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QUERCETIN-INDUCED CHANGES IN FEMORAL BONE MICROSTRUCTURE OF ADULT MALE RABBITS

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ABSTRACT

Flavonoids are a group of plant metabolites with antioxidant effects. One of the most abundant flavonoids in the human diet is quercetin. It is found widely in fruits, vegetables and has a lot of beneficial effects on human health. Quercetin has a positive pharmacological effect on bone metabolism and it prevents the organism against bone loss. However, its impact on the size of basic structural units of the compact bone is still unknown. Therefore, the aim of present study was to investigate the impact of the quercetin on femoral bone microstructure in 5-month-old male rabbits. Five rabbits of Californian broiler line were randomly divided into two groups. In the experimental group (E group; n=3), animals were intramuscularly injected with quercetin at dose 1000 $\mu g.kg^{-1}$ body weight (bw) for 90 days, 3 times per week. Two rabbits without quercetin administration served as a control group (C group). According to our results, intramuscular application of quercetin had an insignificant effect on cortical bone thickness in male rabbits. In these rabbits, changes in qualitative histological characteristics were present in the middle part of the compacta, where primary vascular longitudinal bone tissue was present and expanded there from the periosteum. Also, a lower number of secondary osteons was found in these animals. From the histomorphometrical point of view, significantly decreased sizes of primary osteons' vascular canals and secondary osteons (p <0.05) were found in rabbits administered by quercetin. Our findings indicate that subchronic administration of quercetin at the dose used in our study had considerable impact on both qualitative and quantitative histological characteristics of the compact bone in adult male rabbits.

Keywords: quercetin; femoral bone; histomorphometry; rabbit

INTRODUCTION

Flavonoids are a group of natural polyphenolic substances which consists of two aromatic rings linked by 3 carbons, usually in an oxygenated heterocycle ring **(Liu, 2004)**. These aromatic secondary plant metabolites have been recognized as important bioactive compounds due to their physiological and pharmacological role, and their health benefits **(Hooper and Cassidy, 2006)**. Fruits and vegetables, tea, and cocoa are rich natural sources of flavonoids **(Chen et al., 2010; Egert and Rimbach, 2011; Sekeroğlu and Sekeroğlu, 2012)**. In human diet, one of the most important vegetable with rich content of antioxidant polyphenols is onion. The results by **Kavalcová et al. (2015)** showed that a higher content of polyphenols and thus, a higher antioxidant activity have more colorful varieties of onions. According to **Danihelová and Šturdík (2011)**, average daily intake of flavonoids is strongly dependent on individual, country and culture usages. It is approximately in the range of 150 to 300 mg/day

In nature, flavonoids are most frequently found as conjugates in glycosylated or esterified forms; however, they can occur as aglycones, especially as a result of the effects of food processing **(Aggarwal and Heber, 2014)**. Many flavonoids are shown to have antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention, and anticancer activity **(Yao et al., 2004)**. Their antioxidant capacity is associated with the presence of series of structural characteristics (most probably related to the phenolic hydroxyl groups attached to the ring structure) that allow them to quelate ions of transition metals such as Fe^{2+}, Cu^{2+}, or Zn^{2+} and to catalyze the electron transport **(Braun et al., 2011)**. Moreover, they are able to inhibit lipid peroxidation and platelet aggregation and improve increased capillary permeability and fragility **(Hubbard et al., 2004; Cirico and Omaye, 2006)**.

In the recent past, dietary supplements of flavonoids, as their alternative sources, have become increasingly popular. However, it is important to point out that natural sources of flavonoids contain a complex mixture of secondary plant metabolites and not only flavonoids *per se* **(Crassidy et al., 2011)**. This complex mixture cannot be simply exchanged by single purified substances as dietary supplements. Therefore, it is very essential to evaluate possible adverse effects of purified flavonoids as dietary supplements on human health. Indeed, there is growing evidence that purified flavonoids given in high doses may affect trace element, folate, and vitamin C status. Also, they can exhibit antithyroid and goitrogenic activities **(Egert and Rimbach, 2011)**.

One of the most widely distributed flavonoid in plants is quercetin (3, 3', 4', 5, 7-pentahydroxyflavone; **Liang et al., 2011**). Quercetin is found in many common foods including apples, tea, onions, nuts, berries, cauliflower, cabbage and many other foods **(Lakhanpal and Rai, 2007)**. The normal intake of quercetin is less than 5-40 mg/day. However, people who eat the peel of food with high amounts of quercetin may consume 200-500 mg/day **(Harwood et al., 2007)**. Only 30-50% of ingested quercetin is absorbed, the rest passes through gastro-intestinal tract **(Ross and Kasum, 2002)**.

Quercetin has a broad range of significant health promoting properties **(Agullo et al. 1997; Verhoeyen et al., 2002; Boots et al., 2007)**. According to several authors **(Formica and Regelson, 1995; Manach et al., 1996; Boik, 2001; Satyanarayana et al., 2001; Brookes et al., 2002; Davis et al., 2009; Wein et al., 2013; Wu et al., 2014; Forte et al., 2016)** quercetin has cardioprotective, anticarcinogenogenic, antioxidant, anti-inflammatory, antibacterial and antiapoptic properties. It facilitates apoptosis of tumor cells, in part through depression of an endogenous cytoprotective molecule, heat shock protein 70 **(Hosokawa et al., 1990)**. As well, quercetin may inhibit apoptosis in some nontumorigenic cells. For example, quercetin inhibits hydrogen peroxide (H_2O_2) induced apoptosis of mesangial cells, fibroblasts and epithelial cells **(Ishikawa and Kitamura, 2000)**.

This flavonoid also disposes reactive oxygen species (ROS) and reactive nitrogen species (RNS) scavenging activity **(Heijnen et al., 2001; Nickel et al., 2011; Dehghan and Khoshkam, 2012)** under *in vitro* and *in vivo* conditions **(Choi et al., 2001; Nabavi et al., 2012)**. Therefore, it has often been associated with the reduced risk of oxidative-stress related chronic diseases such as coronary heart disease, stroke and diabetes **(Skibola and Smith, 2000)**.

On the other hand, quercetin has potentially toxic effects, including its mutagenicity, prooxidant activity, mitochondrial toxicity, and inhibition of key enzymes involved in hormone metabolism **(Okamoto, 2005; Zhang et al., 2009)**. **Dunnick and Hailey (1992)** reported that high doses of quercetin over several years might result in the formation of tumors in the kidney of rats. The results by **Rise et al. (2006)** showed that quercetin can modulate ovarian functions by interfering with cell steroidogenic activity and angiogenic activity. Quercetin can also be a potential neurotoxic substance **(Jakubowicz-Gil et al., 2008)**. According to **Robaszkiewicz et al. (2007)**, quercetin-induced antioxidant or prooxidant effects are largely relates to its dose given to biological system. At concentrations > 50 μM, quercetin is able to participate in the oxidation of NADPH in liver cells, shifting the cellular conditions to a more oxidized states **(Buss et al., 2005).**

Regarding the bone, quercetin has a positive pharmacological effect on bone metabolism and it prevents the organism against bone loss **(Boots et al., 2007; Sharan et al., 2011)**. It inhibits mRNA expression of osteoclast-related genes and osteoclast differentiation, thereby reduces bone resorption **(Guo et al., 2012)**.

The studies by **Notoya et al. (2004)** and **Wattel et al. (2004)** revealed that inhibitory potential of quercetin on *in vitro* osteoclastic differentiation is connected via a mechanism involving NF kappa B and activator protein 1 (AP-1). Also, increased alkaline phosphatase activity in MG-63 osteoblasts followed by quercetin application was demonstrated **(Robaszkiewicz et al., 2007)**. **Zhou and Lin (2014)** reported that quercetin could enhance the osteogenic differentiation of adipose-derived stem cells (ASCs) and osteoblastic MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. Moreover, it could stimulate Osterix (Osx), BMP-2, Runx2, OCN, OPN, COL1 and ALP gene expression in ASCs, and increase bone sialoprotein (BSP) and OCN gene expression in osteoblastic MC3T3-E1 cells **(Kim et al., 2006; Satué et al., 2013)**. However, the effect of quercetin on osteoblast function is contradictory **(Yamaguchi and Weitzmann, 2011)**. According to **Prouillet et al. (2004)** it stimulates proliferation and differentiation of rat calvarial osteoblasts and MG-63 osteoblast-like cells. **Braun et al. (2011)** have found protective effect of quercetin on primary human osteoblasts against the toxic influence of cigarette smoke. This fact indicates that a dietary supplementation with quercetin could improve bone structure, skeletal integrity, and even fracture healing in smokers. On the contrary to above findings, **Kanno et al. (2004)** mention that quercetin induces apoptosis of MC3T3-E1 mouse calvarial osteoblasts. **Notoya et al. (2003)** found that it inhibited not only the proliferation but also the differentiation and mineralization of of rat calvarial osteoblast-like cells (ROB cells; **Hagiwara et al., 1996; Partridge et al., 1981)**. Quercetin-induced apoptosis (through a mitochondria-dependent mechanism involving ERK activation) and inhibition of migration (through activation of ERK and p38 pathways) of osteoblasts were also showed in the research by **Nam et al. (2008)**.

The impact of quercetin on histomorphometry of basic structural units of the compact bone is still unknown. Therefore, the aim of our study was to investigate the effect of intramuscular application of quercetin on femoral bone microstructure in adult male rabbits.

MATERIAL AND METHODOLOGY

Our research was carried out on five male rabbits of meat line M91, maternal albinotic line (crossbreed New Zealand White, Buskat rabbit, French Silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big Light Silver) of approximately 5 months of age, with a body weight 4.00 ±0.5 kg. Animals were obtained from an experimental farm of the Animal Production Research Centre in Nitra (Slovak Republic) and were housed in individual flat-deck wire cages. The animals were maintained under constant conditions of light (12-h light/12-h dark), temperature (20-24 °C) and humidity (55% ±10%), with access to food (feed mixture) and drinking water *ad libitum*. The rabbits were randomly assigned into two groups. In the first group (E group; n=3), quercetin was applied intramuscularly in the concentration of 1000 $\mu g.kg^{-1}$bw 3 times per week, for 90 days. The dose of quercetin (reflecting the constant exposure of animals to quercetin in rabbit feed) was chosen based on the literature data **(Choi and Li, 2005; Knab et al., 2011; Lesniak-Walentyn et al., 2013)**. Two rabbits without quercetin application served as a 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 histological analyses. Thin sections from femora were prepared according to the methodology of **Martiniaková et al. (2008)**. The qualitative histological characteristics of compact bone were determined according to the internationally accepted classification systems of **Enlow and Brown (1956) and de Ricqlés et al. (1991),** who classified bone tissue into three broad categories: primary vascular tissue, non-vascular tissue and Haversian bone tissue. 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 minimum and maximum diameters of primary osteons' vascular canals, Haversian canals, and secondary osteons in all fields (i.e., anterior, posterior, medial and lateral) of the thin sections. The 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.

Statistical analysis was performed using SPSS 8.0 software. All data were expressed as mean ±standard deviation (SD). The unpaired t-test was used for establishing statistical significance (p <0.05) between both groups of rabbits.

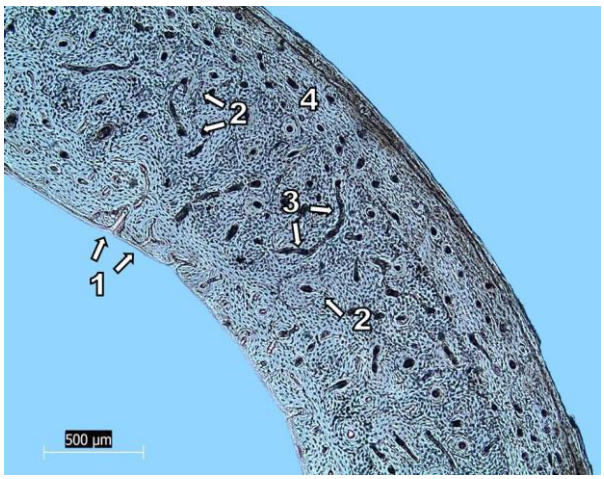
RESULTS AND DISCUSSION

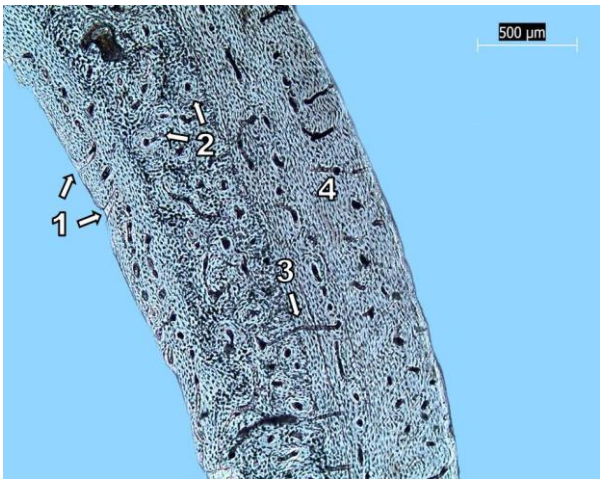
Our results showed an insignificant effect of quercetin on cortical bone thickness in male rabbits (1035.56 ±159.42 μm and 1025.06 ±209.09 μm in rabbits from E and C groups, respectively).

Compact bone microstructure in rabbits from C group (Fig.1) was formed near endosteal bone surfaces by primary vascular radial, irregular Haversian and/or dense Haversian bone tissues. The middle part of the compact bone considered of a layer of irregular and/or dense Haversian bone tissues. Secondary osteons were often connected with Volkmann's canals. The periosteal bone surface mostly consisted of primary vascular longitudinal bone tissue; irregular Haversian bone tissue was observed only in anterior side. These findings are consistent with the results of several authors **(Enlow and Brown, 1958; Martiniaková et al., 2003; Martiniaková et al., 2006)**.

In rabbits from E group, endosteal bone surface was composted by primary vascular radial and irregular Haversian bone tissues. Primary vascular longitudinal bone tissue was in some areas (anterior and posterior) near endosteal surface completely resorbed. The rabbits intramuscularly administered by quercetin had fewer secondary osteons in the middle part of *substantia compacta* because primary vascular longitudinal bone tissue expanded into this part of bone from periosteum. The periosteal border was formed only by primary vascular longitudinal bone tissue (Fig. 2).

Intramuscular administration of quercetin caused evident alterations in femoral bone microstructure of male rabbits. A lower number of secondary osteons in the middle parts of the *substantia compacta* could be associated with accelerated bone resorption. ROS have been reported to play a crucial role in the process of bone resorption **(Halliwell et al., 1992; Yang et al., 1998)**. 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)**. Quercetin has been described as the protector against ROS RNS **(Nickel et al., 2011; Kovacevic and Matulic, 2013)**. However, quercetin has the potential to produce ROS at high doses **(Rahman et al., 1992)**. In the process of scavenging reactive species, quercetin may be converted into potentially harmful oxidation products or subjected to *in vitro* oxidative degradation resulting in the formation of an ortho-quinone and the subsequent production of ROS (i.e., superoxide and hydrogen peroxide; **Boots et al., 2003**). The resultant prooxidant properties of quercetin are responsible for its mutagenic and cytotoxic effects **(Sahu**





1 - primary vascular radial bone tissue, 2 - several secondary osteons form irregular Haversian bone tissue,
3 - Volkmann's canals, 4 - primary vascular longitudinal bone tissue

Figure 1 Microstructure of femoral bone in rabbits from control (C) group.

Figure 2 Microstructure of femoral bone in rabbits from experimental (E) group.

Table 1 Data on vascular canals of primary osteons in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	344.68 ±50.07	66.43 ±4.78	11.48 ±0.99	9.60 ±0.98
E	120	317.35 ±51.82	63.80 ±5.11	11.10 ±1.00	9.13 ±1.00
t-test		***p* <0.05**	***p* <0.05**	**NS**	***p* <0.05**

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

Table 2 Data on Haversian canals in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	322.15 ±65.07	64.25 ±6.53	11.13 ±1.35	9.20 ±1.19
E	120	301.32 ±56.49	62.20 ±6.06	10.82 ±1.37	8.90 ±0.98
t-test		**NS**	**NS**	**NS**	**NS**

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

Table 3 Data on secondary osteons in male rabbits from C and E groups.

Rabbit's group	N	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
C	80	5979.63 ±2816.19	273.19 ±60.51	47.97 ±11.30	38.12 ±9.18
E	120	4629.72 ±1888.92	244.67 ±45.93	43.81 ±8.79	32.86 ±8.00
t-test		***p* <0.05**	***p* <0.05**	**NS**	***p* <0.05**

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

and Washington, 1991; Soria et al., 2010). So, it can indicate toxicity in that case, such as an induction of apoptosis of osteoblasts **(Notoya et al., 2003; Spencer et al., 2003; Son et al., 2008)**.

From histomorphometrical point of view, 200 vascular canals of primary osteons, 200 Haversian canals, and 200 secondary osteons were measured in rabbits from E and C groups. The results are summarized in Tables 1, 2 and 3. The values for measured parameters (area, perimeter, maximum and minimum diameters) of primary osteons' vascular canals and secondary osteons (except for their maximum diameters) were significantly lower (p <0.05) in rabbits from E group. The values from parameters of Haversian canals did not differ between both groups of rabbits. The primary osteons' vascular canals constriction in rabbits from E group can be related to the adverse effect ofquercetin on blood vessels, which provide bone nutrition **(Greenlee and Dunnell, 2010)**. According to **Pries et al. (2005)**, blood vessels are able to adapt its structure (vascular remodeling) as a response to continuous functional changes. *In vitro* studies **(Leikert et al., 2002; Huisman et al., 2004; Wallerath et al., 2005; Jackson and Venema, 2006; Schmitt and Dirsch, 2009)** suggest an adverse effect of quercetin on the enzyme nitric oxidesynthase (eNOS) expression and endothelial nitric oxide (NO) production. NO acts as a potential vasodilator,and itcontributes to the migration and growth of endothelial cells necessary for initiation of angiogenesis *in vivo* **(Carmeliet, 2000; Jackson and Venema, 2006)**. We suppose that alterations in the size of primary osteons' vascular canals in rabbits from E and C groups are connected with an adverse effect of quercetin on the expression of eNOS.

We assume that significant differences (p <0.05) in the size of secondary osteons between rabbits from E and C groups may be associated with the destruction of collagen fibers which are present in the osteons **(Dylevský, 2007)**. **Kang et al. (2001)** found that quercetin significantly inhibited collagens I and III expression and had a growth-inhibitory effect on keloid-derived fibroblasts. The adverse impact of various concentrations of quercetin (20, 40, and 80 $\mu mol.l^{-1}$) on human fibroblasts examined **Stipcevic et al. (2006).** According to these authors, the administration of the highest dose of quercetin leads to significantly decreased collagen concentration (more than 50%) in fibroblasts. We supports that similar effect could also been observed in osteoblasts.

CONCLUSION

The study indicates that intramuscular application of quercetin at dose 1000 $\mu g.kg^{-1}$ bw for 90 days, 3 times per week caused significant changes in qualitative and quantitative histological characteristics of the compact bone tissue in male rabbits. Rabbits exposed to quercetin had a lower number of secondary osteons in the middle part of the *substantia compacta*, and disposed thicker layer of primary vascular longitudinal bone tissue (periost and middle part of the bone). Histomorphometrical evaluations

showed significantly decreased sizes of primary osteons' vascular canals and secondary osteons in males from the E group. Our article provides initial information of the impact of quercetin on femoral bone microstructure in experimental animals.

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