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Royal jelly affects collagen crosslinking in bone of ovariectomized rats

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ABSTRACT

Royal jelly (RJ) is an essential food for queen bees, and it reportedly has estrogen-like activity. The objective of this study was to evaluate the effect of RJ intake on bone quality with a focus on the posttranslational modifications of type I collagen. RJ was fed to ovariectomized (OVX) rats for 12 weeks. RJ intake did not affect OVX-induced reduction in bone volume at the femur epiphysis; however, the reduction of collagen crosslinks (pyridinoline and deoxypyridinoline), which represent an aspect of bone quality, were significantly mitigated. In cultured MC3T3-E1osteoblasts, RJ treatment did not affect cell proliferation, cell differentiation, matrix formation, or mineralization. However, RJ treatment did stimulate expression of *plods*, which encode lysyl hydroxylase isoforms that control the collagen crosslinking pathway, and it also affected collagen crosslinking. These results indicate that oral intake of RJ could improve bone quality by modulating the posttranslational modification of type I collagen.

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1. Introduction

Osteoporosis is a disorder characterized by weakened bone strength, and it results in an increased risk of bone fracture. According to the NIH Consensus Conference held in 2000, bone strength can be defined by bone mineral density (BMD) and bone quality (*Osteoporosis Prevention and Therapy*, 2000). BMD accounts for approximately 70% of the bone strength and the bone quality for the rest. Bone quality encompasses all aspects of micro- and macro-architecture that are related to fracture resistance.

Type I collagen is a major component of the extracellular matrix, and it comprises more than 90% of organic content of bone tissue. Type I collagen molecules are bonded to each other by intra- and inter-molecular crosslinks, making it possible to build a strong three-dimensional structure as a template for bone architecture. Establishment of bone-specific collagen crosslinking requires unique posttranslational modification during biosynthesis of type I collagen (Yamauchi & Shiiba, 2008). There are strong correlations between the composition of collagen crosslinks and the mechanical properties of bone (Saito, Fujii, Soshi, & Tanaka, 2006). Thus, collagen crosslinking is one of the key elements of bone quality.

Lysine hydroxylation is an early and important posttranslational modification of collagen; this modification is catalyzed by lysyl hydroxylases (LHs), which are encoded by *plod* genes. To date, three isoforms of LHs have been identified and partially characterized. LH1 is an LH specific

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Abbreviations: RJ, royal jelly; OVX, ovariectomy; Hyp, hydroxyproline; PYD, pyridinoline; DPD, deoxypyridinoline; LH, lysyl hydroxylase; MRJP, major royal jelly protein

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for triple-helix structures, and LH2 is specific for telopeptides, but LH3 does not have LH activity. We and other groups have reported that various factors, such as mechanical stress (Saito, Soshi, & Fujii, 2003; Shiiba, Arnaud, Tanzawa, Kitamura, & Yamauchi, 2002; Shiiba, Arnaud, Tanzawa, Uzawa, & Yamauchi, 2001), growth factors (Kaku, Mochida, Atsawasuwan, Parisuthiman, & Yamauchi, 2007), and vitamin D (Nagaoka et al., 2008) affect the expression of LHs and subsequent collagen crosslinks.

Honey bee products have been used for traditional medicine since ancient times. Royal jelly (RJ) is a traditional dietary supplement that is produced by mandibular and hypopharyngeal glands of worker honey bees (*Apis mellifera*). RJ is an essential food for queen bees and her larvae; it comprises 60–70% water, 12–15% crude protein, 10–16% sugars, 3–6% lipids, vitamins, minerals, and free amino acids (Sabatini et al., 2009; Takenaka, 1980). RJ has documented antibacterial, antiviral, antioxidant, antitumour, and anti-inflammatory effects (see reviews (Ramadan & Al-Ghamdi, 2012; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2008)). Additionally, RJ is thought to alleviate many aging-related diseases, include postmenopausal symptoms.

A few studies have investigated the effects of RJ on bone metabolism and related cellular activities. Oral administration of RJ resulted in partial recovery from ovariectomy (OVX)-induced osteoporosis in rats (Hidaka et al., 2006; Kafadar, Guney, Turk, Oner, & Silici, 2012). In addition, RJ showed weak estrogen-like activity in cultured osteoblasts (Mishima et al., 2005; Moutsatsou et al., 2010; Suzuki et al., 2008). Post-menopausal osteoporosis is mainly caused by reduced production of estrogen; therefore, RJ's estrogen-like activity may help to alleviate post-menopausal osteoporosis. Furthermore, RJ enhanced the migration of dermal fibroblast and increased collagen production (Fujii et al., 1990; Kim et al., 2010; Park, Cho, Cho, & Kim, 2012). These observations strongly suggest that RJ has positive effects on collagen biosynthesis and bone metabolism. To ensure the essential function of bone as a supporting tissue, it is necessary to have a sufficient quantity and adequate quality of bone. Thus, the objective of this study was to evaluate the effect of RJ intake on bone remodeling, not only on bone architecture and volume, but also on bone quality; we specifically focused on the posttranslational modifications of type I collagen.

2. Material and methods

2.1. Animal study

Wistar/ST female rats ($n = 20$), 12 weeks old (Charles River Laboratories Japan Inc., Yokohama, Japan) were used in this study. Rats were divided into 4 groups, Control (sham operation), OVX with normal diet, OVX with RJ(non-digested (ND)) containing diet and OVX with RJ(enzyme digested (D)) containing diet. The experimental schedule is shown in Fig. 1a. All surgical procedures were carried out under anesthesia, which was induced via intra-peritoneal injection of 0.1% chloral hydrate (0.6 ml per 100 g of body weight). Each rat was subjected to OVX or to a sham operation; all surgeries involved bilateral flank incisions. After a 4-week healing period, rats were fed a dietary powder that contained 1% RJ (either (ND) or (D)); RJ was provided by Yamada bee farm corporation (Okayama, Japan). After 12 weeks on the RJ-containing diet, rats were euthanized. Femur was fixed with 4% formaldehyde for histological analysis and tibia was kept under the −20 °C until used for biochemical analysis. All animal experiments were approved by the Committee for the Guidelines on Animal Experimentation of the Graduate School of Niigata University (Approval number 72-1).

2.2. Micro-computer tomography (micro-CT) analysis

Micro-computer tomography (CT) images of the distal femur epiphysis were taken with 52 kV and 14 μA irradiation by a high-resolution X-ray tomographic system (ELESCAN, Nittetsu Elex, Osaka, Japan). A computer program for bone structure analysis (TRI/3D-Bon, RATOC System Engineering, Tokyo, Japan) was used to reconstruct 3-dimensional (3D) images of the distal femur epiphyses. Bone histomorphometry was performed according to the guideline of American Society for Bone and Mineral Research (Recker, 2009).

2.3. Histology

Following micro-CT analysis, distal femur epiphysis were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 3 weeks, dehydrated, and embedded in paraffin by a standard protocol (Kaku, Uoshima, Yamashita, & Miura, 2005). Histological sections (5 μm thick) were prepared and then stained with Hematoxylin-Eosin (H&E) for morphological observation. Collagenous matrix maturation was analyzed following Picrosirius red staining (1% Sirius red in saturated picric acid in H_2O) and observed under a polarized microscope (Nikon eclipse Ti, Tokyo, Japan). A TRAP-staining kit (Primary Cell Co., Ltd., Sapporo, Japan) was used to perform TRAP staining to detect osteoclasts. Image J software (NIH. Bethesda, MD, USA) was used to measure the osteoclast surface (OS) and bone surface (BS) in the cancellous bone of femur epiphysis, and the OS/BS ratio was calculated (Recker, 2009).

2.4. Crosslink analysis

Each tibia was pulverized and then thoroughly washed first with ice-cold phosphate buffer saline (PBS) and then with distilled water. The collected bone powder was decalcified with 10% EDTA for 3 days at 4 °C and then centrifuge (1500*g*, 30 min); the collagenous component was extracted with an extraction buffer (0.05 M Tris–HCl, pH7.4 containing 4 M guanidine–HCl). The supernatant was dialyzed against distilled water and lyophilized. Dried samples were hydrolyzed with 6 N HCl for 16 h. ELISA Kit for hydroxyproline (Uscn Life Science Inc., Wuhan, China) was used to measure the hydroxyproline (Hyp) content. Collagen amount were calculated based on the contents of Hyp. A dot blot apparatus (Bio-Rad, Hercules, CA, USA) was used to apply the standardized samples to nitrocellulose membrane; each membrane was then incubated with either anti-PYD or anti-DPD antibody (Quidel, San Diego, CA, USA) overnight at 4 °C. Each membrane was then washed several times with tris-buffered

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