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LOW ADMINISTRATION OF BEE POLLEN IN THE DIET AFFECTS BONE MICROSTRUCTURE IN MALE RATS

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ABSTRACT

Bee pollen is often used as a dietary additive because it contains proteins and is rich in vitamins, minerals and phytochemicals. However, its impact on growth characteristics and bone microstructure is still poorly understood. Therefore, the objective of this study was to investigate the effect of low administration of bee pollen on selected growth characteristics and histological structure of femoral bones in rats. For this purpose, 1-month-old male Wistar rats were randomly divided into two groups of 5 animals each. In the control group (CG), rats were fed a commercial diet throughout the experiment (90 days). Rats of experimental group (EG) received standard diets with a 0.2% addition of bee pollen for the same time period of treatment. At the end of the experiment, macroscopical and microscopical structures of femoral bones from all rats were analysed using analytical scales, sliding instrument, polarized light microscopy and atomic absorption spectrophotometry. The statistical analysis of obtained data did not reveal significant differences for body weight, femoral weight, femoral length, and cortical bone thickness between both investigated groups of rats. However, a higher number of primary and secondary osteons was observed in the central area of *substantia compacta* and near periosteal surfaces in rats from the EG group. Histomorphometrical data of primary osteons' vascular canals, Haversian canals and secondary osteons did not differ between rats from both groups. Also, concentrations of Ca, Mg, Fe and Zn in the bones of rats from the EG group were similar to those from CG group. Our results indicate that 0.2% concentration of bee pollen in the diet significantly affects qualitative histological characteristics of femoral bones in rats. On the other hand, it has no impact on the size of primary and secondary osteons and on the content of Ca, Mg, Fe and Zn in the bones of male rats.

Keywords: bee pollen; femoral bone; rat; qualitative parameters; quantitative parameters.

INTRODUCTION

Bee pollen is often used as food for all developmental stages in the hive **(Almeida-Muradian et al., 2005)**. It contains many essential nutritional elements important for growth and development of animals and humans **(Orzaez Villanueva et al., 2002**; **Haščík et al., 2011**; **Petruška et al., 2012)**. Bees use pollen as their nutritional source of proteins (25 - 30%), carbohydrates (30 - 55%), lipids, including fatty acids and sterols (1 - 20%), vitamins and minerals. Furthermore, bee pollen is rich in carotenoids, flavonoids, phytosterols, polyphenols and other beneficial compounds **(Baltrušaityté et al., 2007**; **Estevinho et al., 2008)**.

This natural product is recognized to be a valuable product with potential for medical and nutritional applications **(Almeida-Muradian et al., 2005)**. It has been reported to trigger beneficial effects in the prevention of prostate problems, arteriosclerosis, gastroenteritis, respiratory diseases, allergy desensitization, improving the cardiovascular and digestive systems, body immunity and delaying aging **(Estevinho et al., 2012)**.

The bee pollen also improves bone mineralization due to high vitamin D content, which increases calcium absorption **(Wang et al., 2007)**. According to **Yamaguchi et al. (2004)** and **Hamamoto et al. (2006)**, it has stimulatory effects on bone formation and inhibitory effects on bone resorption. It also stimulates bone calcification.

In general, growth and bone microstructure of animals are affected by numerous factors, e.g. nutritional regime, genetic factors, sex, age, management conditions, production system. Recent years have witnessed an increasing interest in the use of various feed additives and dietary supplements believed to improve growth characteristics of animals. Therefore, the aim of this study was to determine the effect of low administration of bee pollen on selected growth characteristics (body weight, femoral weight, femoral length and cortical bone thickness) and bone microstructure in male rats.

MATERIAL AND METHODOLOGY

Our study was carried out on ten 1-month-old male Wistar rats. The animals were housed individually in plastic containers (Techniplast, Italy) under the same laboratory conditions of temperature (20 - 24 °C) and relative humidity (55 ±10%) with access to food (feed mixture M3, Bonagro, Czech Republic) and drinking water *ad libitum*. All experiments were provided in accordance

with accepted standards of animal care in accredited laboratory (SK PC 50004) of the Slovak University of Agriculture in Nitra.

At the age of four weeks, the young rats were divided into two groups, of 5 animals each. The control group (CG) was fed with the feed mixture without bee pollen additive. Experimental group (EG) was fed with the bee pollen addition (*Brassica napus*) in concentration of 0.2% for a total of 90 days. All procedures were approved by the Animal Experimental Committee of the Slovak Republic.

At the end of the experiment, all animals were killed, weighed and their femora were used for macroscopical and microscopical analyses. Femora were weighed by analytical scales and their length was measured by a sliding instrument.

For histological analysis, the femora were sectioned at the midshaft of the diaphysis and the segments were fixed in HistoChoice fixative (Amresco, USA). The segments were then dehydrated in increasing grades (40 to 100%) of ethanol and embedded in Biodur epoxy resin (Günter von Hagens, Heidelberg, Germany) according to the method described by **Martiniaková et al. (2008)**. Transverse thin sections (70 - 80 μm) were prepared with a sawing microtome (Leitz 1600, Leica, Wetzlar, Germany) and fixed onto glass slides by Eukitt (Merck, Darmstadt, Germany) as previously described **(Martiniaková et al., 2010)**. The qualitative histological characteristics of the compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956)** and **Ricqlés et al. (1991)**. The quantitative (histomorphometrical) variables were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd.). We measured area, perimeter and the minimum and maximum diameters of 264 primary osteons' vascular canals, 216 Haversian canals and 216 secondary osteons in all views (i.e., anterior, posterior, medial and lateral) of the thin sections in order to minimize inter-animal differences. Diaphyseal cortical bone thickness was also measured by Motic Images Plus 2.0 ML software. Twenty random areas were selected, and average thickness was calculated for each femur. The concentrations of Ca, Mg, Fe and Zn were determined by atomic absorption spectrophotometry (Perkin Elmer 4100 ZL) in a graphite furnace **(Stawarz et al., 2003)**. The bone samples were dried at 105 °C until dry mass was obtained. Then all samples were weighed (minimum 2 g) and digested in concentrated nitric acid at 90 °C for 10 h. The samples were diluted to 25 ml with distilled water before analysis **(Martiniaková et al., 2011)**. All metal concentrations were expressed on a dry weight basis in $\mu g.g^{-1}$.

Statistical analysis was performed using SPSS 8.0 software. All data were expressed as mean ± standard deviation (SD). The unpaired Student's t-test was used for establishing statistical significance ($p < 0.05$) between rats from the CG and EG groups.

RESULTS

Our results demonstrate no significant effect of 0.2% administration of bee pollen on body weight, femoral weight, femoral length, and cortical bone thickness in male rats (Table 1).

Endosteal borders of femoral bones in rats from the CG group were formed by non-vascular bone tissue in all views of thin sections. This tissue contained cellular lamellae and osteocytes. Areas of primary vascular radial bone tissue (formed by branching or non-branching vascular canals radiating from the marrow cavity) were also identified in anterior and posterior views. In the middle part of the compact bone, primary and secondary osteons were observed. The periosteal border was again composed of non-vascular bone tissue, mainly in the anterior and posterior views (Figure 1).

The rats treated by 0.2% concentration of bee pollen displayed a similar microarchitecture to that of the control rats, except for the middle part of compact bone in posterior view. In this view, vascular canals expanded into central area of the bone what indicates that primary vascular radial bone tissue was also identified in the central area of the femur. Also, a higher number of primary and secondary osteons was observed in the middle part of *substantia compacta* and near periosteal surface in these rats (Figure 2).

For the quantitative histological analysis, 264 vascular canals of primary osteons, 216 Haversian canals and 216 secondary osteons were measured. The results are summarized in Tables 2, 3 and 4. We found that all measured variables (area, perimeter, maximum and minimum diameters) of the primary osteons' vascular canals, Haversian canals and secondary osteons did not differ between rats from both investigated groups.

Using atomic absorption spectrophotometry, the concentrations of Ca, Mg, Fe and Zn in femoral bones of all rats were determined. The findings are shown in Table 5. We have found that application of 0.2% concentration of bee pollen had not significant impact on the content of selected mineral elements in the bones of male rats.

DISCUSSION

Bee pollen contains a wide spectrum of amino acids, vitamins, hormones, and minerals, as well as enzymes and co-enzymes necessary for good digestion and growth. **Haro et al. (2000)** reported that male rats fed with multifloral bee pollen (10 g/kg of the diet for 10 days) had increased body weight. However, we revealed a non-significant effect of 0.2% administration of bee pollen on the body weight of rats. Moreover, the data observed by **Gálik** (**2012**) indicate a decrease of feed intake in rats fed with bee pollen (at the same level as it was used in our study). The decrease of feed intake in previous study could be explained by the increase in nutrient supply. In fact, nutrients such as minerals and water soluble vitamins could accelerate nutrients metabolism and increase energy digestibility, what can negatively affect feed intake **(Attia et al., 2009)** and subsequently don't influence growth of rats.

Prolonged intake of 0.2% concentration of bee pollen in our study induced changes in microscopic structure of femoral bones in male rats. In general, bone is a metabolically active organ and changes in bone angiogenesis are closely associated with bone remodeling **(Brandi and Collin-Osdoby, 2006)**.

Table 1 Average body weight, femoral weight, femoral length and cortical bone thickness in control (CG) and experimental (EG) groups of rats.

Rat's group	N	Body weight (g)	Femoral weight (g)	Femoral length (cm)	Cortical bone thickness (mm)
CG	5	374.00 ±9.62	1.05 ±0.19	3.82 ±0.09	0.554 ±0.064
EG	5	357.00 ±24.90	1.07 ±0.06	3.76 ±0.05	0.545 ±0.052
T-test		NS	NS	NS	NS

N: number of rats, NS: non-significant changes

Table 2 Data on primary osteons' vascular canals in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	146	390.19 ±94.39	71.23 ±9.23	12.51 ±2.31	9.95 ±1.13
EG	118	381.96 ±93.88	70.65 ±9.59	12.42 ±2.49	9.83 ±1.11
T-test		NS	NS	NS	NS

N: number of rats; NS: non-significant changes

Table 3 Data on Haversian canals in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	109	394.65 ±80.78	73.37 ±20.69	12.89 ±4.26	10.25 ±2.41
EG	107	394.34 ±65.02	71.37 ±5.80	12.34 ±1.42	10.23 ±1.27
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

Table 4 Data on secondary osteons in rats from CG and EG groups.

Rat´s group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
CG	109	6819.98 ±1720.62	295.30 ±37.76	51.27 ±8.17	41.97 ±5.70
EG	107	6758.20 ±1772.16	294.87 ±39.99	51.52 ±8.87	41.40 ±6.25
T-test		NS	NS	NS	NS

N: number of measured structures; NS: non-significant changes

Table 5 Concentrations of selected mineral elements in femoral bones of rats from CG and EG groups.

Rat's group	N	Ca	Mg	Fe	Zn
		$\mu g.g^{-1}$			
CG	5	29784.61 ±8427.94	1328.67 ±228.59	95.74 ±21.15	109.63 ±30.42
EG	5	32631.37 ±8914.33	1406.91 ±247.34	97.16 ±20.19	112.45 ±30.28
T-test		NS	NS	NS	NS

N: number of rats; NS: non-significant changes

The formation of blood vessels serves as a way of transporting circulating osteoblast **(Eghbali-Fatourechi et al., 2005)** and osteoclast precursors **(Kassem et al., 1991)** to the sites undergoing active remodeling. **Yamaguchi et al. (2006)** reported that rats fed by bee pollen (50 or 100 mg/kg body weight) had higher DNA content (an indication of higher cell numbers) and higher alkaline phosphatase activity (marker of bone formation) in the femur in comparison with those from the control group. These results point to increased bone remodeling in rats administered by bee pollen. Therefore, a higher number of primary and secondary osteons could be observed in our rats from the EG group. It is generally known that aged rats lack true Haversian cortical bone remodeling but not cancellous bone remodeling **(Erben, 1996**; **Reim et al., 2008)**, which can be influenced by various factors, e.g. fatigue loading of long bones **(Bentolila et al., 1998)**, treatment with bone anabolic substances such as PTH, IGF-I, prostaglandins, vitamin D analogies **(Ibbotson et al., 1992**; **Lauritzen et al., 1993**; **Uzawa et al., 1995; Weber et al., 2004)**. Bee pollen extract has been found to have anabolic effects on bone components in the femora of rats **(Yamaguchi et al., 2006)**. There is also evidence that bee pollen stimulates bone remodeling due to high vitamin D content **(Zuo and Xu, 2003)**. These facts indicate that endocortical bone remodeling could also be stimulated by bee pollen administration in rats from the EG group. Therefore, some newly formed remodeling units (primary and secondary osteons) located near endosteal borders extended deep into the middle part of compact bone.

Morphometrical measurements of basic structural units of the compact bone (primary and secondary osteons) didn't show significant differences in their size between rats from CG and EG groups. These results suggest that low administration of bee pollen in the diet did not influence histomorphometry of the osteons in rats.

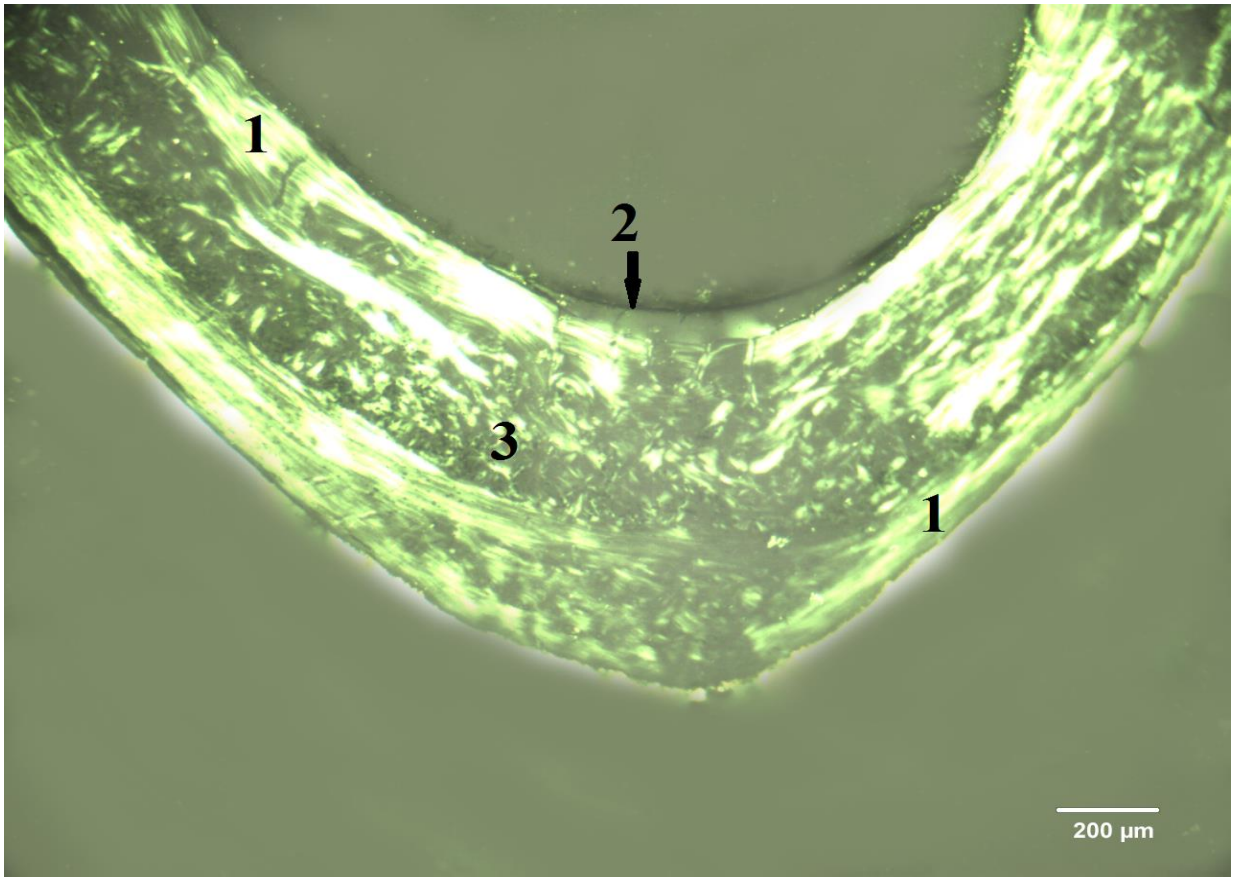

Figure 1 Microstructure of femoral bone in rat from the CG group: 1- non-vascular bone tissue; 2- primary vascular radial bone tissue; 3- primary and secondary osteons.

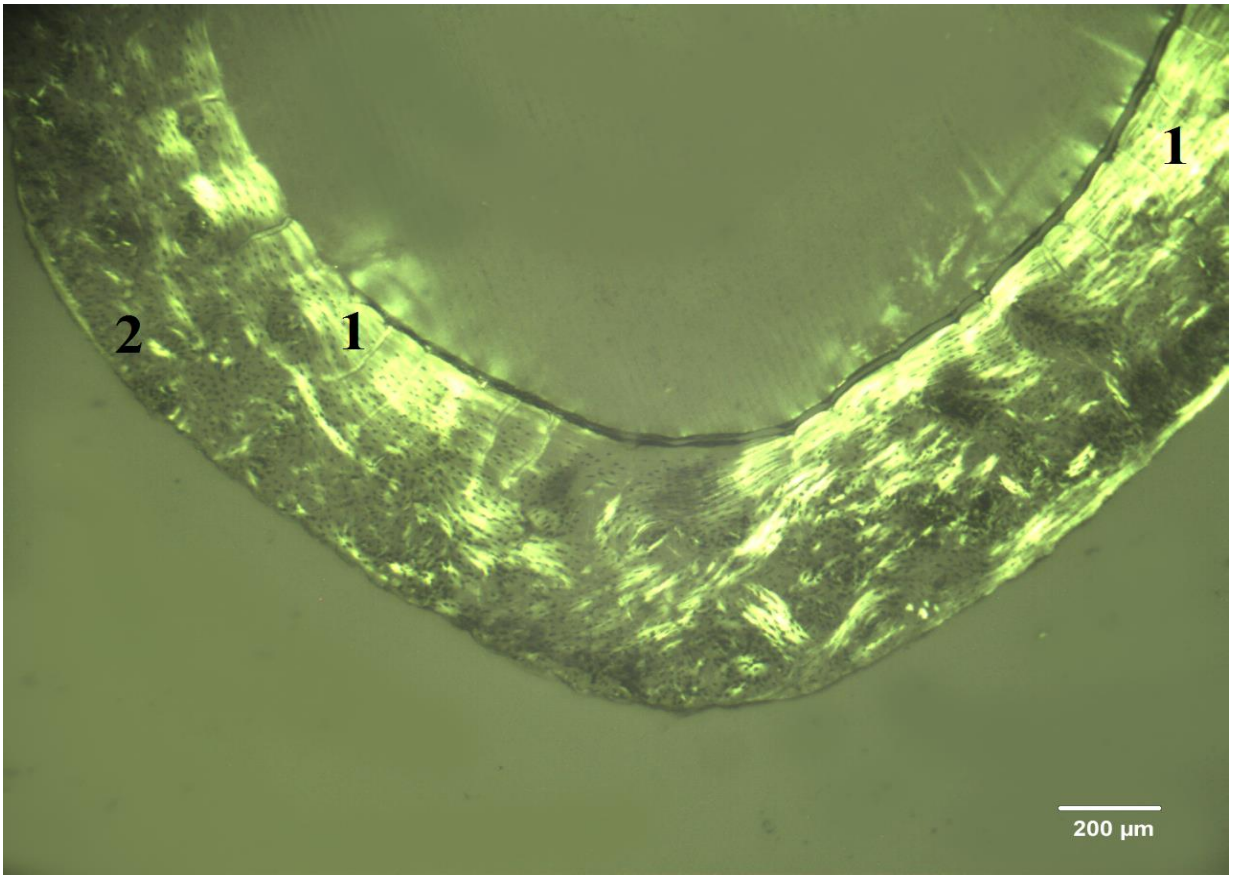

Figure 2 Microstructure of femoral bone in rat from the EG group: 1- primary vascular radial bone tissue; 2- higher number of primary and secondary osteons.

Additionally, our results revealed no demonstrable effect of 0.2% administration of bee pollen on the content of Ca, Mg, Fe and Zn in femoral bones of rats. Bone is generally an important storage organ for essential elements (e.g. Ca, Mg, Fe and Zn). However, their concentrations can be affected by various food additives. Many experimental studies **(Yamaguchi et al., 2006; Wang et al., 2007)** demonstrated positive effects of bee pollen on Ca absorption following its higher deposition in the bone. The better absorption and digestion of Ca in small intestine is attributed to increased contents of vitamin D **(Zuo and Xu, 2003)** and amino acids (e.g. lysine, aspartate, glutamate, ornithine; **Gozálbez, 1984)** in bee pollen. Higher concentration of Ca in femoral bones of rats after peroral administration to 50 and 100 mg/kg bw was observed in the study by **Yamaguchi et al. (2006)**. On the other hand, supplementation with bee pollen in concentrations of 0.5, 1 and 1.5% had no significant impact on Ca content in the bones of broilers **(Oliveira et al., 2013)**, what is consistent with our results. Besides Ca, other minor trace elements (such as Mg, Zn and Fe) can also play important roles in bone metabolism **(O'Neil and Evans, 2004)**. In general, Mg, Zn and Fe are considered to be essential elements for bone formation and bone resorption **(Yamaguchi et al., 1986; D'Haese et al., 1999; Katsumata et al., 2009)**, and they are also primary minerals detected in bee pollen **(Roulston and Cane 2000; Pernal and Currie 2002; Gergen et al., 2006)**. However, we did not observe significant differences related to Mg, Fe and Zn contents in the bones of rats from CG and EG groups. Therefore, it can be concluded that low supplementation with bee pollen (at the concentration used in our study) did not affect Ca, Mg, Fe and Zn levels in femoral bones of male rats.

Additional research dealing with the impact of higher concentrations of bee pollen on bone characteristics is required to gain more information and to verify the results of this study.

CONCLUSION

Our study demonstrates that 0.2% administration of bee pollen in the diet affects the qualitative histological characteristics of femoral bones in male rats. However, it has no significant effect on the size of primary osteons, secondary osteons and on the content of Ca, Mg, Fe and Zn in femoral bones. These results can be applied in experimental studies focusing on the effect of various bee products on bone structure.

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