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A POSITIVE DOSE-RESPONSE OF QUERCETIN ON COMPACT BONE MICROSTRUCTURE OF MALE RABBITS

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ABSTRACT

Quercetin is one of the most widely distributed flavonoids in plants which should have a broad range of significant health promoting properties. Its effect on bone microstructure of males has not been sufficiently investigated. Therefore, our study was aimed to determine the impact of quercetin on femoral bone microstructure in 5-month-old male rabbits. Nine rabbits were intramuscularly injected with following doses of quercetin: 10 µg/kg body weight (bw) (E1 group; n=3), 100 µg/kg bw (E2 group; n=3) and 1000 µg/kg bw (E3 group; n=3) for 90 days, 3 times per week. Three rabbits without quercetin administration served as a control group. An accelerated bone resorption at the endosteal surface and intensive periosteal bone apposition were observed in all rabbits administered quercetin. However, the most evident changes such as a thicker layer of primary vascular longitudinal bone tissue under periosteum and a lower density of secondary osteons were identified in males from the E3 group. Morphometrical evaluations of the compact bone showed significantly decreased sizes of primary osteons' vascular canals and secondary osteons in rabbits exposed to the highest dose of quercetin. Sizes of secondary osteons were also lower in males from the E2 group. Our results demonstrate that quercetin has a positive dose-response on compact bone microstructure in male rabbits.

Keywords: bone microstructure, histology, quercetin, rabbit

INTRODUCTION

Quercetin (3,3',4',5,7-pentahydroxyflavone) is considered to be one of the best characterized flavonoids present in many common fruits and vegetables, beverages, olive oil, and propolis from the bee hives **(Aguirre *et al.*, 2011)**. Its biological activity has been investigated over the last decades with a great interest. Quercetin has anti-inflammatory, anti-allergic, antibacterial and antiapoptotic effects, as well as it protects against cardiovascular diseases, diabetes and stroke **(Wein *et al.*, 2013)**. However, potentially deleterious effects of the quercetin, related to its cytotoxicity and prooxidant activity have also been reported **(Chen *et al.*, 2014)**. According to **Robaszkiewicz *et al.* (2007)**, quercetin-induced antioxidant or prooxidant properties are largely related to its dose given to the biological system.

In general, bone is a rigid organ whose underlying composite structure and chemical composition are responsible for its functionality. Due to very active metabolism, bone microstructure can also be affected by various xenobiotics which either improve or diminish biochemical properties and strength of the bone. According to **Notoya *et al.*, 2004**, quercetin should have protective effects on metabolism of this organ. **Liang *et al.* (2011)** stated a positive impact of quercetin on diabetic osteopenia of rats. Also, it stimulated proliferation and differentiation of osteoblast and MG63 osteoblast-like cells in rats. On the contrary, it decreased osteoclastogenesis of osteoclast precursor 2T-110 **(Forte *et al.*, 2016)**. However, **Kanno *et al.* (2004)** mention that quercetin can also induce apoptosis of MC3T3-E1 mouse calvarial osteoblasts. The same findings have also been observed in the research of **Nam *et al.* (2008)**.

Although quercetin belongs to often analysed flavonoids, its effect on bone microstructure of males has not been sufficiently described yet. Therefore, our study was aimed to determine femoral bone microstructure of adult male rabbits after intramuscular administration of quercetin.

MATERIAL AND METHODS

Our research was conducted on twelve 5-month-old male rabbits of Californian broiler line, which were obtained from an experimental farm of the Animal Production Research Centre in Nitra (Slovak Republic). Animals with a body weight (bw) of 4.00 ± 0.5 kg were housed individually in flat-deck wire cages under constant temperature (20 - 24 °C), humidity (55 % ± 10 %), and 12/12 h cycle of light and darkness with an access to food (feed mixture) and drinking water *ad libitum*. Nine adult rabbits were intramuscularly injected with following doses of quercetin (Sigma-Aldrich, Germany): 10 µg/kg bw (E1 group; n=3), 100 µg/kg bw (E2 group; n=3) and 1000 µg/kg bw (E3 group; n=3) for 90 days, 3 times per week. The doses of quercetin reflected the constant exposure of animals to quercetin in rabbit feed. Three rabbits without quercetin application served as the control group (C group). Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3 and ethics committee.

At the end of experimental period (after 90 days), all rabbits were killed and their femora were used for microscopical analyses. Thin sections from femora were prepared according to the methodology of **Martiniakova *et al.* (2008).** The qualitative characteristics of the compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956)** and **de Ricqles *et al.* (1991)**. The quantitative parameters of the compact bone were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd.). We measured area, perimeter, minimum and

maximum diameters of 320 primary osteons' vascular canals, 320 Haversian canals and 320 secondary osteons in all views (anterior, posterior, medialis and lateralis) of the thin sections.

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc.; Chicago, IL, USA). All data were expressed as mean ± standard deviation. The unpaired Games-Howell's test was used for establishing statistical significance ($P<0.05$) among all groups of rabbits.

RESULTS AND DISCUSSION

Primary vascular longitudinal bone tissue as a basic structural pattern of femora formed the periosteal and endosteal surfaces in rabbits from the C group. This type of the bone tissue was created by vascular canals that run in a direction essentially parallel to the long axis of the bone. Additionally, primary vascular radial bone tissue (formed by branching or non-branching vascular canals radiating from the marrow cavity) was found in some areas near endosteal surfaces. The middle part of the compact bone consisted of irregular and/or dense Haversian bone tissues. Irregular Haversian bone tissue was characterized by an occurrence of isolated and scattered secondary osteons. For dense Haversian bone tissue, a large density of secondary osteons was typical (Figure 1a).

We identified differences in compact bone microstructure of rabbits from the E1, E2 and E3 groups (Figure 1b, c, d). Primary vascular longitudinal bone tissue was completely resorbed in some areas (mainly in anterior and posterior views) near endosteal surfaces. In the other views (medialis and lateralis), a thinner layer of this tissue type was observed. Also, an intensive apposition of primary vascular longitudinal bone tissue under periosteum was identified in all quercetin-exposed rabbits. However, the thicker layer of this bone tissue was found in rabbits from the E3 group (Figure 1d) which also had fewer secondary osteons when compared to the other groups.

Our results from qualitative histological analysis of male rabbits are consistent with those of several authors **(Enlow and Brown, 1958; Martiniakova *et al.* 2006)**. Primary vascular longitudinal, primary vascular radial, dense Haversian and/or irregular Haversian bone tissues were found in all groups of male rabbits. However, the exposure to quercetin leads to dose-dependent changes in these characteristics which were associated with accelerated bone resorption at the endosteal surface and increased periosteal bone appostion. According to **Ahlborg *et al.* (2003)**, excessive endosteal resorption results in increased mechanical stress in the bone tissue, which consequently stimulates periosteal bone formation. Deposition of bone tissue onto the periosteal surface is considered to be a compensative response of the bone against endosteal resorption in an effort to increase bone resistance to loss of bone strength which would lead to microfracture creation **(Seeman, 2007)**. Generally, reactive oxygen species (ROS) are physiologically involved in the process of bone resorption. During this process, osteoclasts produce large amounts of ROS, and their excessive accumulation inhibits bone formation and stimulates further bone resorption **(Baek *et al.*, 2010)**. However, the effect of ROS on bone cells depends on their concentration in the bone tissue **(Brzoska *et al.*, 2011)**. According to **Rahman *et al.* (1992)**, high doses of quercetin contribute to the generation of ROS. Therefore, the thinner layer of primary vascular longitudinal bone tissue near endosteal surfaces, the thicker layer of this tissue type under periosteum and a lower density of secondary osteons in male rabbits from the E3 group could be associated with these aspects.

On the contrary, opposite results have been observed in female rabbits which were administered to identical doses of quercetin **(Babosova *et al.*, 2016)**. The most evident changes in qualitative histological characteristics of compact bone tissue were found in female rabbits exposed to the lowest dose of quercetin. It indicates that quercetin had a negative dose-response on these characteristics in females. We suppose that these variations may be associated with different growth and modeling of the femur through influence by sex-specific steroids, genetic factors and a multitude of other sources. In humans, histological qualitative bone morphology expresses a low sex-related variation. Together with a strong genetic component of qualitative traits they can be used for taxonomic identification of various species **(Martiniakova *et al.*, 2008)**. Remodeling of bones seems to be lower in females, since estrogens slow down bone remodeling and protect against bone loss **(Martiniakova *et al.*, 2003)**. Sex-specific steroids (estrogens in females and androgens in males) play an important role in bone remodeling **(Bord *et al.*, 2000; Manolagas *et al.*, 2013)**. It is known that hormones do not act on bone tissue in isolation **(Hogler *et al.*, 2008)**. Bone tissue must integrate a variety of signals that result from mechanical loads, hormones, and a multitude of other sources. The deficit of these hormones causes changes in the production of regulatory cytokines in the bone marrow **(Aydin *et al.*, 2013)**, which can lead to the increased bone resorption near endosteal surfaces and also increased bone formation near periosteal area **(Feher *et al.*, 2010)**. The results by **Miodini *et al.* (1999)** demonstrated an antagonistic effect of quercetin on the function of estrogen receptor (ER) in breast cancer cell lines MCF-7.

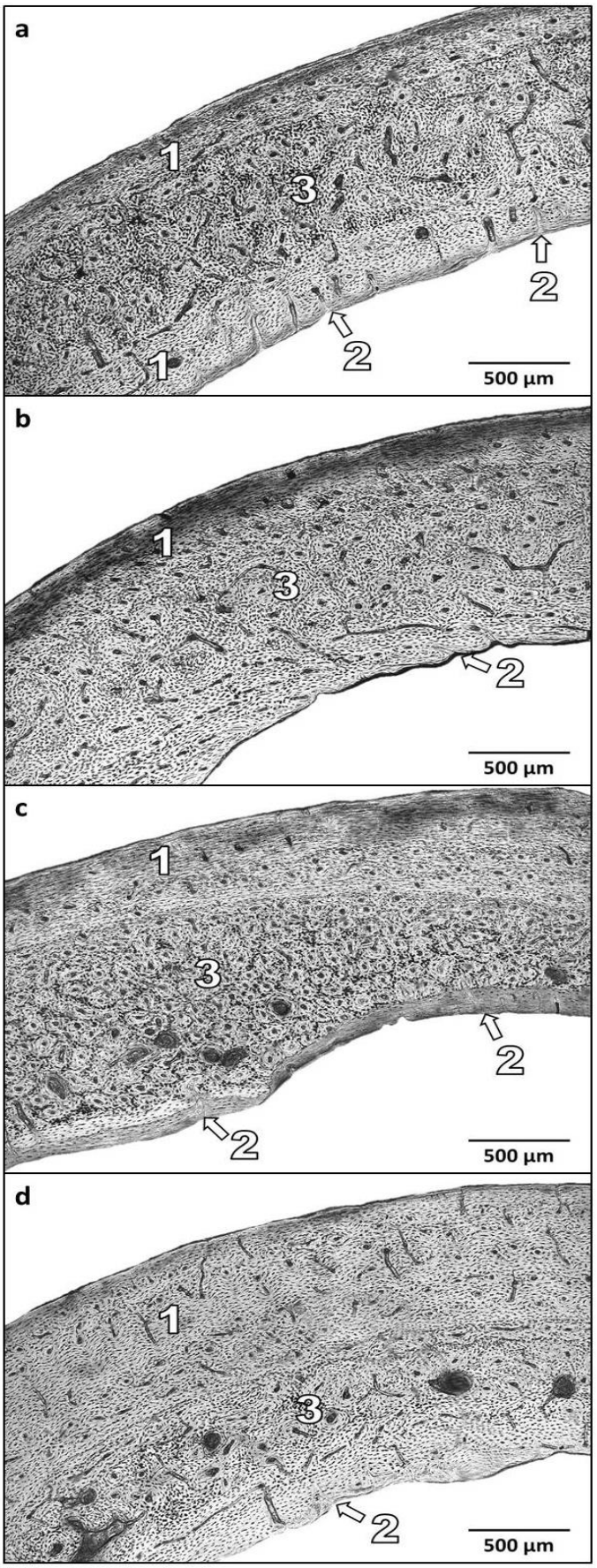

Figure 1 Microscopic structure of compact bone tissue in rabbits from all investigated groups

Legend: a - control group; b - E1 group: 10 µg/kg bw; c - E2 group: 100 µg/kg bw; d - E3 group: 1000 µg/kg bw; 1 - primary vascular longitudinal bone tissue; 2 - primary vascular radial bone tissue; 3 - dense Haversian bone tissue

Krazeisen *et al*. (2001) described an inhibitory effect of quercetin on the 17β-hydroxysteroid dehydrogenase type 5 production which is involved in estrogen and androgen metabolisms. In connection with these facts, **Santini *et al*. (2009)** indicated that quercetin had been found to exhibit both estrogenic and anti-estrogenic actions *in vitro* depending on the dose. Similarly, **Yuan *et al*. (2004)** reported the dose-dependent inhibitory effect of quercetin on the expression and function of the androgen receptor (induction of enzyme c-Jun) in mice. Besides these facts, the lower density of secondary osteons in the middle part of *substantia compacta* of male rabbits from the E3 group leads to weakness of biomechanical properties of their bones.

The results of morphometrical analysis of compact bone tissue in male rabbits from all groups are summarized in Table 1. Measured variables (area, perimeter and minimum diameter) of the primary osteons' vascular canals were significantly decreased in males from the E3 group compared to the C group. Significant differences were also found between E1 and E3 groups (area, perimeter). Haversian canals' values did not differ significantly among all analysed groups. On the other hand, sizes of the secondary osteons (except for maximum diameter) were significantly lower in males from the E2 and E3 groups compared to the C group.

Our results revealed significantly decreased sizes of primary osteons` vascular canals in rabbits from the E3 group. Primary osteons` vascular canals contain blood vessels which provide nutrition for the bone (**Greenlee and Dunnell, 2010). Pries et al. (2005)** showed that blood vessels can adapt its structure (vascular remodeling) in response to continuous functional changes. Vasoconstriction of these canals could be associated with deleterious effect of quercetin on blood vessels. It is known that quercetin may cause decreased production of nitric oxide which leads to vasoconstriction of blood vessels **(Pries *et al*., 2005; Sanchez *et al*., 2006; Lamalice *et al*., 2007; Schmitt and Dirsch, 2009)**.

Significantly reduced sizes of secondary osteons in rabbits from the E2 and E3 groups could be related to the destruction of collagen fibers which are present in these osteons **(Martiniakova *et al*., 2013)**. According to **Ren *et al*. (2016)** quercetin has suppressive effects on the synthesis of collagen types I and III and similarly **Stipcevic *et al*. (2006)** observed a significantly decreased collagen concentration in fibroblasts after quercetin administration.

Babosova *et al*. (2016) found different results in female rabbits exposed to the same doses of quercetin. Significantly decreased sizes of the primary osteons` vascular canals were observed in individuals from E1 and E2 groups. Secondary osteons were significantly decreased in all quercetin administered females. These results indicate that quercetin had not only a positive dose-response on qualitative and quantitative histological characteristics of the compact bone in female rabbits as it would be expected. A positive dose response of quercetin has been identified for sizes of primary osteons` vascular canals and secondary osteons. On the contrary, quercetin had a negative dose-response on qualitative histological characteristics of the compact bone.

In our study, a positive dose-response of quercetin on compact bone tissue microstructure of male rabbits was demonstrated. Therefore, it can be concluded that intramuscular application of quercetin at the doses used in our studies had sex-dependent and also dose-dependent effects on compact bone microstructure in adult rabbits.

Table 1 Morphometrical results of compact and trabecular bone tissues in male rabbits

Measured structures	Group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
Vascular canals of primary osteons	E1 (1)	80	340.70 ± 54.93	66.06 ± 5.38	11.48 ± 1.08	9.48 ± 0.99
	E2 (2)	80	331.47 ± 54.19	65.21 ± 5.21	11.33 ± 1.03	9.35 ± 1.07
	E3 (3)	80	317.35 ± 51.82	63.80 ± 5.11	11.10 ± 1.00	9.13 ± 1.00
	C (4)	80	344.68 ± 50.07	66.43 ± 4.78	11.48 ± 0.99	9.60 ± 0.98
	Games-Howell test		1:3*; 3:4*	1:3*; 3:4*	NS	3:4*
Haversian canals	E1 (1)	80	300.42 ± 63.05	61.94 ± 6.63	10.75 ± 1.38	8.90 ± 1.04
	E2 (2)	80	280.99 ± 48.66	60.04 ± 5.36	10.43 ± 1.21	8.63 ± 0.89
	E3 (3)	80	301.32 ± 56.49	62.20 ± 6.06	10.82 ± 1.37	8.90 ± 0.98
	C (4)	80	322.15 ± 65.07	64.25 ± 6.53	11.13 ± 1.35	9.20 ± 1.19
	Games-Howell test		NS	NS	NS	NS
Secondary osteons	E1 (1)	80	5079.12 ± 1619.61	259.19 ± 44.09	46.74 ± 10.18	34.28 ± 6.29
	E2 (2)	80	4518.80 ± 2169.25	239.66 ± 52.11	43.07 ± 9.65	32.12 ± 8.25
	E3 (3)	80	4629.72 ± 1888.92	244.67 ± 45.93	43.81 ± 8.79	32.86 ± 8.00
	C (4)	80	5979.63 ± 2816.19	273.19 ± 60.51	47.97 ± 11.30	38.12 ± 9.18
	Games-Howell test		2:4*; 3:4*	2:4*; 3:4*	NS	1:4*; 2:4*; 3:4*

Legend: N - number of measurements; E1 group: 10 μg/kg bw; E2 group: 100 μg/kg bw; E3 group: 1000 μg/kg bw; NS - non-significant differences; P<0.05 (*)

CONCLUSION

The results indicate that subchronic intramuscular administration of quercetin has a positive dose-response on compact bone microstructure in male rabbits. The most evident changes in both qualitative and quantitative histological characteristics were observed in males exposed to the highest dose of quercetin. On the contrary, quercetin had not only a positive dose-response on these characteristics in female rabbits as it was documented in our previous study. These results could extend our knowledge related to quercetin's impact on bone microstructure in experimental animals of both genders.

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