Original Article

Femoral Bone Microstructure in 1-Month-Old Non-transgenic versus Transgenic Rabbits with the *WAP-hFVIII* Gene Construct

(*WAP-hFVIII* transgenic rabbit / femur / histomorphometry)

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Abstract. Differences in microscopic structure of the femur between 1-month-old transgenic rabbits carrying the *hFVIII* gene and non-transgenic rabbits were investigated. Bone microstructure was evaluated from the point of view of qualitative and quantitative histological characteristics. We identified fibrolamellar bone tissue only in the transgenic animals. Measured values for area, perimeter of the Haversian canals and minimum diameter of the primary osteons' vascular canals were higher in 1-month-old transgenic individuals ($P < 0.05$; $P < 0.001$). We also observed lower concentrations of Ca, P, K, solids, and total mineral content in femora of transgenic rabbits. A statistically significant difference was observed for the concentration of Ca ($P < 0.05$). Our results indicate evident changes in both qualitative and quantitative histological characteristics of the femur, which result especially in better blood supply and slightly reduced mineralization process in 1-month-old transgenic rabbits.

Introduction

Transgenic rabbits represent an alternative way to produce biologically active proteins. However, several problems are commonly encountered with their production, including transgene integration, stability and expression. The results of our previous studies indicate that transgenic technology can produce possible changes also in other tissues than in the target ones.

We identified a new type of bone tissue – fibrolamellar tissue – in 2.5-month-old transgenic rabbits carrying the *WAP-hFVIII* gene construct (Martiniaková et al., 2005a). This type of woven bone has not been observed in non-transgenic rabbits even in any ontogenetic stages to date. According to Currey (2002), fibrolamellar bone tissue is found particularly in large mammals whose bones have to grow in diameter rather quickly. It has been reported in *Canis* (dog), *Ovis* (sheep), *Sus* (pig), *Bison* (buffalo), and *Bos* (cattle), and also in fossil bones including *Pharahippus blackburgi* (primitive horse), *Kannemeyeria* (herbivorous mammal-like reptiles), and *Brachiosaurus* and *Plateosaurus* (extinct herbivorous dinosaurs) (Mori et al., 2005). Fibrolamellar bone, which is primary, seems to be stronger, particularly when loaded along the grain, than Haversian bone, which in many animals replaces it (Currey, 1959). In recent years, it has been widely accepted that richly vascularized fibrolamellar bone tissue is deposited at a higher rate than parallel-fibered or lamellar bone (Currey, 2003; Ponton et al., 2004). The way fibrolamellar bone is laid down means that there are, in effect, alternating layers of parallel-fibered or woven bone and lamellar bone tissue extending, quite often, for many millimeters, or even centimeters, in the radial direction (Currey, 2002). The appearance of fibrolamellar bone tissue in 2.5-month-old transgenic rabbits carrying the *hFVIII* gene referred to changes in bone modelling and subsequently remodelling in these animals. In detail, we identified increased bone modelling in transgenic individuals (femoral bone length was higher in transgenic rabbits in comparison with the non-transgenic ones) and also better blood supply (area, perimeter, minimum diameter of the Haversian canals were higher in transgenic rabbits; the differences were statistically significant) (Martiniaková et al., 2006). In an effort to explain these differences we compared the cytogenetic profile of bone marrow cells between transgenic

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and non-transgenic rabbits. A significantly higher rate of aneuploidy was obtained in transgenic rabbits (62 %) than in non-transgenic individuals (37 %) ($P < 0.001$; Martiniaková et al., 2005a). We suggested that a significant difference in chromosomal aneuploidy between 2.5-month-old transgenic and non-transgenic rabbits could result from genetic manipulations.

It signals that the above-mentioned results brought some new questions about microstructural differences in compact bone tissue between non-transgenic and transgenic rabbits. One of the most important is at what age of rabbits the differences already appear. Therefore, the aim of the present study was to compare femoral bone microstructure in younger (1-month-old) non-transgenic versus transgenic rabbits carrying the *hFVIII* gene. We tried to find out whether some changes in histological structure of the examined bones are also present in these rabbits.

Material and Methods

In our experiments, 14 clinically healthy 1-month-old rabbits (six females and eight males) were analysed. The animals were obtained from an experimental farm of the Slovak Agricultural Research Centre in Nitra (Slovakia). They were housed in individual flat-deck wire cages, under a constant photoperiod of 14 h of daylight. The temperature and humidity of the building were recorded continually by means of a thermograph positioned at the same level as the cages. The rabbits were fed *ad libitum* with a commercial diet and water was provided *ad libitum* with nipple drinkers. The breeding conditions were similar to intensive industrial conditions. We used New Zealand White transgenic rabbits carrying the human blood clotting factor VIII gene and non-transgenic animals. The transgene was under transcriptional control of the whey acidic protein (WAP) promoter. The rabbits were kept and euthanized especially for other investigations at the Slovak Agricultural Research Centre in Nitra (Slovakia). In addition to these investigations, bone samples were collected for our study. All procedures were conducted with the approval of the Animal Experimental Committee of the Slovak Republic. Transgenic versus non-transgenic individuals were identified by the PCR method. Total DNA was isolated from the ear issue of newborn rabbits. Conditions for PCR amplification of the *hFVIII* transgene were the same as reported by Chrenek et al. (2005), using primers hFVIII-F: 5'-GTA GAC AGC TGT CCA GAG GAA-3' and hFVIII-R: 5'-GAT CTG ATT TAG TTG GCC CAT-3', which define a 578-bp region of human FVIII cDNA.

Fourteen right femora were studied by histological analysis (seven from transgenic and seven from non-transgenic rabbits). Each of the bones was sectioned at the midshaft of its diaphysis. The obtained segments were macerated and degreased (Martiniaková et al., 2005b). Later, the samples were embedded in epoxy resin Biodur (Günter von Hagens, Heidelberg Germany). Transverse thin sections (70–80 μm) were prepared with a sawing microtome (Leitz 1600, Wetzlar, Germany) and affixed to glass slides with Eukitt (Merck, Darmstadt, Germany). The qualitative histological characteristics of the bone tissue were determined according to the classification systems by Enlow and Brown (1956) and Ricqlès et al. (1991), the quantitative were assessed using computer software Scion Image (Scion Corporation, Frederick, MD) in anterior, posterior, medial and lateral views of thin section. The osteon size can vary in the views mentioned above (Skedros et al., 2007); therefore, we had to measure the area, perimeter, and the minimum and maximum diameter of primary osteons' vascular canals, Haversian canals, and secondary osteons in all views of the thin section in order to correct individual differences in the statistics. The *t*-test was used to distinguish differences in quantitative histological characteristics between transgenic and non-transgenic individuals (Statistica 4.3, 1993).

The concentrations of mineral elements (Ca, P, Mg, Na, K, Fe, Mn, Zn, Cu) were determined using the method of atomic absorption spectrophotometry. We used left femora from analysed rabbits for the mineral concentration. The samples were ashed at 550°C for 6 h. The ash was dissolved in 10 ml of HCl (1 : 3). The concentrations of mineral elements were analysed using an atomic absorption spectrophotometer (Unicam, Cambridge, UK). The flame conditions were those recommended by the instrument manufacturer for Ca, Mg, Na, K, Fe, Mn, Zn and Cu (wavelength 422.7 nm, 285.2 nm, 589 nm, 766.5 nm, 248.3 nm, 279.5 nm, 213.9 nm and 324.8 nm, respectively). In the case of P determination, the analysis was done by photometric assay (wavelength 430 nm) after reaction of the sample with solution of molybdenum and vanadium (Mikrochem, Pezinok, Slovak Republic). All concentrations were expressed on a wet weight basis in $g.kg^{-1}$. From the final data, the arithmetic mean and standard deviation were calculated.

Results and Discussion

Integration of the *WAP-hFVIII* gene was detected in seven out of 14 analysed rabbits. Representative results are shown in Fig. 1. The distribution of sexes in both

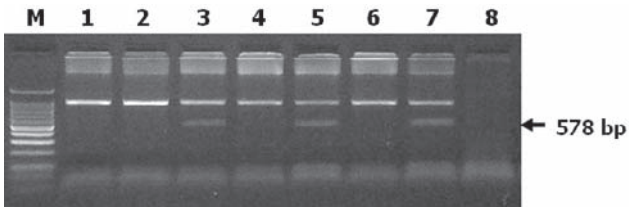

Fig. 1. Detection of *hFVIII* insert by PCR method.
M – marker 100 bp; 3, 5, 7 – positive (transgenic) rabbits; 1, 2, 4, 6 – negative (non-transgenic) rabbits; 8 – negative control

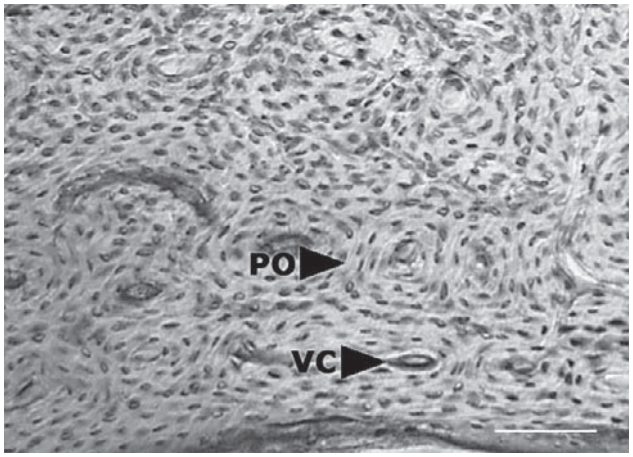

Fig. 2. Femoral bone microstructure in 1-month-old non-transgenic rabbit. Bar represents 100 μm.
PO – longitudinally oriented primary osteons; VC- vascular canals of primary osteons

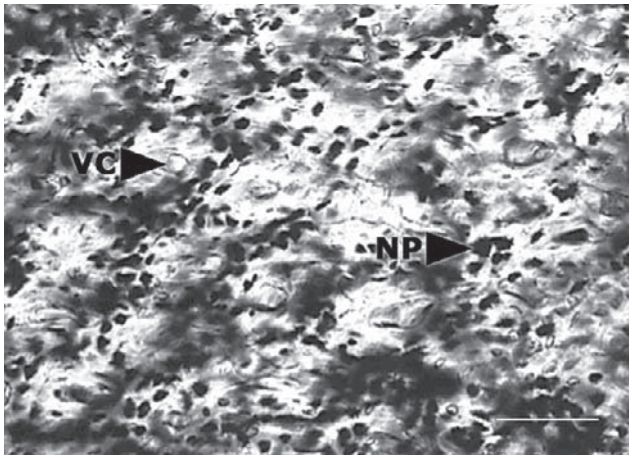

Fig. 3. Fibrolamellar bone tissue with many non-mineralized parts of *substantia compacta* in 1-month-old transgenic rabbit.
Bar represents 100 μm. NP- non-mineralized parts of compact bone; VC- vascular canals of primary osteons

transgenic and non-transgenic rabbits was four males and three females.

In histological analysis we found that in general, femoral bone tissue displayed primary vascular longitudinal structure in both groups of 1-month-old rabbits (Fig. 2). However, fibrolamellar bone tissue was again identified only in the transgenic individuals (mainly at periosteal border). In addition, the fibrolamellar tissue possessed more non-mineralized parts of *substantia compacta* (Fig. 3) as compared to 2.5-month-old transgenic rabbits. This fact indicates that the process of mineralization might be considerably reduced in 1-month-old transgenic individuals. To this aim, mineral content was also assessed in the analysed bones of rabbits (transgenic vs. non-transgenic). The results are shown in Table 1. In concordance with our results, lower concentrations of Ca, P, K, solids and also total mineral content were found in transgenic individuals. On the contrary, transgenic rabbits display higher concentrations of Fe, Zn, Cu, Mg and Na. A statistically significant difference was observed for the concentration of Ca ($P < 0.05$). It suggests that the process of mineralization is slightly reduced in 1 month-old transgenic individuals in comparison with the non-transgenic ones.

In general, a lower number of secondary osteons was identified in 1-month-old rabbits as compared to the 2.5-month-old animals. We suppose that this fact is caused by different age of the individuals. Rajtová and Globočník (1978), Martiniaková et al. (2005b) have found that circumferential lamellae are substituted by the secondary osteons with increased age in mice and rabbits, respectively. Besides, Currey (2002) mentions that formation of secondary osteons tends to lead to the production of more secondary osteons during the individual's life. Therefore, we observed more secondary osteons in 2.5-month-old rabbits.

All in all, 565 vascular canals of primary osteons, 77 Haversian canals, and 77 secondary osteons of transgenic and non-transgenic rabbits were measured. The results are summarized in Table 2. We found that all variables of the Haversian canals were higher in transgenic individuals. Statistically significant differences were recorded for their area ($P < 0.05$) and perimeter ($P < 0.001$). Similarly, a higher value for minimum diameter of the primary osteons' vascular canals was observed in transgenic individuals ($P < 0.05$). On the other hand, non-transgenic rabbits displayed higher values for

Table 1. Mineral content ($g.kg^{-1}$) in the femur of 1-month-old transgenic and non-transgenic rabbits

rabbits	element					
	Ca	P	Mg	Na	K	solids (%)
transgenic	138.54±2.12	90.19±6.42	3.49±0.26	4.22±0.31	4.16±0.84	44.03±2.57
non-transgenic	142.87±2.69+	94.82±6.12	3.25±0.55	4.18±0.13	4.50±0.27	44.81±1.06
	element					
	Fe	Mn	Zn	Cu	total mineral content (%)	
transgenic	0.145±0.034	0.005±0.0005	0.173±0.014	0.008±0.002	48.82±1.24	
non-transgenic	0.142±0.024	0.005±0.0005	0.168±0.007	0.007±0.001	50.24±1.65	

$P < 0.05$ (+)

Table 2. Results of quantitative histological analysis in 1-month-old transgenic versus non-transgenic rabbits

rabbits	n	measured values	variables	x±SD	v (%)
transgenic	7	vascular canals of primary osteons	area (μm²)	220.06±85.01	38.63
			perimeter (μm)	40.59±10.67	26.29
			max. diameter (μm)	19.07±5.97	31.31
			min. diameter (μm)	6.38±1.84^{+}	28.91
non-transgenic	7	vascular canals of primary osteons	area (μm²)	228±84.78	38.72
			perimeter (μm)	44.08±11.50	26.09
			max. diameter (μm)	21.15±6.54	30.91
			min. diameter (μm)	6.31±1.62	25.71
transgenic	7	Haversian canals	area (μm²)	481.97±183.51^{+}	38.08
			perimeter (μm)	65.67±15.85^{+++}	24.13
			max. diameter (μm)	30.32±9.95	32.81
			min. diameter (μm)	10.08±2.70	26.77
non-transgenic	7	Haversian canals	area (μm²)	370.02±115.22	31.14
			perimeter (μm)	57.72±14.85	25.73
			max. diameter (μm)	25.56±9.58	34.75
			min. diameter (μm)	9.04±2.40	26.54
transgenic	7	secondary osteons	area (μm²)	7705±2059.28	26.72
			perimeter (μm)	240.06±51.70	21.54
			max. diameter (μm)	114.90±37.02	32.22
			min. diameter (μm)	37.25±6.12	16.43
non-transgenic	7	secondary osteons	area (μm²)	8292.76±2620.91	31.69
			perimeter (μm)	258.91±61.78	23.26
			max. diameter (μm)	119.87±36.74	30.65
			min. diameter (μm)	44.21±9.67^{+++}	21.88

x - mean, SD - standard deviation, v - coefficient of variance, $P < 0.05$ (+), $P < 0.001$ (+++)

the other variables of primary osteons' vascular canals and all variables of the secondary osteons, except for their maximum diameter. The value for minimum diameter of the secondary osteons was significant in statistics ($P < 0.001$).

Although the analysis of quantitative histological characteristics of the femur in 1-month-old rabbits revealed a lower number of significant differences between transgenic and non-transgenic individuals in comparison with the 2.5-month-old ones, it is evident that changes in compact bone tissue are also present in these individuals. However, higher values for measured variables of the Haversian canals were identified in transgenic individuals in both cases. This means that juvenile transgenic rabbits carrying the *hFVIII* gene in general display improved process of blood supplying due to the presence of fibrolamellar bone tissue. Reid (1997) mentions that fibrolamellar tissue is well vascularized, and most of the bone matrix is deposited in abundant primary osteons that produce a fibrous, woven appearance. According to Starck and Chinsamy (2002), fibrolamellar bone is more richly vascularized as compared to lamellar bone with less intense vascularization. Moreover, Currey (2002) reports that the blood vessels anastomose in three dimensions and each is surrounded by more or less concentric layers of lamellar bone. In addition, Ray et al. (2005) note that this type of the bone tissue is considered to indicate rapid osteogenesis and suggest overall fast growth connected with intensive modelling of the bone tissue. On the other hand, higher values for variables of the primary osteons' vascular canals and the secondary osteons were recorded in non-transgenic individuals for both age categories. In our opinion, it could be caused by different length of investigated bones between transgenic and non-transgenic individuals. Jowsey (1966) found that values of the secondary osteons are higher in individuals with longer bones. According to our results the femoral bone length was higher in 1-month-old non-transgenic rabbits (4.786 ± 1.366 cm) as compared to the transgenic animals (4.476 ± 1.44 cm). This suggests that the appearance of fibrolamellar bone tissue in our transgenic rabbits does not indicate still increased bone modelling as it was observed in older (2.5-month-old) transgenic rabbits in our previous study (Martiniaková et al., 2006). We speculate that it is caused by different localization of fibrolamellar

bone between 1- and 2.5-month-old transgenic rabbits (fibrolamellar bone tissue was found mainly at periosteal border in 1-month-old transgenic rabbits; the tissue was identified especially in the middle part of *substantia compacta* in 2.5-month-old transgenic individuals). Another possible reason might be the presence of many non-mineralized parts of the bone in fibrolamellar bone tissue of 1-month-old transgenic rabbits. To this aim, the mineralization process is slightly reduced, and bone modelling could not be as much increased as expected. Reduction of non-mineralized parts of *substantia compacta* in fibrolamellar bone tissue of 2.5-month-old transgenic rabbits led up namely to increased bone modelling in these rabbits.

In conclusion, changes of femoral bone microstructure in 1-month-old transgenic versus non-transgenic rabbits were identified. Evident changes in both qualitative and quantitative histological characteristics result especially in better blood supply and reduced mineralization process in the examined individuals. Although it is difficult to explain these changes (mainly the appearance of fibrolamellar bone tissue only in transgenic rabbits) since genetic and environmental factors are taken into account (Martiniaková et al., 2005a), our results indicate that they could be caused by genetic manipulations. In other words, fibrolamellar bone tissue has not been identified in rabbits at all. Additionally, it is known that microstructural changes in the bone tissue between individuals of the same species are widely conditional to different age (Martiniaková et al., 2005b), length of investigated bone (Jowsey, 1966), and also to genetic factors (Beamer et al., 2001). However, the investigated animals were examined at the same age and they were kept under standard conditions. Femoral bone length indeed displayed higher value in 1-month-old non-transgenic rabbits, but we presume that it is a result of the reduced mineralization process in transgenic animals. With increasing age of transgenic individuals, higher bone length has been observed in these rabbits due to intensive bone modelling. In any case, some changes in the bone length were found between transgenic and non-transgenic individuals. Nevertheless, changes in bone length identified between individuals of the same species have never led to the appearance of a new type of bone tissue in these animals; they were correlated mainly to different bone geometry and mechanical properties (Brianza et al., 2007). In order to obtain more reliable results in this direction, more individuals have to be examined.

Our study seems to be the first report about changes in femoral bone microstructure between 1-month-old non-transgenic and transgenic rabbits carrying the *hFVIII* gene. Further research will need to extend the number of analysed skeletal elements in transgenic versus non-transgenic individuals and to verify the results that were obtained from our bone samples in successive ontogenetic stages of rabbits. It is a subject of current research. With the information about confirmed changes in histological structure of the rabbit femur for both transgenic and non-transgenic individuals we would be able to perform more precise assessment of the effects of environmental and genetic factors on bone tissue formation in several age groups of rabbits.

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