due to an increasingly aging population, osteoporosis has become a major health issue requiring innovative disease management. Proteins are important for bone by providing building blocks and by exerting specific regulatory function. This is why adequate protein intake plays a considerable role in both bone development and bone maintenance. More specifically, since an increase in the overall metabolism of collagen can lead to severe dysfunctions and a more fragile bone matrix and because orally administered collagen can be digested in the gut, cross the intestinal barrier, enter the circulation and become available for metabolic processes in the target tissues, one may speculate that a collagen-enriched diet provides benefits for the skeleton. Collagen-derived products such as gelatin or hydrolyzed collagen (HC) are well acknowledged for their safety from a nutritional point of view, however what is their impact on bone biology? In this manuscript, we critically review the evidence from literature for an effect of HC on bone tissues in order to determine whether HC may represent a relevant alternative in the design of future nutritional approaches to manage osteoporosis prevention.

Abbreviation: Hydrolyzed Collagen (HC)

Keywords: Collagen · Hydrolyzed collagen · Bone · Osteoporosis

1. A need for alternative osteoporosis treatments

Osteoporosis is considered as a major cause of morbidity, disability and as an important contributor to medical care costs in many regions of the world. Its prevalence increases with age and the disease is twice more common in women than in men because of the hormonal changes that occur during menopause (Kanis, 1994). It has been defined as a skeletal disorder characterized by a low bone mineral density and micro-architectural alterations of bone tissue predisposing to an increased risk of fracture (1993; NIH, 2000). Several drugs are available for the treatment of osteoporosis such as bisphosphonates or parathyroid hormone derivatives. However, it has been highlighted that compliance to such therapy is usually poor and that the benefit does not continue after the end of treatment. This is why there is an increasing rationale to focus on early prevention to avoid or delay limitations of skeletal functions, rather than to curative strategies. However, classical prophylaxis with hormone replacement therapy is restricted due to concerns about an increased risk for cancer and cardiovascular disease. This is why health professionals strongly advocate the implementation of new strategies with proven scientific and clinical value for the prevention of osteoporosis (Coxam, Davicco, & Demigne, 2008). In this light, food has multiple assets for good compliance. Over the past 30 years research in nutrition has led to an exciting progress supporting the hypothesis that dietary intervention, including dietary supplements, can modulate specific target functions in the body and thus reduce the risk of disease. In this line dietary intervention may offer an effective means to deal with the problem of osteoporosis and its consequential health costs. A nutritional approach has been shown to be a cost-effective way of reducing calcium and vitamin D insufficiency, and thereby improving bone health and reducing fracture risk (Lotters et al., 2013). The primary goal of a nutritional strategy for the prevention of bone loss is to provide a sufficiently bioavailable amount of constitutive elements such as calcium, proteins as well as nutrients endowed with specific bone sparing properties (proteins, some fatty acids, micronutrients...) (Coxam et al., 2008; Nieves, 2013). Based on this concept and because proteins play a major role in bone by providing building blocks and by exerting specific regulatory functions collagen may provide a new option for aging consumers to maintain good health. Nevertheless, the scientists need to provide a high level of proof based on clinical trials, preclinical investigations and mechanistic studies to establish a health claim.

2. Collagen and bone biology

2.1. Collagen structure is associated with bone mechanical properties

Collagen comprises of three polypeptide strands (alpha-chains) which form a unique triple-helical structure (Fig. 1). To wind into a triple helix, the chains must contain glycine as every third residue thus presenting the repeating structure Gly-X-Y (Exposito, Valcourt, Cluzel, & Lethias, 2010), in which X and Y are mainly proline (Pro) and hydroxyproline (Hyp) (Gelse, 2003). The resulting Gly-Pro-Hyp triplet is the most frequent (10.5%) (Ramshaw, Shah, & Brodsky, 1998). In addition, the amino-acids Lys, Gln and Arg show a periodic distribution of 18 residues (Ottani, Martini, Franchi, Ruggeri, & Raspanti, 2002; Ramshaw et al., 1998). Collagens represent 30% of the total protein mass in the body (Ricard-Blum, 2011) and are therefore the most abundant proteins in mammals. They are the major structural element in the extracellular matrix of all connective tissues, including bone where they represent about 80% of the total protein (Tzaphlidou, 2005). While the mineral content mainly determines bone stiffness and rigidity, collagens provide skeletal toughness. Basically, they form the scaffold for the attachment of cells and the anchorage of macromolecules, defining the shape of the tissue. Collagen fibers in bone are organized in concentric layers providing maximal resistance against torsional and compressive stress (Bailey, 2001). Within the fibers, the collagen molecules are precisely aligned in a quarter-staggered end-overlap manner. This arrangement provides holes within the fiber for the nucleation of calcium apatite crystals.

As a matter of fact, the term “collagen” comprises a large and still growing family of proteins. They all share the same feature: a right-handed triple helix composed of alpha-chains assembled into a rope-like figure bordered by the C- and N-propeptides (Shoulders & Raines, 2009) (Fig. 1). However, if the average collagen molecule measures 300 nm in length (corresponding to about 1000 amino acids) and 1.5 nm in diameter, the length of the triple helical part varies considerably between the different collagen types (Exposito et al., 2010; Ottani et al., 2002). Collagen types, their distribution and composition are listed in Table 1S (supplemental data). In bone approximately 95% is type I collagen (a heterotrimer formed by two identical $\alpha 1(I)$ - chains and one $\alpha 2(I)$ -chain) providing viscoelastic strength, torsional stiffness, and load bearing capacity while also presenting nucleation sites for crystalline deposition. Type II collagen is also involved in bone formation, even though it is mainly found in cartilage (Álvarez et al., 2000; Aszódi, Chan, Hunziker, Bateman, & Fässler, 1998). Types III, V (Kahai, Vary, Gao, & Seth, 2004), VI (Keene, Sakai, & Burgeson, 1991), and X (Rosati et al., 1994) are present in bone at a very low level. Unlike type I, collagen type III fibrils are less ordered, thinner, and always combined with other collagen types. Association of types III and VI are characteristic for some regions of mature bone (for example rat proximal femur) (Luther, Saino, Carter, & Aaron, 2003). The function of type V collagen

1
2
3 is not well defined (Niyibizi & Eyre, 1994). Type VI is a microfibrillar collagen which seems to line the matrix
4 surrounding the osteocytes and their canaliculi (Keene et al., 1991). Finally, according to Hjorten *et al.*, type
5 XXVII collagen is found during cartilage calcification and the transition of cartilage into bone during
6 osteogenesis as well as in cartilage modeling during endochondral bone formation (Hjorten et al., 2007).
7
8
9

10 11 12 2.2. Collagen network alterations lead to bone fragility

13
14 In bone, collagen plays an important role in the force transmission and tissue structure maintenance.
15 Importantly, it determines the amount of mineral deposition. Thus the capacity of bone to resist mechanical
16 forces and fractures depends not only on the quantity of bone tissue (mineralization) but also on its quality
17 (organization of the collagen framework) (Currey, 2003; Viguet-Carrin, Garnero, & Delmas, 2006).
18
19

20
21 During aging, changes in the collagen network reduce bone mechanical strength and elasticity, which
22 contributes to the occurrence of osteoporotic fractures (Wang, Shen, Li, & Agrawal, 2002). In postmenopausal
23 osteoporosis there is growing evidence that at the material level, the volume fraction of mineral and the relative
24 amounts of mature and immature collagen crosslinks are affected by the tissue turnover rate, thus contributing to
25 bone fragility (Viguet-Carrin et al., 2006). Indeed, estrogen deficiency has been shown to affect collagen
26 stability by decreasing its maturation rate (Sanada et al., 1978). Luther *et al.* observed a disconnection of the
27 collagen fibers after ovariectomy (Luther et al., 2003). In the same line, Kafantari's group reported structural
28 changes in fibril architecture as well as diameter due to altered crosslinks and hydroxylation in the
29 ovariectomized rat (Kafantari, Kounadi, Fatouros, Milonakis, & Tzaphlidou, 2000). Moreover, in inflammation-
30 mediated osteoporosis, severe alterations were detected at the ultrastructural level in bone and skin collagen
31 fibrils in rabbits (Fountos, Kounadi, Tzaphlidou, Yasumura, & Glaros, 1998).
32
33
34
35
36
37
38
39
40
41

42 Regarding the mechanisms involved in ageing, Knott *et al.* highlighted an increase in the overall
43 metabolism of collagen which may account for impaired post-translational modifications, leading to severe
44 dysfunctions in the collagen network and a more fragile bone matrix (Knott & Bailey, 1998). Altered post-
45 translational modifications hamper the formation of cross-links between collagen molecules based on aldehyde
46 formation from specific telopeptide hydroxylysine or lysine residues (Knott & Bailey, 1998) and include an
47 abnormal increase in lysyl hydroxylation or glycosylation, which are key to sustain the structural and mechanical
48 integrity of the collagen network (M. Saito & Marumo, 2010; Yeowell & Pinnell, 1993). These alterations lead
49 to thinner fibrils and higher bone fragility. Another age-related non-enzymatic modification of collagen is the
50 formation of advanced glycation end products (AGE) via the so-called Maillard reaction, due to the
51
52
53
54
55
56
57
58
59
60

1
2
3 accumulation of reducible sugars in bone tissue (Viguet-Carrin et al., 2006). In addition, racemization
4 (spontaneous conversion of the L-enantiomeric form to the biologically rare D-form) and isomerization (transfer
5 of the peptide backbone from the aspartyl residue alpha-carboxyl group to the side chain beta- or gamma-
6 carboxyl group) occur during aging, resulting in structurally altered forms of the collagen molecule with
7 disrupted function (Viguet-Carrin et al., 2006).
8
9

10
11 The knowledge of certain genetic diseases further emphasizes the importance of correctly formed
12 collagen. The replacement of just one glycine residue by another amino acid can lead to pathologies such as
13 osteogenesis imperfecta and the Ehlers-Danlos Syndrome which are characterized by bone fragility, weak
14 tendons and thin skin (Gautieri, Buehler, & Redaelli, 2009). Subtypes of the Ehlers-Danlos Syndrome are linked
15 to mutations in type I or type III collagens, lysyl hydroxylase, or procollagen N-proteinase (Yeowell & Pinnell,
16 1993). Type VI collagen deficiency results in a disorganized collagen arrangement suggesting that collagen type
17 VI contributes to maintain bone mass (Izu et al., 2012). Mutations in COL1A1 coding for the $\alpha 1(I)$ -chain) and
18 COL1A2 (coding for the $\alpha 2(I)$ -chain) are linked to osteogenesis imperfecta, a group of brittle bone diseases.
19 Further, a polymorphism in the Sp1 binding site of the COL1A1 gene results in the synthesis of altered collagen
20 with a possible association to both decreased bone strength and bone mineral density and has thus been
21 postulated to play a role in osteoporosis (Mann et al., 2001). In summary, mutations in genes that encode
22 individual chains of triple-helical bone collagens as well as in genes encoding proteins involved in the
23 intracellular and extracellular modifications of the molecule are associated with heritable diseases of the skeletal
24 tissues and the development of skeletal abnormalities (Arnold & Fertala, 2013). These data emphasize the major
25 role of collagen quantity and quality in bone remodeling.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43

44 **3. Collagen in nutrition and food supplements**

45 Collagen-derived ingredients (gelatin and HC) are widely used in food, cosmetic and pharmaceutical
46 industries or tissue engineering thanks to their gelling capacity (gel formation, texturizing, thickening and water
47 binding capacities) as well as their surface (emulsion, foam formation and stabilization, adhesion and cohesion,
48 protective colloid function and film forming capacity) and hydration properties (swelling and solubility). The
49 terms “hydrolyzed gelatin”, “collagen hydrolysate”, “hydrolyzed collagen” or sometimes “collagen peptides”
50 used in publications designate the same product. Gelatin is obtained by a partial thermal hydrolysis of collagen
51 which (partially) separates the chains by destroying the crosslinks (Fig. 2). Subsequently, gelatin is extracted,
52
53
54
55
56
57
58
59
60

1
2
3 purified and dried (Karim & Bhat, 2009). Two types of gelatin with different characteristics can be
4 manufactured. Type A gelatin is produced from acid-treated collagen, while alkali-treatment forms type B
5 gelatin. The extraction process (temperature, time, and pH) can influence the length of the polypeptide chains
6 and the functional properties of the gelatin. This is why the quality of a gelatin preparation depends on the
7 manufacturing method, but also from which species or tissue it is extracted (Gómez-Guillén, Giménez, López-
8 Caballero, & Montero, 2011). For instance, shark gelatin has different characteristics than pig gelatin
9 (Yoshimura et al., 2000).

10
11 To form HC, gelatin is submitted to an enzymatic hydrolysis, the most commonly used proteases being
12 trypsin, chymotrypsin, pepsin, alcalase, properase E, pronase, collagenase, bromelain and papain (Gómez-
13 Guillén et al., 2011). HC is usually presented as a white powder with good solubilisation properties, commonly
14 used as a dietary supplement or included in various foodstuffs. Like for gelatin, HC molecular weight
15 distribution, structure and composition, and subsequently functional properties, depend on the processing
16 conditions as well as the raw material and the specificity of the enzyme used to hydrolyze the gelatin (Denis et
17 al., 2008). The average molecular weight of HC ranges between 2,000 and 6,000 Daltons (Moskowitz, 2000).
18 The most abundant sources of gelatin or HC are derived from mammals such as pig skin (46%), bovine hide
19 (29.4%) and pork and cattle bones (23.1%) (Gómez-Guillén et al., 2011). However, the demand for alternative
20 sources has increased after the bovine spongiform encephalopathy (BSE) crisis and for religious and cultural
21 reasons (Karim & Bhat, 2008; Mhd Sarbon, Badii, & Howell, 2013). Production from non-mammalian species,
22 for instance from fish, is thus of growing importance (Mhd Sarbon et al., 2013; Nagai & Suzuki, 2000; Singh,
23 Benjakul, Maqsood, & Kishimura, 2011; Zeng et al., 2012).

24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 *3.1. Safety*

43 Gelatin, and by extension HC, have been approved as Generally Recognized As Safe (GRAS) by the US Food
44 and Drug Administration (USFDA) Center for Food Safety and Nutrition. Indeed, there is no documented
45 evidence of a deleterious effect from the ingestion of HC other than a rare allergy, sensation of unpleasant taste
46 or feeling of heaviness in the stomach. In a multicenter, randomized, parallel, placebo-controlled clinical trial, of
47 389 patients who were orally treated with 10g HC or placebo over a period of 6 months, only 12 dropped out due
48 to side effects and among those 9 had received the placebo (Moskowitz, 2000). Comparably, in a multicenter,
49 randomized, parallel, double-blind study carried out on one hundred male and female volunteers aged ≥ 40 years
50 with knee osteoarthritis, HC was well tolerated (Trc & Bohmova, 2011). Recorded adverse events were similar
51
52
53
54
55
56
57
58
59
60

whether the volunteers were given 10g HC daily or glucosamine sulphate for 90 consecutive days (Trc & Bohmova, 2011). HC tolerability has also been assessed in various animal studies. Acute, subacute, mutagenic and teratogenic toxicity analyses have not indicated any health risk. Indeed, Takeda *et al.* studied the acute and subacute toxicity of collagen from bovine derm, showing no marked deleterious effect except for local irritation which was seen only after parenteral administration (U. Takeda et al., 1982). In the same line, Wu *et al.* described the high safety of oral HC administration in a rat model when given 1660 mg/kg body weight per day (corresponding to about 10 times the currently used doses in humans). Notwithstanding, rats could exhibit kidney hypertrophy at a dose of 100 times the recommended daily intake (166 mg/kg body weight per day) (Wu, Fujioka, Sugimoto, Mu, & Ishimi, 2004). Schauss *et al.* also conducted two acute and subchronic oral toxicity investigations in rats with hydrolyzed chicken sternal cartilage which contains mostly type II collagen (Schauss, Merkel, Glaza, & Sorenson, 2007). With a single dose of 5000 mg/kg, all the animals survived without any major pathological lesions, exhibiting a normal body weight gain throughout the study. Regarding subchronic toxicity, all the animals survived and showed no significant changes in body weight or histopathology, whether they were administered 0, 30, 300 or 1000 mg/day of the test product per kg of body weight for over 90 days. Additionally, the risk for chronic toxic effects was not higher in marine HC-treated rats at concentrations of 2.25%, 4.5%, 9% and 18% (wt/wt) in the diet (equivalent to 1063, 2216, 4609 and 8586 mg/kg·body weight/day for females, and 0907, 1798, 3418, 6658 mg/kg·bw/day for males, respectively), than in those fed the basal rodent diet (Liang et al., 2012). Note however that cardiac arrhythmias have been observed in 3 of 6 subjects receiving 300 kcal/ day as HC (equivalent to 75 g/day, ie all the protein intake in the form of collagen) supplemented with triptophane, calcium, magnesium, phosphorus, potassium, vitamins (Lantigua, Amatruda, Biddle, Forbes, & Lockwood, 1980). Deaths have even been registered in obese adults who were reducing their body weight by means of diets that provided same amounts of collagen or gelatin hydrolysates (300 to 500 kcal/day) without any supplementation in micronutrients (Van Itallie & Yang, 1984).

3.2. Bioavailability

Orally administered HC are digested in the gut, cross the intestinal barrier, enter the circulation and become available for metabolic processes in the target tissues. Even though HC does not contain all the essential amino acids (tryptophan is not present, and cysteine only in small amounts), it is often used to supplement other proteins because of its high digestibility, good consumer tolerance and its specific amino-acid content (high Hyp, Pro and Gly) (Iwai et al., 2005; Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). As a matter of fact, ingestion of a

1
2
3 protein hydrolysate, as opposed to its intact form, accelerates the digestion and the absorption from the gut,
4
5 increases postprandial amino acid bioavailability and tends to improve the incorporation rate of dietary amino
6
7 acids into target tissues (Koopman et al., 2009). This concept was confirmed by Urao *et al.* who found that
8
9 intestinal permeability followed a different pattern for small molecular weight particles than for large molecules
10
11 in rabbits, suggesting there may be different mechanisms of intestinal transport for molecules of different size
12
13 (Urao, Okuyama, Drongowski, Teitelbaum, & Coran, 1997). It has been proposed that HC peptides are only
14
15 digested to a certain degree within the gastrointestinal tract, with a proportion of intact high-molecular-weight
16
17 proteins reaching the blood by passing through the enterocyte (transcytosis) at a level of approximately 10%
18
19 (Moskowitz, 2000) (Fig. 3). Oesser *et al.* demonstrated that 95% of orally applied HC were resorbed within 6
20
21 hours from the gastro-intestinal tract of mice (Oesser, Adam, Babel, & Seifert, 1999). Just one hour after oral
22
23 administration already 47% had been absorbed. Iwai *et al.* provided evidence in humans, that oral ingestion of
24
25 HC significantly increased the peptide form of Hyp in blood with a maximum level after 1-2 hours and a
26
27 decrease to half of the maximum level after 4 hours (Iwai et al., 2005).

28
29 Subsequent to oral ingestion of HC in rodents and humans, various studies have shown that HC-derived
30
31 amino acids, as well as di- and tripeptides can be detected in blood as well as in various target tissues such as
32
33 cartilage (Oesser et al., 1999), skin (Kawaguchi, Nanbu, & Kurokawa, 2012) or kidney (Watanabe-Kamiyama et
34
35 al., 2010). The major collagen-derived dipeptide found in plasma is Pro-Hyp (Ichikawa et al., 2010; Iwai et al.,
36
37 2005; Shigemura et al., 2011). It is highly resistant to hydrolysis and is not digestible by peptidase (Aito-Inoue,
38
39 Lackeyram, Fan, Sato, & Mine, 2007) like other Hyp-containing peptides (Ohara et al., 2007). In addition, small
40
41 amounts of other di- and tripeptides such as Ala-Hyp, Ala-Hyp-Gly, Leu-Hyp, Ile-Hyp, Phe-Hyp, Pro-Hyp-Gly,
42
43 (Iwai et al., 2005) and Gly-Pro-Hyp can be detected (Ichikawa et al., 2010; Watanabe-Kamiyama et al., 2010).
44
45 Another peptide, Hyp-Gly, was more recently discovered in human plasma upon collagen ingestion (Shigemura
46
47 et al., 2011; Sugihara, Inoue, Kuwamori, & Taniguchi, 2012). It is noticeable that the average plasma
48
49 concentration of those peptides is dose-dependent: Hyp-containing peptides reach maximum levels of 6.43,
50
51 20.17 and 32.84 nmol/mL following ingestion of 30.8, 153.8 and 384.6 mg doses of HC, respectively
52
53 (Shigemura et al., 2011; Shigemura, Kubomura, Sato, & Sato, 2014). Moreover, the quantity and structure of
54
55 such Hyp-containing peptides in human blood after oral administration of HC depends on the source; for
56
57 example, Ala-Hyp-Gly and Ser-Hyp-Gly were detected only from fish scale gelatin hydrolysate, Ala-Hyp and
58
59 Pro-Hyp-Gly from fish scale or fish skin gelatin hydrolysates, whereas Leu-Hyp, Ile-Hyp and Phe-Hyp appeared
60
61 after ingestion of fish and to a lower level porcine skin originated HC (Ohara et al., 2007). Finally, synergistic

1
2
3 effects with the food matrix can occur and improve HC absorption, for instance when HC is provided within
4 fermented milk (Walrand, Chiotelli, Noirt, Mwewa, & Lassel, 2008).
5
6
7

8 3.3. *Hydrolyzed collagen reaches the bone tissue*

9
10 To synthesize a single picogram of collagen type II, more than 1 billion glycine molecules and 620
11 million proline molecules are required. In the absence of these amino acids, the anabolic phase of collagen
12 metabolism can be impaired (Clark, 2007). Proline and Hydroxyproline serves to stabilize the collagen triple
13 helix, their structure constrain the rotation of the polypeptide collagen chain and creates and strengthens the
14 helical characteristics of the molecule. Proline biosynthesis is related to both the citric acid cycle and the urea
15 cycle. In looking at other proline biosynthetic pathways, the arginine / ornithine / glutamic semialdehyde / proline
16 pathway looks the most promising (Barbul, 2008). As a matter of fact, orally consumed HC has not only been
17 shown to be well absorbed in the intestine, but also to accumulate in target tissues. *Kawaguchi et al.* studied the
18 biodistribution of orally administered [¹⁴C]Pro-Hyp in rats using autoradiography (Kawaguchi et al., 2012). They
19 observed a wide distribution of radioactivity at 30 min post-dose and a cellular uptake of radioactivity in
20 osteoblasts and osteoclasts as well as in dermal fibroblasts, epidermal cells, synovial cells and chondrocytes after
21 24 h. In addition, according to Watanabe's group, absorption of low molecular weight HC in the ovariectomized
22 rat was associated with an increased content of the organic substance in bone (Watanabe-Kamiyama et al.,
23 2010). Finally, Barbul has shown that during the early phases of wound healing, wound fluid proline levels are at
24 least 50% higher than plasma levels, suggesting active import of proline into the wound (Barbul, 2008).
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

42 4. **Hydrolyzed collagen affects bone biology**

43 4.1. *Evidence from animal models*

44
45 Growth models: young growing rats are potential models to study factors that can influence bone mass
46 accrual and thereby affect peak bone mass (table 1). In growing male rats, HC supplementation has been
47 described to promote the development of long bones (Xu, Han, & Li, 2010). The reported increase in size, dry
48 weight, ash weight, bone mineral density and both stiffness and toughness of femurs was likely related to an
49 increased osteoblast activity rather than a decreased rate of bone resorption, since higher serum osteocalcin and
50 bone-specific alkaline phosphatase (BALP) content was observed with no significant difference in N-terminal
51 telopeptide of type I collagen (NTX). Leem *et al.* confirmed a dose-dependent effect of a selected HC with a
52
53
54
55
56
57
58
59
60

1
2
3 molecular weight of <3 kDa on longitudinal bone growth and height of the growth plate in adolescent male rats,
4
5 whereas gelatin as such failed to produce the same effect (Leem, Lee, Jang, & Kim, 2013). Insulin like growth
6
7 factor -1 (IGF1) and bone morphogenetic protein-2 were highly expressed in the growth plate in the treated
8
9 group animals. Accordingly, Takeda *et al.* demonstrated that moderate HC consumption (20% protein in the diet
10
11 of which 30% was HC) increased bone mass during growth in rats and that running exercise further promoted
12
13 the effect. No further beneficial effect on bone mass was elicited with a higher HC intake (40% protein in the
14
15 diet of which 30% was HC) (S. Takeda, Park, Kawashima, Ezawa, & Omi, 2013). Finally, in the work published
16
17 by Wu *et al.* (Wu *et al.*, 2004), carried out in growing male rat, collagen peptides given at amounts equal to the
18
19 currently used doses in humans (166 mg/kg body weight per day) or at a 10 or even a 100 higher dose (1660 or
20
21 16660 mg/kg body weight per day), bone mineral density of the femur was significantly higher in the animals
22
23 given the highest dose.

24
25 Bone loss models: Most of the studies set up to study the effect of HC on bone loss have been carried out in
26
27 young OVX animals (table 1). Although, the only small animal model recommended by the FDA (Thompson,
28
29 Simmons, Pirie, & Ke, 1995) for preclinical evaluation of postmenopausal bone loss is the aged OVX rat model
30
31 because in marked contrast to postmenopausal women, growing rodents have very little, if any, bone remodeling
32
33 (Erben, 1996), young growing rats can provide useful information on the short-term effects of drugs on bone
34
35 resorption, calcium kinetics and balance, or calciotropic hormone levels. They can also be used to evaluate the
36
37 effects of interventions aimed at increasing osteoblastic recruitment and bone formation (Bonjour, Ammann,
38
39 & Rizzoli, 1999). As a matter of fact, ovariectomized (OVX) rodents are currently used as animal models to
40
41 study postmenopausal osteoporosis. Estrogen deficiency results in disorganized bone collagen fibrils of smaller
42
43 diameter in both trabecular and cortical bone (Garcia-Moreno *et al.*, 1995). In inflammation-mediated
44
45 osteoporosis (similar to senile osteoporosis), severe alterations at the ultrastructural level in bone and skin
46
47 collagen fibrils were detected in rabbits (Fountos *et al.*, 1998). A growing body of evidence demonstrates the
48
49 potential of collagen intake to prevent bone loss in models of estrogen deficiency.

50
51 Nomura *et al.* demonstrated the efficacy of shark skin gelatin to increase type I collagen and glycosaminoglycan
52
53 content as well as bone mineral density in the femur of OVX rats to a level comparable in the sham operated
54
55 group (Nomura, Oohashi, Watanabe, & Kasugai, 2005). In the same line, Han and colleagues tested cod gelatin
56
57 for 90 days in 3-month old female Sprague-Dawley OVX rats observing a preserved femoral neck bone mineral
58
59 density and trabecular microarchitectural properties in OVX rats fed a gelatin compared to a control diet (2009).
60

1
2
3 The beneficial effect was partly attributed to a significant reduction of pro-inflammatory cytokines (IL-1beta, IL-
4 6 and TNF-alpha) and a decreased urinary excretion of resorption markers (NTX, C telopeptides of type I
5 collagen (CTX) and deoxypyridinoline). As mentioned above, HC ingestion can increase the content of organic
6 substance in bone (Watanabe-Kamiyama et al., 2010). In OVX rats, HC supplementation at a level 10 times
7 higher than the human recommendations (i.e. 10g/day) unequivocally contributed to the conservation of
8 vertebral mass, protein content (including osteocalcin) and mechanical strength, not seen when gelatin was used
9 as a supplement (De Almeida Jackix, Cuneo, Amaya-Farfan, de Assuncao, & Quintaes, 2010). In the same
10 experimental model, Kim *et al.* observed a prevention of trabecular bone loss and improved microarchitecture of
11 the lumbar vertebrae (H. K. Kim, Kim, & Leem, 2013). Finally, Guillerminet *et al.* demonstrated that HC
12 administration to 3- month old OVX C3H/HeN mice increased bone mineral density and bone strength
13 (Guillerminet et al., 2010). The fact that plasma concentrations of CTX were lower while BALP levels were
14 higher under HC treatment suggested that collagen can improve bone remodeling. These data allow to test for
15 evidence of heterogeneity of bone turnover in such a condition of bone loss, and to attempt to devise an
16 'uncoupling index' by using the relationship between bone-specific biochemical markers of bone formation and
17 bone resorption. Indeed, where turnover markers are reported, bone formation and degradation markers should
18 always be reported in tandem (Eastell et al., 1993). In the present case, increased bALP levels, while CTX
19 decreased may indicate a net benefit to bone.
20
21
22
23
24
25
26
27
28
29
30
31
32
33

34
35
36 That is, it cannot be determined whether bone formation increased to a greater degree than resorption, suggesting
37 a net benefit to bone, or to a lesser degree, suggesting net harm to bone, or to a similar degree, suggesting bone
38 turnover remains tightly coupled.
39
40

41
42 A second study by the same group showed that the HC administration for 3 or 6 months significantly prevented
43 bone loss in OVX mice (Guillerminet et al., 2012). The authors further demonstrated that HC ingestion for 3
44 months is as efficient as raloxifene to protect 3-month-old OVX mice from bone loss. Such a bone sparing effect
45 was also seen as soon as 1 month post-surgery in a follow-up study (Daneault, Coxam, Fabien Soulé, & Wittrant,
46 2014). Finally, in a mice model of protein undernutrition, have shown that gelatin has differential effects on bone
47 mineral density compared to casein (6% casein + 4% gelatin having a greater effect than a 10% casein diet)
48 (Koyama et al., 2001).
49
50
51
52
53

54 Bone healing models: Tsuruoka *et al.* have shown that oral administration of HC to rats with femur
55 damage accelerated the fracture healing (Tsuruoka, Yamato, Sakai, Yoshitake, & Yonekura, 2007) (table 1).
56
57
58
59
60

1
2
3 Accordingly, a 3-week oral supplementation with High Advanced-Collagen Tripeptide (HACP), a soluble
4 powder containing about 20% of Gly-X-Y was beneficial for the bone healing process after a cortical bone
5 defect in rats (Hata et al., 2008).
6
7

8
9 Altogether these results from preclinical models provide a solid body of evidence that HC has a
10 promising potential to maintain a balanced bone turnover in different physiological settings (growth, bone loss,
11 healing) by promoting bone formation (the ratio of bone formation to resorption biomarkers being used to
12 represent the state of bone turnover). In those studies, the significant difference in such a ratio denotes an
13 improvement in bone turnover in favor of bone formation resulting from HC supplementation. Consequently,
14 like postulated by Elam et al. (Elam et al., 2014), HC may serve as an effective supplement for preventing bone
15 loss by significantly enhancing the organic substance content of bone. This could be explained by a
16 downregulation of the production of pro-inflammatory molecules such as interleukins- 1b and - 6, and tumor
17 necrosis factor- α . Because these cytokines in particular are responsible for upregulation of receptor activator for
18 nuclear factor kappa-B ligand (RANKL) for osteoclast recruitment, this may explain the noteworthy impairment
19 of bone loss
20
21
22
23
24
25
26
27

28 The key emerging question is whether these results can be extrapolated to the human situation.
29
30
31

32 4.2. Evidence from clinical trials 33

34 If HC has already been used as a food supplement to sooth pain in patients suffering from osteoarthritis,
35 to date very few clinical studies have evaluated its effects on bone metabolism (Bagchi et al., 2002; Bruyere,
36 Zegels, et al., 2012; Fujita, Ohue, Fujii, Miyauchi, & Takagi, 2002; Henrotin, Lambert, Couchourel, Ripoll, &
37 Chiotelli, 2011; Moskowitz, 2000; Trc & Bohmova, 2011) (table 2). In most of the studies, HC is applied in
38 association with other compounds like drugs or food supplements (Elam et al., 2014; Hooshmand et al., 2013).
39
40
41
42

43 In a first clinical investigation, the effects of calcitonin alone or in combination with a HC-rich diet
44 were studied on bone metabolism in postmenopausal women. The results revealed that a daily ingestion of 10g
45 HC associated with intra-muscular injection of calcitonin (100 UI) twice a week for 24 weeks enhanced and
46 prolonged the effect of the drug as shown by a fall in urinary pyridinoline cross-link levels (Adam, Spacek,
47 Hulejova, Galianova, & Blahos, 1996). Next, Fujita *et al.* evaluated the effect of a daily supplementation with
48 900 mg absorbable algal calcium, 3.5 g collagen and other matrix components, including glucosamine (Fujita et
49 al., 2002). Urinary excretion of NTX was decreased in the supplemented group. In addition to the calcium-
50 mediated suppression of parathyroid hormone, collagen degradation was reduced by the inhibition of cytokine-
51
52
53
54
55
56
57
58
59
60

1
2
3 induced metalloproteinase release, including collagenase. Consistently, another study reported, that in osteopenic
4 post-menopausal women consumption of 5 g calcium/collagen mix (containing 500 mg of calcium carbonate and
5 5 µg vitamin D) for 3 months enhanced bone mass by orienting bone turnover towards formation rather than
6 resorption (increased BALP/TRAP5b ratio), compared to control volunteers (given 500 mg of calcium carbonate
7 and 5 µg vitamin D daily) (Hooshmand et al., 2013). However, in another investigation the daily ingestion of
8 only HC (10 g/d) for 24 weeks in osteopenic post-menopausal women did not produce any significant effect on
9 bone metabolism as assessed by resorption or formation biomarkers such as osteocalcin and BALP (Cuneo,
10 Costa-Paiva, Pinto-Neto, Morais, & Amaya-Farfan, 2010). The authors noticed that the majority of patients
11 exhibited an excess body weight (it is thus possible that they did not receive a sufficient dose) as well as
12 inadequate calcium intake, which could have been limiting for the HC effect. More recently, Elam et al (2014)
13 reported that long-term calcium collagen chelate supplementation together with vitamin D, may provide
14 protection against excessive bone loss and turnover (for which calcium and vitamin D alone could not prevent),
15 in postmenopausal women (Elam et al., 2014).

16
17
18
19
20
21
22
23
24
25
26
27 Finally, since the bone mass at a given age also depends on the peak bone mass acquired during growth,
28 investigating the effect of HC in children is of interest. Martin-Bautista *et al.* demonstrated in a 4-months
29 randomized double-blind study, that a daily intake of HC (with or without calcium) at key stages of growth and
30 development had a beneficial effect on bone remodeling (Martin-Bautista et al., 2011). The bone formation
31 factors Insulin-like growth factor 1 (IGF1) and BALP were higher in the group receiving HC when compared to
32 the placebo group.

33
34
35
36
37
38 Although the existing data on HC effects on bone health in humans is promising, the Group for the Respect of
39 Ethics and Excellence in Science has comprehensively outlined (Bruyere, Rizzoli, et al., 2012), that further, well
40 designed studies are warranted to strengthen the scientific evidence, also with regard to the pathways that
41 mediate HC effects on bone health.

42 43 44 45 46 47 4.3. Mechanisms involved in collagen effects on bone

48
49 Changes in bone cell behavior: Studies investigating the *in vitro* effect of hydrolyzed collagen provide
50 interesting data even though we must stay aware of the limitations of such approaches (table 3). As a matter of
51 fact, in the body, the bone cells are never exposed to collagen as usually used in these studies. Indeed, digestion
52 of dietary collagen in the gastro intestinal tract is followed by first-pass metabolism during absorption, and
53 bioactive molecules (i.e., proteins, peptides...) will appear in the circulation (Fig. 4). Therefore testing the effect
54
55
56
57
58
59
60

1
2
3 of serum from animals fed specific enriched diets on cellular outcomes should provide better information for
4 evaluation of dietary effect on specific organ. It should be noted that only Tsuruoka et al. (Tsuruoka et al., 2007)
5 considered a physiological form (collagen tripeptide).
6
7

8
9 Most of the studies investigating the effect of HC on bone cell metabolism have focused on bone
10 forming cells (osteoblasts) (Fig. 5). In 1998, Komori *et al.* reported that bone marrow stromal cells differentiate
11 into osteoblasts when cultured with type I collagen matrix (Komori & Kishimoto, 1998). Andrianarivo and
12 collaborators demonstrated concurrent biochemical changes in the human cell line MG-63 in response to type I
13 collagen exposure involving increased specific activity of cell-associated alkaline phosphatase and increased
14 secretion of osteonectin (up to 2.5-fold for each protein) (1992). Using osteoblasts derived from rat calvaria and
15 grown on collagen type I films, Lynch *et al.* defined the critical role of type I collagen in mediating the signaling
16 cascade for the expression of a mature osteoblast phenotype and the mineralization of the extracellular matrix in
17 a physiological manner (Lynch, Stein, Stein, & Lian, 1995). They described the temporal expression of genes
18 characterizing distinct periods of growth and differentiation. During the initial proliferation period, expression of
19 fibronectin, beta 1 integrin, and actin was decreased by 50 to 70% in cells grown on collagen. In contrast,
20 alkaline phosphatase enzyme activity was elevated during the proliferation period, while mRNA levels remained
21 low, suggesting a post-transcriptional regulation. In the postproliferative period, osteonectin, osteocalcin, and
22 osteopontin were up-regulated. These results strongly support that collagen I from bone extracellular matrix may
23 play an important role in osteoblastic differentiation and phenotypic expression.
24
25

26
27 Regarding HC, Fu *et al.* observed that salmon skin gelatin hydrolysates were capable to induce cell proliferation,
28 accelerated cell cycle progression and to inhibit cell apoptosis in human hFOB1.19 cells, especially when skin
29 HC were hydrolyzed with papain compared to other proteases (Fu & Zhao, 2013). Kim *et al.* confirmed the dose-
30 dependent effect of HC on human osteoblast proliferation (H. K. Kim et al., 2013; H. K. Kim, Kim, & Leem,
31 2014a) and recent data from our lab have provided evidence of both enhanced osteoblast differentiation and
32 proliferation as well as improved cell survival and viability by bovine HC (Daneault et al., 2014). In parallel, Liu
33 *et al.* demonstrated that bovine HC promotes osteoblast differentiation and mineralized bone matrix formation
34 (Liu et al., 2014). Accordingly, HC dose-dependently stimulates type I collagen mRNA expression and protein
35 production (H. K. Kim et al., 2014a; Tsuruoka et al., 2007; Yamada, Yoshizawa, et al., 2013) as well as alkaline
36 phosphatase activity (Guillerminet et al., 2010; H. K. Kim et al., 2013; Yamada, Nagaoka, et al., 2013).
37
38 Incubation of human osteoblasts with 0.1% fish HC increased osteocalcin, osteopontin, BMP-2 and integrin β
39 mRNA expression and accelerated matrix mineralization as compared to untreated cells (Yamada, Yoshizawa, et
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 al., 2013). Consequently, this translated into increased calcium disposal or mineralization in either human or
4 murine osteoblasts (Liu et al., 2014; Tsuruoka et al., 2007; Yamada, Nagaoka, et al., 2013; Yamada, Yoshizawa,
5 et al., 2013) (Fig. 5). In addition to an effect on osteoblasts, the impact of HC on osteoclast biology was
6 investigated. A significant inhibition of osteoclast formation and activity in cell lines and in primary cultures was
7 observed when incubated with bovine and porcine HC (Guillerminet et al., 2010) or with shark protein
8 hydrolysates (Uehara, Takahashi, Watanabe, & Nomura, 2014). Consistently, we recently found a higher
9 OPG/RANKL ratio after incubation of MC3T3 cells with bovine HC reflecting an unfavorable metabolic
10 orientation for osteoclast differentiation (Daneault et al., 2014). Similar to HC, in human osteoblastic MG-63
11 cells, other peptides such as egg yolk-derived peptides have been shown to stimulate early stages of the
12 osteogenic differentiation via the MAPK/ELK1 signaling pathway (up-regulation of genes responsible for bone
13 formation such as *ALPL*, *COL1A1*, and *SPPI*) and accelerate mineralization by hastening mineralization
14 initiation, subsequently leading to an increase in the extent of calcium deposition (H. K. Kim, Kim, & Leem,
15 2014b).

16
17
18
19
20
21
22
23
24
25
26
27 *Molecular mechanisms:* Interaction of the Asp-Gly-Glu-Ala amino acid domain of type I
28 collagen with the alpha2beta1 integrin receptor on the cell membrane was proven to be an important signal for
29 bone marrow cell differentiation towards an osteoblastic phenotype (Mizuno & Kuboki, 2001). Additionally,
30 HC-induction of the bone-specific transcription factor osterix was associated with the up-regulation of type I
31 collagen expression, thus providing insights into the molecular basis of HC action on osteoblasts (H. K. Kim et
32 al., 2014a; Tsuruoka et al., 2007; Yamada, Yoshizawa, et al., 2013). Bovine HC was shown to stimulate
33 osteoblast differentiation, mineralized bone matrix formation, ALP activity, and osteocalcin production through
34 increased Runx2 expression and activity (Liu et al., 2014). Activation of ERK1/2, JNK1/2, p38, and ELK1
35 phosphorylation in the human osteoblast cell line MG-63 by HC was correlated with increased COL1A1,
36 alkaline phosphatase, osteocalcin and osteopontin gene expression (H. K. Kim et al., 2014a). Extracellular
37 signal-regulated kinase (ERK) inhibitor abolished the HC-induced COL1A1 expression, thus supporting the
38 importance of the ERK/MAPK signaling pathway in mediating HC effects on osteoblast cells (H. K. Kim et al.,
39 2013). Furthermore, it cannot be excluded that, due to its richness in aromatic amino-acids (HYP), hydrolyzed
40 collagen can induce IGF1 production which consequently activate a calcium sensing receptor and in turn exert
41 an anabolic effect on bone as previously shown (Conigrave, Brown, & Rizzoli, 2008; Dawson-Hughes, Harris,
42 Rasmussen, & Dallal, 2007). Finally, HC appears to greatly impact osteoblast biology but the mechanisms
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 underlying their action are only partially understood. Besides, the impact of HC on osteoclasts remains to be
4 further investigated.
5
6
7

8 Other effects of hydrolyzed collagen: In addition to a direct modulation of bone cells, HC has been
9 shown to improve calcium absorption, another very important mechanism for preserving bone capital (G. H.
10 Kim et al., 1998). Indeed, epidemiological, isotopic and meta-analysis studies suggest that dietary protein works
11 synergistically with calcium to improve calcium retention and bone metabolism (Kerstetter, Kenny, & Insogna,
12 2011). For example, brush border membrane vesicle Ca uptake studies suggest that higher protein intake
13 improves Ca absorption, at least in part, by increasing transcellular Ca uptake (Gaffney-Stomberg et al., 2010).
14 Jung *et al.* isolated fish-bone peptides with a high affinity to calcium and a high content of phosphopeptide
15 (Jung, Lee, & Kim, 2006). Using ovariectomized rats, they observed a higher calcium retention and a
16 preservation of both bone mineral density and bone strength when rodents were supplemented with those
17 peptides. HC from both fish and shrimp were described to contain both, a biologically related calcitonin and/or
18 calcitonin-gene-related peptide (Fouchereau-Peron et al., 1999). Nevertheless this observation requires further
19 investigation with regards to the role of these peptide fragments in bone biology. Besides, HC derived from
20 chicken bones has been shown to reduce proinflammatory-cytokine production in mice (Zhang et al., 2010) and
21 recent works support natural antioxidative properties of HC peptides (Alemán, Giménez, Pérez-Santín, Gómez-
22 Guillén, & Montero, 2011; Ao & Li, 2012). In addition, as bone tissue function is closely linked to lipid
23 metabolism, it is worth noting that the Pro-Hyp peptide reduces the size of lipid droplets in mouse 3T3-L1
24 preadipocytes (Minaguchi et al., 2012) and that fish HC was found to affect lipid absorption and metabolism in
25 rats resulting in a lower transient increase of plasma triglycerides and associated inflammation (Masataka Saito,
26 Kiyose, Higuchi, Uchida, & Suzuki, 2009). Finally, it cannot be excluded that immunomodulation can be
27 involved. *In vitro* and *in vivo* studies have shown that certain peptide fractions in fish protein hydrolysates may
28 stimulate the nonspecific immune defense system (Khora, 2013). Indeed, according to Gómez-Guillén et al.
29 collagen and gelatin-derived peptides have numerous bioactivities, among which antimicrobial activity, mineral
30 binding capacity, a lipid-lowering effect, immunomodulatory activity and beneficial effects on skin, bone or joint
31 health (Gómez-Guillén et al., 2011).
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57

58 **5. Conclusion**

59
60

1
2
3 A growing body of evidence demonstrates that HC owns bioactive properties beneficial for bone tissue,
4 including stimulation of bone forming cells, improvement of calcium absorption, anti-inflammatory and
5 antioxidant capacities. Those properties render HC a new and innovative candidate for putative dietary
6 intervention in the prevention of osteoporosis. Still, many questions remain to be answered: what is the optimal
7 form of HC, what is the optimal dose? To date, we recently started to address these questions and identified that
8 origin and length of hydrolyzed collagen may play an important role in mediating positive action on bone
9 (unpublished data). In parallel, investigations of the signaling pathways involved in the bone sparing effect are
10 now required to further support these conclusions. Altogether, in the light of the increasing prevalence of
11 osteoporosis related to the worldwide increasing longevity, good candidates for dietary prevention are of
12 particular relevance. As such, HC could offer additional values to calcium and vitamin D, thus responding to the
13 growing demand for primary prevention.
14
15
16
17
18
19
20
21
22
23

24 25 26 **6. References**

- 27
28
29 Adam, M., Spacek, P., Hulejova, H., Galianova, A., & Blahos, J. (1996). [Postmenopausal osteoporosis.
30 Treatment with calcitonin and a diet rich in collagen proteins]. *Cas Lek Cesk*, 135(3), 74-78.
31
32 Aito-Inoue, M., Lackeyram, D., Fan, M. Z., Sato, K., & Mine, Y. (2007). Transport of a tripeptide, Gly-Pro-Hyp,
33 across the porcine intestinal brush-border membrane. *J Pept Sci*, 13(7), 468-474. doi: 10.1002/psc.870
34
35 Alemán, A., Giménez, B., Pérez-Santin, E., Gómez-Guillén, M., & Montero, P. (2011). Contribution of Leu and
36 Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin
37 hydrolysate. *Food Chemistry*, 125(2), 334-341.
38
39 Álvarez, J., Balbín, M., Santos, F., Fernández, M., Ferrando, S., & López, J. M. (2000). Different bone growth
40 rates are associated with changes in the expression pattern of types II and X collagens and collagenase 3 in
41 proximal growth plates of the rat tibia. *Journal of Bone and Mineral Research*, 15(1), 82-94.
42
43 Andrianarivo, A. G., Robinson, J. A., Mann, K. G., & Tracy, R. P. (1992). Growth on type I collagen promotes
44 expression of the osteoblastic phenotype in human osteosarcoma MG-63 cells. *J Cell Physiol*, 153(2), 256-265.
45
46 Ao, J., & Li, B. (2012). Amino acid composition and antioxidant activities of hydrolysates and peptide fractions
47 from porcine collagen. *Food Science and Technology International*, 18(5), 425-434.
48
49 Arnold, W. V., & Fertala, A. (2013). Skeletal diseases caused by mutations that affect collagen structure and
50 function. *Int J Biochem Cell Biol*, 45(8), 1556-1567.
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Aszódi, A., Chan, D., Hunziker, E., Bateman, J. F., & Fässler, R. (1998). Collagen II is essential for the removal
4 of the notochord and the formation of intervertebral discs. *J Cell Biol*, 143(5), 1399-1412.
5
6 Bagchi, D., Misner, B., Bagchi, M., Kothari, S. C., Downs, B. W., Fafard, R. D., & Preuss, H. G. (2002). Effects
7 of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic
8 exploration. *Int J Clin Pharmacol Res*, 22(3-4), 101-110.
9
10 Bailey, A. J. (2001). Molecular mechanisms of ageing in connective tissues. *Mechanisms of ageing and*
11 *development*, 122(7), 735-755.
12
13 Barbul, A. (2008). Proline precursors to sustain Mammalian collagen synthesis. *J Nutr*, 138(10), 2021S-2024S.
14
15 Bonjour, J. P., Ammann, P., & Rizzoli, R. (1999). Importance of preclinical studies in the development of drugs
16 for treatment of osteoporosis: a review related to the 1998 WHO guidelines. *Osteoporos Int*, 9(5), 379-393. doi:
17 10.1007/s001980050161
18
19 Bruyere, O., Rizzoli, R., Coxam, V., Avouac, B., Chevalier, T., Fabien-Soule, V., . . . Reginster, J. Y. (2012).
20 Assessment of health claims in the field of bone: a view of the Group for the Respect of Ethics and Excellence in
21 Science (GREES). *Osteoporos Int*, 23(1), 193-199. doi: 10.1007/s00198-011-1561-x
22
23 Bruyere, O., Zegels, B., Leonori, L., Rabenda, V., Janssen, A., Bourges, C., & Reginster, J. Y. (2012). Effect of
24 collagen hydrolysate in articular pain: a 6-month randomized, double-blind, placebo controlled study.
25 *Complement Ther Med*, 20(3), 124-130. doi: 10.1016/j.ctim.2011.12.007
26
27 Clark, K. L. (2007). Nutritional considerations in joint health. *Clin Sports Med*, 26(1), 101-118. doi:
28 10.1016/j.csm.2006.11.006
29
30 Conference, C. D. (1993). Diagnosis, prophylaxis, and treatment of osteoporosis. *Am J Med*, 94(6), 646-650.
31
32 Conigrave, A. D., Brown, E. M., & Rizzoli, R. (2008). Dietary protein and bone health: roles of amino acid-
33 sensing receptors in the control of calcium metabolism and bone homeostasis. *Annu Rev Nutr*, 28, 131-155. doi:
34 10.1146/annurev.nutr.28.061807.155328
35
36 Coxam, V., Davicco, M. J., & Demigne, C. (2008). Nutrition et métabolisme osseux. In Lavoisier (Ed.),
37 *Aliments fonctionnels* (pp. 729-798).
38
39 Cuneo, F., Costa-Paiva, L., Pinto-Neto, A. M., Morais, S. S., & Amaya-Farfan, J. (2010). Effect of dietary
40 supplementation with collagen hydrolysates on bone metabolism of postmenopausal women with low mineral
41 density. *Maturitas*, 65(3), 253-257. doi: 10.1016/j.maturitas.2009.10.002
42
43 Currey, J. D. (2003). Role of collagen and other organics in the mechanical properties of bone. *Osteoporos Int*,
44 14 Suppl 5, S29-36. doi: 10.1007/s00198-003-1470-8
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Daneault, A., Coxam, V., Fabien Soulé, V., & Wittrant, Y. (2014). Hydrolyzed collagen contributes to osteoblast
4 differentiation in vitro and subsequent bone health in vivo. Paper presented at the OARSI World Congress, Paris,
5 France.
6

7
8 Dawson-Hughes, B., Harris, S. S., Rasmussen, H. M., & Dallal, G. E. (2007). Comparative effects of oral
9 aromatic and branched-chain amino acids on urine calcium excretion in humans. *Osteoporos Int*, 18(7), 955-961.
10 doi: 10.1007/s00198-006-0320-x
11

12
13 De Almeida Jackix, E., Cuneo, F., Amaya-Farfan, J., de Assuncao, J. V., & Quinaes, K. D. (2010). A food
14 supplement of hydrolyzed collagen improves compositional and biodynamic characteristics of vertebrae in
15 ovariectomized rats. *J Med Food*, 13(6), 1385-1390. doi: 10.1089/jmf.2009.0256
16

17
18 Denis, A., Brambati, N., Dessauvages, B., Guedj, S., Ridoux, C., Meffre, N., & Autier, C. (2008). Molecular
19 weight determination of hydrolyzed collagens. *Food Hydrocolloids*, 22(6), 989-994.
20

21
22 Eastell, R., Robins, S. P., Colwell, T., Assiri, A. M., Riggs, B. L., & Russell, R. G. (1993). Evaluation of bone
23 turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption.
24 *Osteoporos Int*, 3(5), 255-260.
25

26
27 Elam, M. L., Johnson, S. A., Hooshmand, S., Feresin, R. G., Payton, M. E., Gu, J., & Arjmandi, B. H. (2014). A
28 Calcium-Collagen Chelate Dietary Supplement Attenuates Bone Loss in Postmenopausal Women with
29 Osteopenia: A Randomized Controlled Trial. *J Med Food*. doi: 10.1089/jmf.2014.0100
30

31
32 Erben, R. G. (1996). Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? *Anat Rec*,
33 246(1), 39-46. doi: 10.1002/(SICI)1097-0185(199609)246:1<39::AID-AR5>3.0.CO;2-A
34

35
36 Exposito, J. Y., Valcourt, U., Cluzel, C., & Lethias, C. (2010). The fibrillar collagen family. *Int J Mol Sci*, 11(2),
37 407-426. doi: 10.3390/ijms11020407
38

39
40 Fouchereau-Peron, M., Duvail, L., Michel, C., Gildberg, A., Batista, I., & Le Gal, Y. (1999). Isolation of an acid
41 fraction from a fish protein hydrolysate with a calcitonin-gene-related-peptide-like biological activity.
42 *Biotechnol Appl Biochem*, 29(1), 87-92.
43

44
45 Fountos, G., Kounadi, E., Tzaphlidou, M., Yasumura, S., & Glaros, D. (1998). The effects of inflammation-
46 mediated osteoporosis (IMO) on the skeletal Ca/P ratio and on the structure of rabbit bone and skin collagen.
47 *Applied radiation and isotopes*, 49(5), 657-659.
48

49
50 Fu, Y., & Zhao, X.-H. (2013). In vitro responses of hFOB1.19 cells towards chum salmon (*Oncorhynchus keta*)
51 skin gelatin hydrolysates in cell proliferation, cycle progression and apoptosis. *Journal of Functional Foods*, 5(1),
52 279-288. doi: 10.1016/j.jff.2012.10.017
53
54
55
56
57
58
59
60

- 1
2
3 Fujita, T., Ohue, M., Fujii, Y., Miyauchi, A., & Takagi, Y. (2002). The effect of active absorbable algal calcium
4 (AAA Ca) with collagen and other matrix components on back and joint pain and skin impedance. *J Bone Miner*
5 *Metab*, 20(5), 298-302. doi: 10.1007/s007740200043
6
7
8 Gaffney-Stomberg, E., Sun, B. H., Cucchi, C. E., Simpson, C. A., Gundberg, C., Kerstetter, J. E., & Insogna, K.
9 L. (2010). The effect of dietary protein on intestinal calcium absorption in rats. *Endocrinology*, 151(3), 1071-
10 1078. doi: 10.1210/en.2009-0744
11
12
13 Garcia-Moreno, C., Calvo, O. M., Herrero, S., Martin, E., Suquia, B., San Roman, J. I., . . . del Pino, J. (1995).
14 Heterogeneous decrease of bone mineral density in the vertebral column of ovariectomized rats. *Bone*, 16(4
15 Suppl), 295S-300S.
16
17
18 Gautieri, A., Buehler, M. J., & Redaelli, A. (2009). Deformation rate controls elasticity and unfolding pathway
19 of single tropocollagen molecules. *J Mech Behav Biomed Mater*, 2(2), 130-137. doi:
20 10.1016/j.jmbbm.2008.03.001
21
22
23 Gelse, K. (2003). Collagens—structure, function, and biosynthesis. *Advanced Drug Delivery Reviews*, 55(12),
24 1531-1546. doi: 10.1016/j.addr.2003.08.002
25
26
27 Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011). Functional and
28 bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids*, 25(8),
29 1813-1827. doi: <http://dx.doi.org/10.1016/j.foodhyd.2011.02.007>
30
31
32 Guillerminet, F., Beaupied, H., Fabien-Soule, V., Tome, D., Benhamou, C. L., Roux, C., & Blais, A. (2010).
33 Hydrolyzed collagen improves bone metabolism and biomechanical parameters in ovariectomized mice: an in
34 vitro and in vivo study. *Bone*, 46(3), 827-834. doi: 10.1016/j.bone.2009.10.035
35
36
37 Guillerminet, F., Fabien-Soule, V., Even, P. C., Tome, D., Benhamou, C. L., Roux, C., & Blais, A. (2012).
38 Hydrolyzed collagen improves bone status and prevents bone loss in ovariectomized C3H/HeN mice.
39 *Osteoporos Int*, 23(7), 1909-1919. doi: 10.1007/s00198-011-1788-6
40
41
42 Han, X., Xu, Y., Wang, J., Pei, X., Yang, R., Li, N., & Li, Y. (2009). Effects of cod bone gelatin on bone
43 metabolism and bone microarchitecture in ovariectomized rats. *Bone*, 44(5), 942-947. doi:
44 10.1016/j.bone.2008.12.005
45
46
47 Hata, S., Hayakawa, T., Okada, H., Hayashi, K., Akimoto, Y., & Yamamoto, H. (2008). Effect of Oral
48 Administration of High Advanced-Collagen Tripeptide (HACP) on Bone Healing Process in Rat. *Journal of hard*
49 *tissue biology*, 17(1), 17-21.
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Henrotin, Y., Lambert, C., Couchourel, D., Ripoll, C., & Chiotelli, E. (2011). Nutraceuticals: do they represent a
4 new era in the management of osteoarthritis? - a narrative review from the lessons taken with five products.
5 Osteoarthritis Cartilage, 19(1), 1-21. doi: 10.1016/j.joca.2010.10.017
6
7
8 Hjorten, R., Hansen, U., Underwood, R. A., Telfer, H. E., Fernandes, R. J., Krakow, D., . . . Pace, J. M. (2007).
9 Type XXVII collagen at the transition of cartilage to bone during skeletogenesis. *Bone*, 41(4), 535-542. doi:
10 10.1016/j.bone.2007.06.024
11
12
13 Hooshmand, S., Elam, M. L., Browne, J., Campbell, S. C., Payton, M. E., Gu, J., & Arjmandi, B. H. (2013).
14 Evidence for bone reversal properties of a calcium-collagen chelate, a novel dietary supplement. *Journal of Food*
15 & *Nutritional Disorders*, 2, 1. doi: doi:10.4172/2324-9323.1000102
16
17
18 Ichikawa, S., Morifuji, M., Ohara, H., Matsumoto, H., Takeuchi, Y., & Sato, K. (2010). Hydroxyproline-
19 containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of
20 gelatin hydrolysate. *Int J Food Sci Nutr*, 61(1), 52-60. doi: 10.3109/09637480903257711
21
22
23 Iwai, K., Hasegawa, T., Taguchi, Y., Morimatsu, F., Sato, K., Nakamura, Y., . . . Ohtsuki, K. (2005).
24 Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *J*
25 *Agric Food Chem*, 53(16), 6531-6536. doi: 10.1021/jf050206p
26
27
28 Izu, Y., Ezura, Y., Mizoguchi, F., Kawamata, A., Nakamoto, T., Nakashima, K., . . . Noda, M. (2012). Type VI
29 collagen deficiency induces osteopenia with distortion of osteoblastic cell morphology. *Tissue Cell*, 44(1), 1-6.
30 doi: 10.1016/j.tice.2011.08.002
31
32
33 Jung, W.-K., Lee, B.-J., & Kim, S.-K. (2006). Fish-bone peptide increases calcium solubility and bioavailability
34 in ovariectomised rats. *British Journal of Nutrition*, 95(01), 124-128.
35
36
37 Kafantari, H., Kounadi, E., Fatouros, M., Milonakis, M., & Tzaphlidou, M. (2000). Structural alterations in rat
38 skin and bone collagen fibrils induced by ovariectomy. *Bone*, 26(4), 349-353. doi: 10.1016/s8756-
39 3282(99)00279-3
40
41
42 Kahai, S., Vary, C. P., Gao, Y., & Seth, A. (2004). Collagen, type V, alpha1 (COL5A1) is regulated by TGF-
43 beta in osteoblasts. *Matrix Biol*, 23(7), 445-455. doi: 10.1016/j.matbio.2004.09.004
44
45
46 Kanis, J. A. (1994). Assessment of fracture risk and its application to screening for postmenopausal osteoporosis:
47 synopsis of a WHO report. WHO Study Group. *Osteoporos Int*, 4(6), 368-381.
48
49
50 Karim, A., & Bhat, R. (2008). Gelatin alternatives for the food industry: recent developments, challenges and
51 prospects. *Trends in food science & technology*, 19(12), 644-656.
52
53
54
55
56
57
58
59
60

- 1
2
3 Karim, A., & Bhat, R. (2009). Fish gelatin: properties, challenges, and prospects as an alternative to mammalian
4 gelatins. *Food Hydrocolloids*, 23(3), 563-576.
5
6 Kawaguchi, T., Nanbu, P. N., & Kurokawa, M. (2012). Distribution of prolylhydroxyproline and its metabolites
7 after oral administration in rats. *Biol Pharm Bull*, 35(3), 422-427.
8
9
10 Keene, D. R., Sakai, L. Y., & Burgeson, R. E. (1991). Human bone contains type III collagen, type VI collagen,
11 and fibrillin: type III collagen is present on specific fibers that may mediate attachment of tendons, ligaments,
12 and periosteum to calcified bone cortex. *Journal of Histochemistry & Cytochemistry*, 39(1), 59-69. doi:
13 10.1177/39.1.1983874
14
15
16 Kerstetter, J. E., Kenny, A. M., & Insogna, K. L. (2011). Dietary protein and skeletal health: a review of recent
17 human research. *Curr Opin Lipidol*, 22(1), 16-20. doi: 10.1097/MOL.0b013e3283419441
18
19
20 Khora, S. S. (2013). Marine fish-derived bioactive peptides and proteins for human therapeutics. *International*
21 *Journal of Pharmacy and Pharmaceutical Sciences*, 5.
22
23
24 Kim, G. H., Jeon, Y. J., Byun, H. G., Lee, Y. S., Lee, E. H., & Kim, S. K. (1998). Effect of calcium compounds
25 from oyster shell bound fish skin gelatin peptide in calcium deficient rats. *Journal Of The Korean Fisheries*
26 *Society*, 31 (2), 149-159.
27
28
29 Kim, H. K., Kim, M. G., & Leem, K. H. (2013). Osteogenic activity of collagen peptide via ERK/MAPK
30 pathway mediated boosting of collagen synthesis and its therapeutic efficacy in osteoporotic bone by back-
31 scattered electron imaging and microarchitecture analysis. *Molecules*, 18(12), 15474-15489. doi:
32 10.3390/molecules181215474
33
34
35 Kim, H. K., Kim, M. G., & Leem, K. H. (2014a). Collagen hydrolysates increased osteogenic gene expressions
36 via a MAPK signaling pathway in MG-63 human osteoblasts. *Food Funct*, 5(3), 573-578. doi:
37 10.1039/c3fo60509d
38
39
40 Kim, H. K., Kim, M. G., & Leem, K. H. (2014b). Effects of egg yolk-derived peptide on osteogenic gene
41 expression and MAPK activation. *Molecules*, 19(9), 12909-12924. doi: 10.3390/molecules190912909
42
43
44 Knott, L., & Bailey, A. J. (1998). Collagen cross-links in mineralizing tissues: a review of their chemistry,
45 function, and clinical relevance. *Bone*, 22(3), 181-187.
46
47
48 Komori, T., & Kishimoto, T. (1998). Cbfa1 in bone development. *Current opinion in genetics & development*,
49 8(4), 494-499.
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Koopman, R., Crombach, N., Gijzen, A. P., Walrand, S., Fauquant, J., Kies, A. K., . . . van Loon, L. J. (2009).
4 Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when
5 compared with its intact protein. *Am J Clin Nutr*, 90(1), 106-115. doi: 10.3945/ajcn.2009.27474
6
7
8 Koyama, Y., Hirota, A., Mori, H., Takahara, H., Kuwaba, K., Kusubata, M., . . . Irie, S. (2001). Ingestion of
9 gelatin has differential effect on bone mineral density and body weight in protein undernutrition. *J Nutr Sci*
10 *Vitaminol (Tokyo)*, 47(1), 84-86.
11
12 Lantigua, R. A., Amatruda, J. M., Biddle, T. L., Forbes, G. B., & Lockwood, D. H. (1980). Cardiac arrhythmias
13 associated with a liquid protein diet for the treatment of obesity. *N Engl J Med*, 303(13), 735-738. doi:
14 10.1056/NEJM198009253031305
15
16
17
18
19 Leem, K. H., Lee, S., Jang, A., & Kim, H. K. (2013). Porcine skin gelatin hydrolysate promotes longitudinal
20 bone growth in adolescent rats. *J Med Food*, 16(5), 447-453. doi: 10.1089/jmf.2012.2461
21
22
23 Liang, J., Pei, X. R., Zhang, Z. F., Wang, N., Wang, J. B., & Li, Y. (2012). A chronic oral toxicity study of
24 marine collagen peptides preparation from chum salmon (*Oncorhynchus keta*) skin using Sprague-Dawley rat.
25 *Mar Drugs*, 10(1), 20-34. doi: 10.3390/md10010020
26
27
28
29 Liu, J., Zhang, B., Song, S., Ma, M., Si, S., Wang, Y., . . . Guo, Y. (2014). Bovine collagen peptides compounds
30 promote the proliferation and differentiation of MC3T3-E1 pre-osteoblasts. *PLoS One*, 9(6), e99920. doi:
31 doi:10.1371/journal.pone.0099920
32
33
34 Lotters, F. J., Lenoir-Wijnkoop, I., Fardellone, P., Rizzoli, R., Rocher, E., & Poley, M. J. (2013). Dairy foods
35 and osteoporosis: an example of assessing the health-economic impact of food products. *Osteoporos Int*, 24(1),
36 139-150. doi: 10.1007/s00198-012-1998-6
37
38
39
40 Luther, F., Saino, H., Carter, D. H., & Aaron, J. E. (2003). Evidence for an extensive collagen type III/VI
41 proximal domain in the rat femur. *Bone*, 32(6), 652-659. doi: 10.1016/s8756-3282(03)00094-2
42
43
44 Lynch, M. P., Stein, J. L., Stein, G. S., & Lian, J. B. (1995). The influence of type I collagen on the development
45 and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of
46 expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. *Exp Cell Res*,
47 216(1), 35-45.
48
49
50
51 Mann, V., Hobson, E. E., Li, B., Stewart, T. L., Grant, S. F., Robins, S. P., . . . Ralston, S. H. (2001). A COL1A1
52 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. *J Clin*
53 *Invest*, 107(7), 899-907.
54
55
56
57
58
59
60

- 1
2
3 Martin-Bautista, E., Martin-Matillas, M., Martin-Lagos, J. A., Miranda-Leon, M. T., Munoz-Torres, M., Ruiz-
4 Requena, E., . . . Campoy, C. (2011). A nutritional intervention study with hydrolyzed collagen in pre-pubertal
5 spanish children: influence on bone modeling biomarkers. *J Pediatr Endocrinol Metab*, 24(3-4), 147-153.
6
7
8 Mhd Sarbon, N., Badii, F., & Howell, N. K. (2013). Preparation and characterisation of chicken skin gelatin as
9 an alternative to mammalian gelatin. *Food Hydrocolloids*, 30(1), 143-151.
10
11
12 Minaguchi, J., Tometsuka, C., Koyama, Y.-i., Kusubata, M., Nagayasu, A., Sawaya, S., . . . Takehana, K. (2012).
13 Effects of collagen-derived oligopeptide prolylhydroxyproline on differentiation of mouse 3T3-L1
14 preadipocytes. *Food Science and Technology Research*, 18(4), 593-599.
15
16
17 Mizuno, M., & Kuboki, Y. (2001). Osteoblast-related gene expression of bone marrow cells during the
18 osteoblastic differentiation induced by type I collagen. *J Biochem*, 129(1), 133-138.
19
20
21 Moskowitz, R. W. (2000). Role of collagen hydrolysate in bone and joint disease. *Semin Arthritis Rheum*, 30(2),
22 87-99. doi: 10.1053/sarh.2000.9622
23
24
25 Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material—skin, bone and fins. *Food*
26 *Chemistry*, 68(3), 277-281.
27
28
29 Nieves, J. (2013). Skeletal effects of nutrients and nutraceuticals, beyond calcium and vitamin D. *Osteoporosis*
30 *International*, 24(3), 771-786.
31
32
33 NIH. (2000). Osteoporosis prevention, diagnosis, and therapy. *NIH Consens Statement*, 17(1), 1-45.
34
35
36 Niyibizi, C., & Eyre, D. R. (1994). Structural characteristics of cross-linking sites in type V collagen of bone.
37 Chain specificities and heterotypic links to type I collagen. *Eur J Biochem*, 224(3), 943-950.
38
39
40 Nomura, Y., Oohashi, K., Watanabe, M., & Kasugai, S. (2005). Increase in bone mineral density through oral
41 administration of shark gelatin to ovariectomized rats. *Nutrition*, 21(11-12), 1120-1126. doi:
42 10.1016/j.nut.2005.03.007
43
44
45 Oesser, S., Adam, M., Babel, W., & Seifert, J. (1999). Oral administration of (14)C labeled gelatin hydrolysate
46 leads to an accumulation of radioactivity in cartilage of mice (C57/BL). *J Nutr*, 129(10), 1891-1895.
47
48
49 Ohara, H., Matsumoto, H., Ito, K., Iwai, K., & Sato, K. (2007). Comparison of quantity and structures of
50 hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different
51 sources. *J Agric Food Chem*, 55(4), 1532-1535. doi: 10.1021/jf062834s
52
53
54 Ottani, V., Martini, D., Franchi, M., Ruggeri, A., & Raspanti, M. (2002). Hierarchical structures in fibrillar
55 collagens. *Micron*, 33(7-8), 587-596.
56
57
58
59
60

- 1
2
3 Ramshaw, J. A., Shah, N. K., & Brodsky, B. (1998). Gly-X-Y tripeptide frequencies in collagen: a context for
4 host-guest triple-helical peptides. *J Struct Biol*, 122(1-2), 86-91. doi: 10.1006/jsbi.1998.3977
5
6 Ricard-Blum, S. (2011). The collagen family. *Cold Spring Harb Perspect Biol*, 3(1), a004978. doi:
7 10.1101/cshperspect.a004978
8
9
10 Rosati, R., Horan, G. S., Pinero, G. J., Garofalo, S., Keene, D. R., Horton, W. A., . . . Behringer, R. R. (1994).
11 Normal long bone growth and development in type X collagen-null mice. *Nat Genet*, 8(2), 129-135.
12
13 Saito, M., Kiyose, C., Higuchi, T., Uchida, N., & Suzuki, H. (2009). Effect of collagen hydrolysates from
14 salmon and trout skins on the lipid profile in rats. *J Agric Food Chem*, 57(21), 10477-10482.
15
16 Saito, M., & Marumo, K. (2010). Collagen cross-links as a determinant of bone quality: a possible explanation
17 for bone fragility in aging, osteoporosis, and diabetes mellitus. *Osteoporos Int*, 21(2), 195-214. doi:
18 10.1007/s00198-009-1066-z
19
20
21 Sanada, H., Shikata, J., Hamamoto, H., Ueba, Y., Yamamuro, T., & Takeda, T. (1978). Changes in collagen
22 cross-linking and lysyl oxidase by estrogen. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 541(3),
23 408-413.
24
25
26 Schauss, A., Merkel, D., Glaza, S., & Sorenson, S. (2007). Acute and subchronic oral toxicity studies in rats of a
27 hydrolyzed chicken sternal cartilage preparation. *Food and chemical toxicology*, 45(2), 315-321.
28
29
30 Shigemura, Y., Akaba, S., Kawashima, E., Park, E. Y., Nakamura, Y., & Sato, K. (2011). Identification of a
31 novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column
32 derivatisation with phenyl isothiocyanate. *Food Chemistry*, 129(3), 1019-1024. doi:
33 10.1016/j.foodchem.2011.05.066
34
35
36 Shigemura, Y., Kubomura, D., Sato, Y., & Sato, K. (2014). Dose-dependent changes in the levels of free and
37 peptide forms of hydroxyproline in human plasma after collagen hydrolysate ingestion. *Food Chemistry*, 159,
38 328-332.
39
40
41 Shoulders, M. D., & Raines, R. T. (2009). Collagen structure and stability. *Annu Rev Biochem*, 78, 929-958.
42 doi: 10.1146/annurev.biochem.77.032207.120833
43
44
45 Singh, P., Benjakul, S., Maqsood, S., & Kishimura, H. (2011). Isolation and characterisation of collagen
46 extracted from the skin of striped catfish (< i> Pangasianodon hypophthalmus</i>). *Food Chemistry*, 124(1), 97-
47 105.
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Sugihara, F., Inoue, N., Kuwamori, M., & Taniguchi, M. (2012). Quantification of hydroxyprolyl-glycine (Hyp-
4 Gly) in human blood after ingestion of collagen hydrolysate. *J Biosci Bioeng*, 113(2), 202-203. doi:
5 10.1016/j.jbiosc.2011.09.024
6
7
8 Takeda, S., Park, J. H., Kawashima, E., Ezawa, I., & Omi, N. (2013). Hydrolyzed collagen intake increases bone
9 mass of growing rats trained with running exercise. *J Int Soc Sports Nutr*, 10(1), 35. doi: 10.1186/1550-2783-10-
10 35
11
12
13 Takeda, U., Odaki, M., Yokota, M., Sasaki, H., Niizato, T., Kawaoto, H., . . . Hayasaka, H. (1982). Acute and
14 subacute toxicity studies on collagen wound dressing (CAS) in mice and rats. *The Journal of toxicological*
15 *sciences*, 7, 63-91.
16
17
18
19 Thompson, D. D., Simmons, H. A., Pirie, C. M., & Ke, H. Z. (1995). FDA Guidelines and animal models for
20 osteoporosis. *Bone*, 17(4 Suppl), 125S-133S.
21
22
23 Trc, T., & Bohmova, J. (2011). Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs. glucosamine
24 sulphate (GS) in the treatment of knee osteoarthritis (KOA). *Int Orthop*, 35(3), 341-348. doi: 10.1007/s00264-
25 010-1010-z
26
27
28 Tsuruoka, N., Yamato, R., Sakai, Y., Yoshitake, Y., & Yonekura, H. (2007). Promotion by Collagen Tripeptide
29 of Type I Collagen Gene Expression in Human Osteoblastic Cells and Fracture Healing of Rat Femur.
30 *Bioscience, Biotechnology, and Biochemistry*, 71(11), 2680-2687. doi: 10.1271/bbb.70287
31
32
33 Tzaphlidou, M. (2005). The role of collagen in bone structure: an image processing approach. *Micron*, 36(7),
34 593-601.
35
36
37 Uehara, K., Takahashi, A., Watanabe, M., & Nomura, Y. (2014). Shark protein improves bone mineral density in
38 ovariectomized rats and inhibits osteoclast differentiation. *Nutrition*, 30(6), 719-725. doi:
39 10.1016/j.nut.2013.11.005
40
41
42
43 Urao, M., Okuyama, H., Drongowski, R. A., Teitelbaum, D. H., & Coran, A. G. (1997). Intestinal permeability
44 to small- and large-molecular-weight substances in the newborn rabbit. *J Pediatr Surg*, 32(10), 1424-1428.
45
46
47 Van Itallie, T. B., & Yang, M. U. (1984). Cardiac dysfunction in obese dieters: a potentially lethal complication
48 of rapid, massive weight loss. *Am J Clin Nutr*, 39(5), 695-702.
49
50
51 Viguet-Carrin, S., Garnero, P., & Delmas, P. D. (2006). The role of collagen in bone strength. *Osteoporos Int*,
52 17(3), 319-336. doi: 10.1007/s00198-005-2035-9
53
54
55
56
57
58
59
60

- 1
2
3 Walrand, S., Chiotelli, E., Noirt, F., Mwewa, S., & Lassel, T. (2008). Consumption of a functional fermented
4 milk containing collagen hydrolysate improves the concentration of collagen-specific amino acids in plasma. *J*
5 *Agric Food Chem*, 56(17), 7790-7795. doi: 10.1021/jf800691f
6
7
8 Wang, X., Shen, X., Li, X., & Agrawal, C. M. (2002). Age-related changes in the collagen network and
9 toughness of bone. *Bone*, 31(1), 1-7.
10
11 Watanabe-Kamiyama, M., Shimizu, M., Kamiyama, S., Taguchi, Y., Sone, H., Morimatsu, F., . . . Komai, M.
12 (2010). Absorption and effectiveness of orally administered low molecular weight collagen hydrolysate in rats. *J*
13 *Agric Food Chem*, 58(2), 835-841. doi: 10.1021/jf9031487
14
15
16 Wu, J., Fujioka, M., Sugimoto, K., Mu, G., & Ishimi, Y. (2004). Assessment of effectiveness of oral
17 administration of collagen peptide on bone metabolism in growing and mature rats. *J Bone Miner Metab*, 22(6),
18 547-553. doi: 10.1007/s00774-004-0522-2
19
20
21 Xu, Y., Han, X., & Li, Y. (2010). Effect of marine collagen peptides on long bone development in growing rats.
22 *J Sci Food Agric*, 90(9), 1485-1491. doi: 10.1002/jsfa.3972
23
24
25
26 Yamada, S., Nagaoka, H., Terajima, M., Tsuda, N., Hayashi, Y., & Yamauchi, M. (2013). Effects of fish
27 collagen peptides on collagen post-translational modifications and mineralization in an osteoblastic cell culture
28 system. *Dent Mater J*, 32(1), 88-95.
29
30
31 Yamada, S., Yoshizawa, Y., Kawakubo, A., Ikeda, T., Yanagiguchi, K., & Hayashi, Y. (2013). Early gene and
32 protein expression associated with osteoblast differentiation in response to fish collagen peptides powder. *Dent*
33 *Mater J*, 32(2), 233-240.
34
35
36
37 Yeowell, H. N., & Pinnell, S. R. (1993). The Ehlers-Danlos syndromes. *Semin Dermatol*, 12(3), 229-240.
38
39
40 Yoshimura, K., Terashima, M., Hozan, D., Ebato, T., Nomura, Y., Ishii, Y., & Shirai, K. (2000). Physical
41 properties of shark gelatin compared with pig gelatin. *J Agric Food Chem*, 48(6), 2023-2027.
42
43
44 Zeng, S., Yin, J., Yang, S., Zhang, C., Yang, P., & Wu, W. (2012). Structure and characteristics of acid and
45 pepsin-solubilized collagens from the skin of cobia (< i> *Rachycentron canadum*</i>). *Food Chemistry*, 135(3),
46 1975-1984.
47
48
49 Zhang, Y., Kouguchi, T., Shimizu, K., Sato, M., Takahata, Y., & Morimatsu, F. (2010). Chicken collagen
50 hydrolysate reduces proinflammatory cytokine production in C57BL/6. KOR-ApoEshl mice. *J Nutr Sci*
51 *Vitaminol (Tokyo)*, 56(3), 208-210.
52
53
54
55
56
57
58
59
60

Figures' list:

Figure 1: The collagen triple helix

Figure 2: From collagen to gelatin and hydrolyzed collagen

Figure 3: Mechanisms of hydrolyzed collagen intestinal absorption

Figure 4: Integrated viewpoint of hydrolyzed collagen effects on bone remodeling process

Figure 5: Supposed cellular and molecular mechanisms of hydrolyzed collagen action on bone

Tables' list:

Table 1: *In vivo* studies on HC in bone biology

Table 2: Clinical studies on HC in bone biology

Table 3: *In vitro* studies on HC in bone biology

Table 1-S: Characteristics of the different collagen families

Table 1 : Overview of *in vivo* studies on HC in bone biology

Model/ species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
Growth models	n= 24 male Wistar rats (5-week-old) 4 groups	Collagen peptide MW +/- 5 kDa	Porcine skin	166 mg/kg body weight/day (C1), 1600 mg/kg body weight /day (C10), and 16600 mg/kg body weight /day (C100)	4 weeks	BMD of the femur: C100 group > the other groups Kidneys hypertrophy in the C100 group	[48]
	n=80 Sprague–Dawley (3-week-old) 4 groups	Marine collagen peptides (MCP)	Chum salmon	1125, 2250 or 4500 mg/kg body weight	1 month	Bone size, mineral density, dry weight, ash weight: /	[68]
	n= 59 male Wistar rats (5-week-old) 5 groups	HC peptides MW: 1 kDa	Fish	20% casein group vs. 40% casein group vs. 20% HC group vs. 40% HC group	11 weeks	HC intake (20%): / bone mass during growth and exercise period Higher HC intake has no more beneficial effect	[70]
Bone loss models	n= 32 SHRSP-Izumo rats (8-week-old) 3 groups: control (SHAM-operated) vs. OVX vs. OVX with HC	HC MW : 800 Da.	Chicken foot	10% HC in distilled water	20 weeks	\ bone loss by increasing collagen bone content	[59]
	n = 60 female Wistar rats (1-month-old) 4 groups	Gelatin	Shark skin	10, 20, or 40 mg/100 g of body weight per day	2 weeks	/ BMD femur epiphysis in treated ovariectomized rats (> SHAM) / contents of type I collagen and glycosaminoglycan in the epiphysis	[75]
	n= 84 female Wistar rats (3-month-old) 7 groups: control (SHAM) vs OVX vs 5 groups OVX-HC	Gelatin	Cod bone	0.375, 0.75, 1.5, 3, 6 mg/kg body weight	90 days	Prevention of BMD loss in proximal tibia and femoral neck at 3 g/kg. \ RANKL/OPG mRNA ratio at 3 g/kg and 6 g/kg	[76]
	n= 48 female Wistar rats (3-month-old) 6 groups: OVX vs SHAM vs intact +/- gelatin vs. HC	HC	-	gelatin or HC equivalent from 1000 mg to 100g/day for a 60-kg person	8 weeks	/ levels of protein and osteocalcin content (HC group) preservation of vertebral mass, protein content and mechanical strength (HC)	[77]
	n = 84 female Sprague Dawley rats SHAM vs. OVX: ovariectomized group administrated with 0%-4.0% HC; casein: OVX+casein (1%) group.	HC MW: 3 kDa	Porcine	0%, 0.1%, 0.3%, 1%, 4.0% HC	12 weeks	BMD of whole body and lumbar vertebrae: / (in a dose dependant manner). Femur BMD is not affected by HC treatment.	[78]
	n= 32 female C3H/HeN mice (3-month-old) 3 groups: 8 animals / group control (SHAM-operated) vs. OVX vs. OVX with HC (OVX-CH)	HC MW: 5 kDa	Porcine	1000 mg/kg or 2500 mg/kg of the diet was used. Intake of about 3500 mg diet/day. Ingestion of 1000 mg HC /kg or and 2500 mg HC/kg body weight .	12 weeks	/ BMD for OVX (25g/kg of HC) \ CTX level / bone strength (OVX mice 25g/kg HC)	[79]
	n= 64 female C3H/HeN mice (6-week-old) 2 SHAM groups and 6 OVX groups (3-month-old and 6-month-old)	HC MW: 5 kDa	Porcine	1000 mg/kg or 2500 mg/kg of the diet was used. Intake of about 3500 mg diet/day. Ingestion of 1000 mg HC /kg or and 2500 mg HC/kg body weight .	26 weeks	Ingestion of 25 g/kg of HC for 3 or 6 months reduced bone loss significantly in, respectively, 3 and 6-month-old OVX mice. The lowest HC concentration was less efficient. HC reduces bone loss in OVX mice by increasing the diameter of the cortical areas of femurs.	[81]
	n= 70 female C3H/HeN mice (3-month-old) 2 SHAM groups and 5 OVX groups	HC MW: 5 kDa	Porcine	0 or 2500 mg/kg	12 weeks + 4 additional weeks	HC ingestion for 3 months is as efficient as raloxifene to protect 3-month-old OVX mice from bone loss. HC ingestion before surgery prevented the BMD decreases.	[81]
n= 50 female C3H/HeN mice (9-week-old) 2 groups (SHAM) and 3 groups OVX	HC MW: 2 kDa and 5 kDa	Bovine	2.5% of the diet 15% casein, 17.5% casein or 15% casein plus 2.5% HC	8 weeks	BMD of the femur: / with HC 2kDa	[82]	
Bone healing models	n= 30 IGS male rats (7-week-old) 3 groups	Collagen tripeptide (Ctp)	Porcine skin	0, 80, or 500 mg/kg/day	12 weeks	Fracture healing acceleration.	[84]
	n=8 female Wistar/ST rats (11 week-old)	High Advanced-Collagen Tripeptide (HACP)	Hog skin	80mg/2ml/kg body weight	3 weeks	Beneficial effect on bone healing process.	[85]

Table 2 : Overview of clinical studies on HC in bone biology

	Model/ species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
Clinical studies	Post menopausal women	n=94 Calcitonin alone vs Calcitonin+HC	HC	-	10g / day	24 weeks	Levels of urinary pyridinoline cross-links: ↗	[91]
		n= 71 Control vs HC	HC	-	10g / day	24 weeks	No effect The majority of patients : inadequate calcium intake + excess body weight	[93]
		n=29 Control vs. CC	Calcium-collagen chelate (CC)	-	5 g of CC + 500 mg calcium carbonate + 5 µg vitamin D (Control: 500 mg calcium carbonate + 5 µg vitamin D)	3 months	bAPL/TRAP5b ratio: ↗ Total BMD: ↗	[92]
		n=39 Control vs. CC	Calcium-collagen chelate (CC)	-	5 g of CC + 500 mg calcium carbonate + 5 µg vitamin D (Control: 500 mg calcium carbonate + 5 µg vitamin D)	12 months	Whole body BMD: ↗ in CC group ↘ sclérostin ↘ Trap5b ↗ bALP ↗ bALP/Trap5b ratio	[86]
	Pre-pubertal children	n=60 Control (G-I) vs. Collagen (G-II) vs. Collagen + calcium (G-III)	Partially HC (gelatine)	-	250mL of the product/day Gelatine content is not specified	4 months	serum IGF-1: G-III > G-II or G-I ALP: G-III > G-I (p < 0.05)	[94]

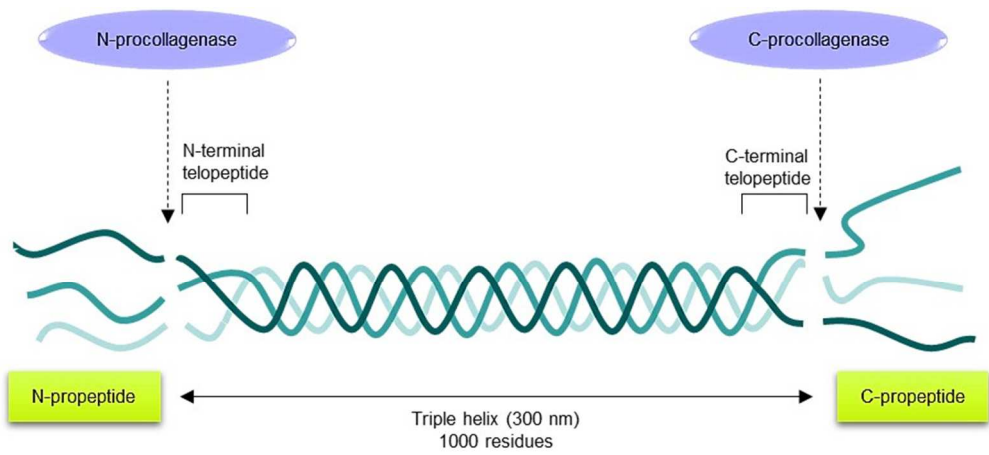
Table 3 : Overview of *in vitro* studies on HC in bone biology

Model/ species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
Osteoblasts	Human osteoblastic MG-63 cells	HC <3 kDa: selected MW: 1.4 kDa	-	0, 10, 25, 50 et 100 µg/mL	-	Cell proliferation: ✓ (dose-dependent) ALP activity: ✓ (dose-dependent) Collagen synthesis and collagen, type1, alpha1 (COL1A1) gene expression: ✓ CH-induced COL1A1 gene expression was completely abolished by extracellular signal-regulated kinase (ERK) inhibitor.	[78]
	Primary tissue co-culture	HC MW: 2 and 5 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	Cell growth : = ALP activity: ✓ (dose-dependent)	[79]
	MC3T3-E1 cells (subclone 4)	HC MW: 2 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	Absence of cytotoxicity Cell proliferation: ✓ ALP activity: ✓ Ca/P nodule formation: ✓ in MC3T3-E1 cultures.	[82]
	hFOB1.19	Collagen tripeptide (Ctp)	Porcine skin	10 µg/ml	-	Calcification: ✓ Type I collagen protein production + its mRNA levels: ✓ Ctp upregulated Osterix.	[84]
	hFOB1.19	Gelatin hydrolysate degree of hydrolysis of 4,7-13,5%	Chum salmon skin	0, 0.005, 0.01, 0.02, 0.05, 0.1 mg/mL	-	Cell growth proliferation Cell cycle progression acceleration Cell apoptosis inhibition Cell proliferation: ✓ with gelatin prepared with papain protease	[99]
	Human osteoblastic MG-63 cells	HC	-	-	-	Cell proliferation: ✓ ALP activity: ✓ Collagen synthesis: ✓ Calcium deposition: ✓ HC activated ERK1/2, JNK1/2, p38, and ELK1 phosphorylation except cJUN. COL1A1, ALPL, BGLAP, and osteopontin gene expressions : ✓	[100]
	MC3T3-E1 cells (subclone 4)	Collagen peptide MW: 0.6–2.5 kDa	Bovine	0.1–6 mg/mL Best concentration :3 mg/mL.	-	Cell proliferation : ✓ Percentage of MC3T3-E1 cells in G2/S phase: ✓ Runx2 expression, ALP activity, and OC production: ✓ Mineralization: ✓	[101]
	NOS-1 human osteosarcoma	Collagen peptide MW: 3kDa (0.5–10kDa)	Cod skin and bone	ALP: 0.0005, 0.005, 0.05, 0.1 and 0.5% Best concentration: . 0.1%	-	ALP activity : ✓ Cell proliferation: ✓ Osteocalcin, osteopontin, BMP-2 and integrin β3 mRNAs expression: ✓ Osteopontin and integrin β3 expression levels: ✓ Osteocalcin, osteopontin and integrin β3 proteins: ✓ Matrix mineralization: ✓	[102]
MC3T3-E1 cells (subclone 4)	Collagen peptide MW: ≈ 2-8 kDa hydroxyproline content:≈5%	Fish	RT-PCR: 0.05, 0.1, 0.2 and 0.5% (w/v) of FCP Best concentration: 0.2%	-	Lysine hydroxylation, levels of hydroxylysine-aldehyde derived cross-links and cross-link maturation : ✓ Collagen synthesis, quality and mineralization: ✓	[103]	
Osteoclasts	Primary tissue co-culture	HC MW: 2 and 5 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	No effects on cell growth ALP activity: ✓ (dose-dependent)	[79]

Table 1-S: Characteristics of the different collagen families

	Type	Molecular composition	Genes	Tissue distribution
<i>Fibril forming collagens</i>				
	I	$[\alpha 1(I)]_2, \alpha 2(I)$	COL1A1, COL1A2	Bone, dermis, tendon, ligaments, cornea
	II	$[\alpha 1(II)]_3$	COL2A1	Cartilage, vitreous body, nucleus pulposus
	III	$[\alpha 1(III)]_3$	COL3A1	Skin, vessel, reticular fibres of most tissues (lungs, liver, spleen etc.)
	V	$\alpha 1(V) \alpha 2(V) \alpha 3(V)$	COL5A1, COL5A2, COL5A3	Lung, cornea, bone, fetal membranes; together with type I
	XI	$\alpha 1(XI) \alpha 2(XI) \alpha 3(XI)$	COL11A1, COL11A2, COL11A3 (COL2A1)	Cartilage, vitreous body
<i>Fibrillar-like collagens (type C clade):</i>				
	XXIV	-	COL24A1	Coexpressed with type I collagen in the developing bone and eye
	XXVII	-	COL27A1	Cartilage and epithelial cell layers in developing tissues including stomach, lung, gonad, skin, cochlear, and tooth
<i>Microfibrillar collagens</i>				
	VI	$\alpha 1(VI) \alpha 2(VI) \alpha 3(VI)-\alpha 6(VI)$	COL6A1, COL6A2, COL6A3, COL6A5 (COL29A1), COL6A6	Dermis, epidermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
<i>Anchoring fibrils</i>				
	VII	$[\alpha 1(VII)]_3$	COL7A1	Skin, dermal-epidermal junctions; oral mucosa, cervix
<i>FACIT collagens (fibril-associated collagens with interrupted triple helices)</i>				
	IX	$\alpha 1(IX) \alpha 2(IX) \alpha 3(IX)$	COL9A1, COL9A2, COL9A3	Cartilage, vitreous humor, cornea
	XII	$[\alpha 1(XII)]_3$	COL12A1	Perichondrium, ligaments, tendon
	XIV	$[\alpha 1(XIV)]_3$	COL14A1	Dermis, tendon, vessel wall, placenta, lungs, liver, embryonic heart
	XX	-	COL20A1	Corneal epithelium, embryonic skin, sternal cartilage, tendon
	XXVI	-	COL26A1	Testis, ovary
<i>FACIT-like collagens:</i>				
	XVI	$[\alpha 1(XVI)]_3$	COL16A1	Fibroblasts, amnion, keratinocytes
	XIX	-	COL19A1	Human rhabdomyosarcoma
	XXI	-	COL21A1	Blood vessel
	XXII	-	COL22A1	Tissue junction: myotendinous junction in skeletal and heart muscle, the articular cartilage-synovial fluid junction, or the border between the anagen hair follicle and the dermis in the skin
<i>Basement membrane collagens</i>				
	IV	$[\alpha 1(IV)]_2, \alpha 1(IV)-\alpha 6(IV)...$	COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6	Basement membranes
<i>MACIT collagens (membrane-associated collagens with interrupted triple helices)</i>				
	XIII	-	COL13A1	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
	XVII	$[\alpha 1(XVII)]_3$	COL17A1	Dermal-epidermal junctions
	XXIII	-	COL23A1	Lung, cornea, brain, skin, tendon, and kidney
	XXV	-	COL25A1	Brain
<i>Hexagonal network-forming collagens</i>				
	VIII	$[\alpha 1(VIII)]_2, \alpha 2(VIII)$	COL8A1, COL8A2	Endothelial cells, Descemet's membrane
	X	$[\alpha 3(X)]_3$	COL10A1	Hypertrophic cartilage
<i>Multiplexin family (multiple triple helix domains with interruptions)</i>				
	XV	$[\alpha 1(XV)]_3$	COL15A1	Highly expressed in heart, skeletal muscles and the placenta and moderately expressed in the adrenal gland, kidney and pancreas
	XVIII	-	COL18A1	Lungs, liver
<i>Collagen containing von Willebrand factor type A domains</i>				
	XXVIII	-	COL28A1	Adult sciatic nerve

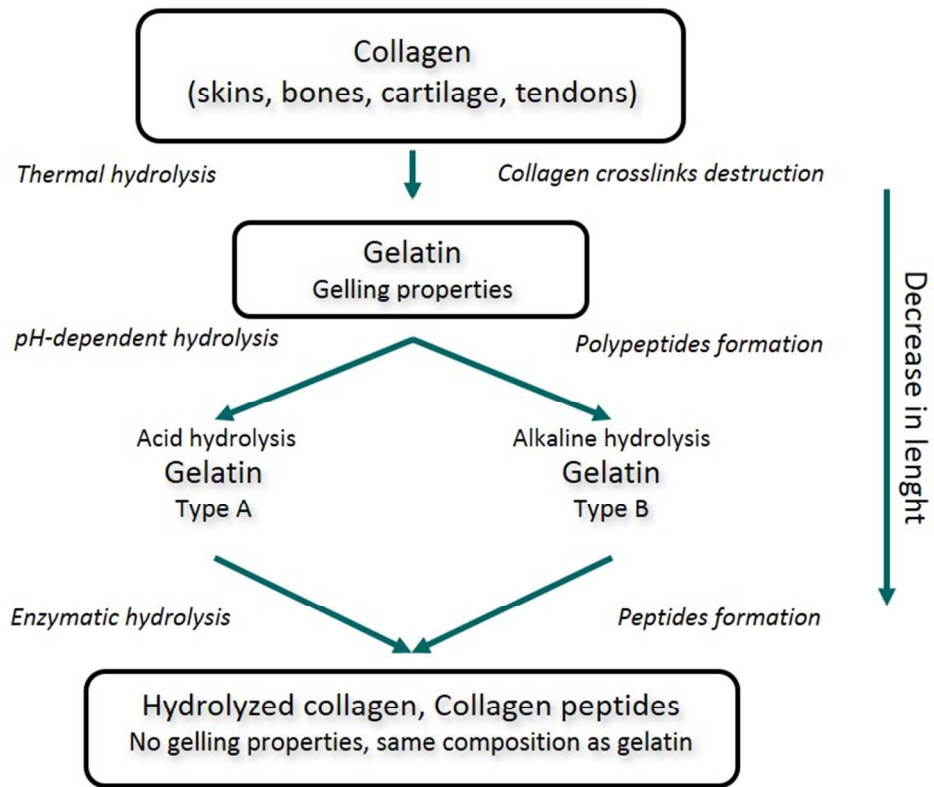
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



The collagen triple helix
320x146mm (72 x 72 DPI)

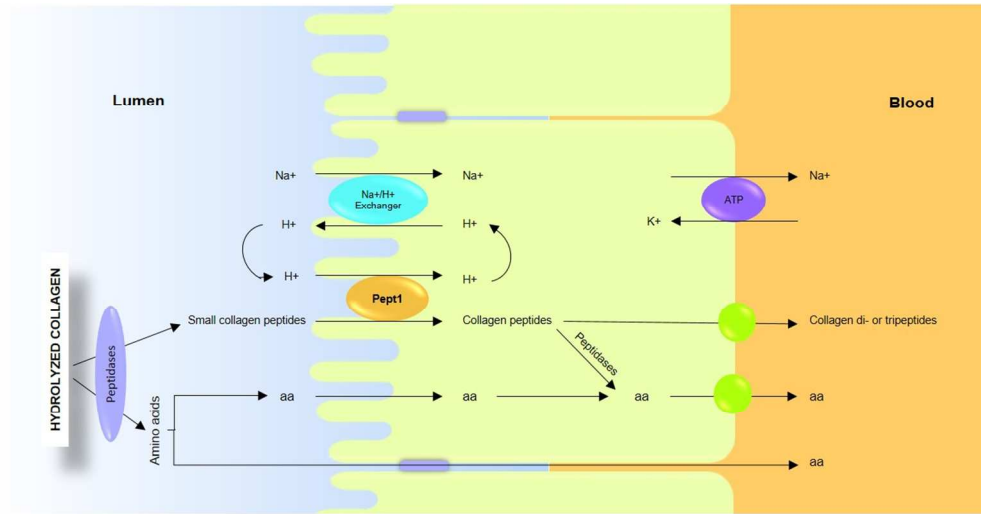
er Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



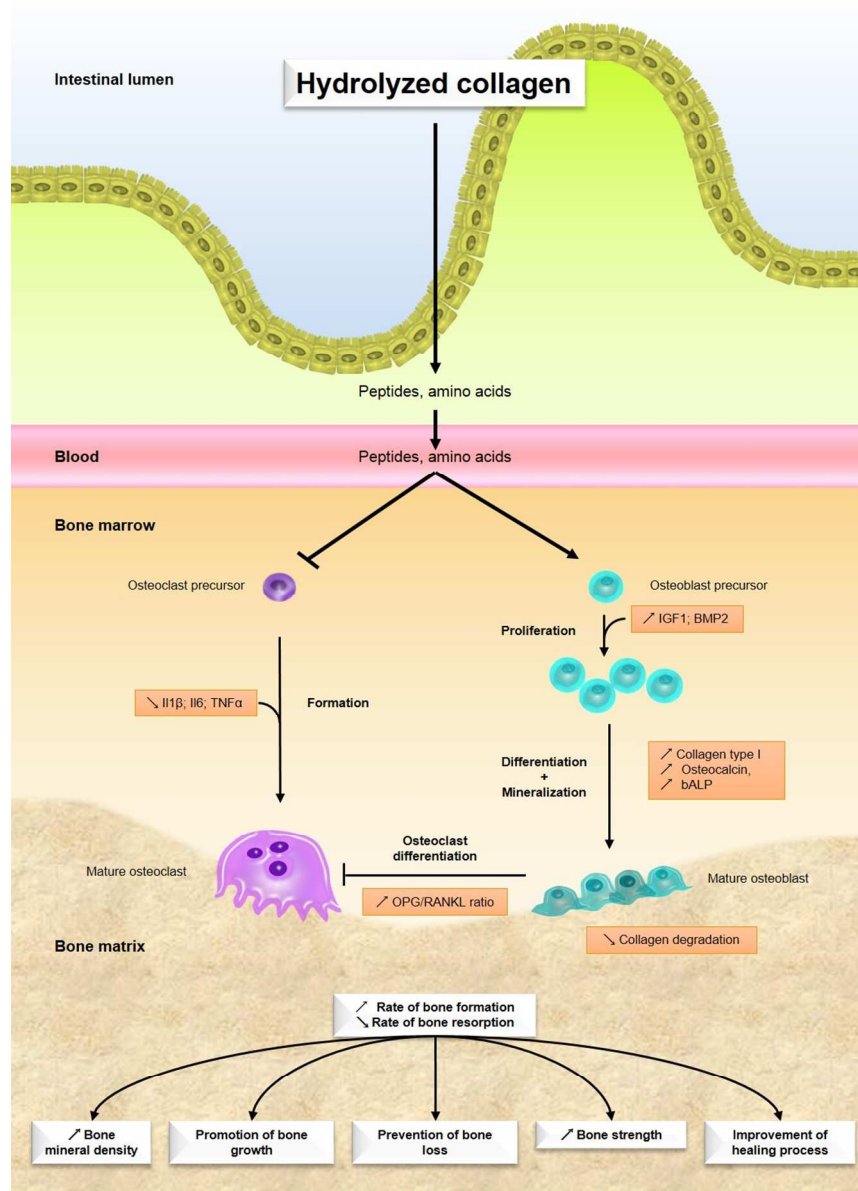
From collagen to gelatin and hydrolyzed collagen
292x239mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

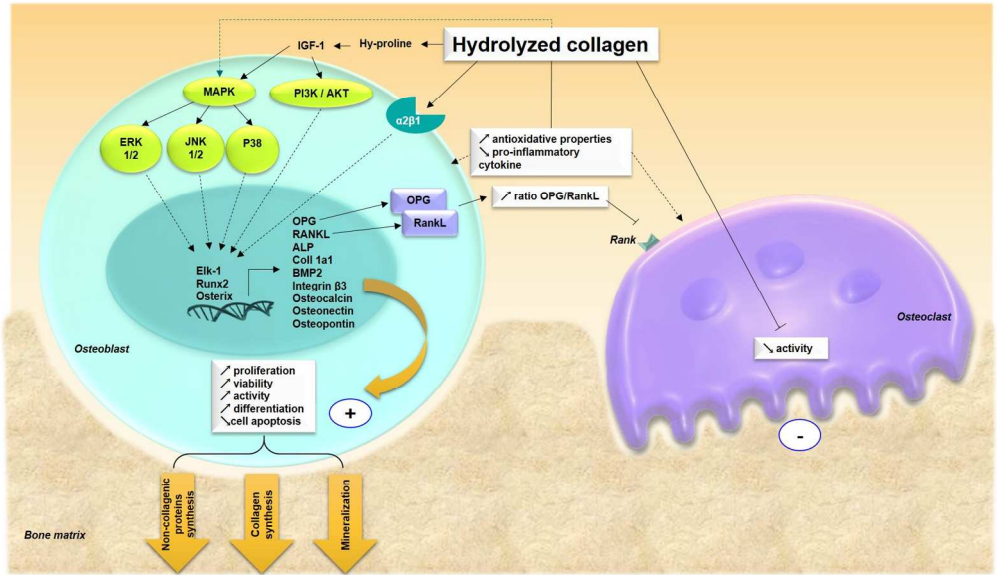


Mechanisms of hydrolyzed collagen intestinal absorption
460x235mm (72 x 72 DPI)

Review Only



Integrated viewpoint of hydrolyzed collagen effects on bone remodeling process
400x560mm (72 x 72 DPI)



Supposed cellular and molecular mechanisms of hydrolyzed collagen action on bone
655x379mm (72 x 72 DPI)

Review Only