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HISTOCHEMICAL ANALYSIS OF SKELETAL MUSCLES IN NORMAL AND LUXANT RABBITS

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ABSTRACT

The objective of this work was to compare the structure of MTB (*musculus triceps brachii*), MLL (*musculus longissimus lumborum*) and MBF (*musculus biceps femoris*) muscles in normal and luxant rabbits of New Zealand White breed. Live weight of luxant rabbits at the age of 84 days was 1950 g, which was by 518 g less compared with normal rabbits ($P<0.05$). Average thickness of muscular fibers in MTB, MLL and MBF was balanced without statistical differences ($P>0.05$) in normal rabbits. The highest thickness of muscular fibers in luxant rabbits was for MTB (42.16 μm) and the lowest for MBF (37.66 μm) and this difference was significant ($P<0.05$). Total area of interstitial connective tissue in muscles of healthy rabbits was 6.82 %, and 6.52 % in luxant rabbits ($P>0.05$). In muscles of normal and luxant rabbits an area of α white fibers was prevailing. Luxant rabbits had the highest area representation of red fibers in MBF (16.66 %), and in normal animals it was 13.76 %; this difference was significant ($P\leq0.05$). We registered giant fibers in muscles of healthy as well as luxant rabbits during subjective evaluation. These fibers were of oval form, enveloped in thicker layer of endomysium. They were located predominantly on the periphery of bundles, and with increased number created the fibers fascicles in the direction of centre of the bundle. These fibers were characterized by clear multiplication of nuclei, and their location is directed spiral-like from the periphery to the centre of the fibre. Bounds of fibre splitting were observed in places of nuclei grouping. Average thickness of giant fibers in muscles of healthy animals was 69.10 μm, and 76.00 μm in luxant animals, the difference 6.9 μm being statistically significant ($P<0.05$). Frequency of giant fibers in muscles of normal rabbits was 1:100, in luxant rabbits was found the highest frequency of these fibers in MBF from 1:10 up to 1:25. The lowest occurrence of giant fibers from 1:100 up to 1:120 was in MLL in both groups of studied rabbits. These results document that the average thickness of muscular fibers of the three studied muscles (MTB, MLL, MBF) in normal rabbits is very balanced, with the same tendency for thickness of red and α white fibers.

Key words: rabbit, luxant, muscle, histochemistry

INTRODUCTION

Pathology of skeletal muscles concerns a broad spectrum of medical problems, and it is classified variably and it transforms into various clinical disciplines. A part of morphological changes in skeletal muscles are also different changes in size and structure of muscular fibres. From the viewpoint of functional and causal pathology a heterogeneous group is under question, the structural changes being mostly of focal arrangement, multifocal character without predilection of type, but they can be bound also to a certain type of muscular fibres that correspond to disorders in metabolism of skeletal muscles and to signs of primary degeneration of skeletal muscles.

Wohlfart (1937) described the large giant hypertrophic fibres “giant fibres” in microscopic image for the first time. Since their manifestation is connected

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with splitting of muscle fibres the subject of interest is to describe more detailed this process and its impact on the organization of the muscle. From the histopathological viewpoint, first of all morphological changes on the level of muscle fibres come into prominence. Comparison of normal and giant muscle fibres shows the disproportions in their thickness in light microscope image that manifests itself by apparent change in the variability of thickness of individual muscle fibres (Linke, 1972; Fenichel, 1963; Bednář, 1984). Ferrer et al. (1992) and Handel and Strickland (1968) state that the mentioned changes represent particularly fibres, that are hypertrophic, dense, containing homogenous structure and without visible myofibrils. It is characteristic that muscle fibres are fragmented and often vacuolized (Dutson et al., 1978). Microscopic finding in muscles is characterized with clear staining already representing continuity of the process, the end stage of which is an elimination of a part of muscle fibre by histiocytes and T-lymphocytes, as described by Bednařik (2001). Muscle nuclei in normal muscle fibres are localized immediately under the sarcolemma, but in giant muscle fibres internally localized nuclei are also observed besides them (Cabello and Ricoy-Campo, 2003). In addition to increasing number of centrally localized nuclei, Taverna et al. (1998) also report a vacuolisation of muscle fibres and multiplication of interstitial connective tissue around them, which is also confirmed in the work of Fazarinc et al. (2002). Multiplication or formation of nuclei aggregates is a common event in the process of neurogenic lesions and the skeletal muscle itself can macroscopically look like enlarged (Brozman and Ondruš, 1968). As described by Gayathri et al. (2000), central nuclei in more than 3 % fibres often present in neurogenic lesions. In case if their frequency exceeds 30 %, chronic muscular dystrophies may be supposed; but if their frequency exceeds 60 %, a central nuclear myopathy or myotonic dystrophy can be diagnosed. Presence of internal nuclei is, according to Adams et al. (1962), connected to splitting of muscular fibres. Since the muscular nuclei aggregate inside the sarcoplasm of giant fibres, in light microscopic picture the central formation of chain-like belt-like structures can be observed resulting in splitting of such affected giant fibre into a number of smaller muscular fibres (Jeannet et al., 2004). According to Remignon et al. (2000) and Fiedler et al. (2001) fissures create in muscle fibres in the place of splitting that are located in the centre of fibres or they penetrate upright from the periphery to their centre.

Hereditary diseases of muscles, various myopathies are described in fact in all farm animals. There are various diseases of muscles that can, but need not, to be manifested during the life of animals but they can affect their growth and deteriorate meat quality. The objective of our work was a comparison of histological structure in selected muscles of normal and luxant rabbits.

MATERIAL AND METHODS

New Zealand White breed belongs to representatives of meat breeds of rabbits; for some lines of this breed quite frequent occurrence of luxant individuals is characteristic. Therefore, in our experiment we used animals whose the left and right hind leg was luxated (luxant individuals), and we compared them with healthy animals originating from the same litter and were males. We used 5 males of "Z" line (luxants) and 5 normal males. Animals involved into the experiment were bred up to the age of 84 days in the Slovak Agricultural Research Centre in standard microclimate conditions, housed in wire mesh cages.

Anatomical and histochemical analysis of experimental animals was done at the Department for Veterinary Disciplines, Slovak Agricultural University, Nitra. The experimental animals were anaesthesized by chloroform; after exsanguination and skinning samples were taken from muscles *musculus longissimus lumborum* (MLL), *musculus triceps brachii* (MTB) and *musculus biceps femoris* (MBF). After extirpation of the whole muscle a sample was taken from the same place, at the size 1 x 1 x 1 cm, and the samples were processed immediately and frozen in liquid nitrogen at the temperature –196°C in Dewar's vessel. Subsequently serial sections at 8-10μm were cut in the cryostat (Slee cryostat MTC, Raymonid a lamb INC, Durham, North America) at constant temperature –17°C. To determine individual types of muscle fibres the activity of succinate dehydrogenase in sections was measured as described by Lojda and Papoušek (1978). Red muscle fibres in studied muscles were evaluated as sum of α and β red fibres. Percentage of individual types of muscle fibres and interstitial connective tissue were determined by means of grid method using Lanameter device (Carl Zeiss, Jena, Germany) as described by Uhrín and Kulíšek (1980). Also average thickness of all muscle fibres and thickness of individual types of muscle fibres were determined by this method. Another series of sections were stained by haematoxylin-eosin after a short incubation (5 min) for subjective evaluation. Neutral lipids were analysed in next series of sections by means of oil red staining "0". Light microscope Olympus Provis (Olympus optical CO, LTD, Tokyo, Japan) was used for subjective evaluation of preparations. Thickness of giant fibres was assessed by means of ocular micrometer, and their frequency was detected by reckoning the normal and giant fibres in primary muscle bundle.

The results obtained in our experiment were evaluated using the SAS programme, where we calculated basic statistical characteristics (arithmetical mean, standard deviation, mean standard deviation, coefficient of variation). To determine significance of differences between normal and luxant rabbits the t-test with subsequent Scheffe test were used.

RESULTS

On the basis of obtained results we can state that at the age of 84 days rabbits with luxated extremities (luxants) had markedly lower live weight (1950.60 g), i.e. by 518.90 g less compared with normal individuals that achieved live weight 2469.50 g. From the viewpoint of statistics significant difference was found in favour of the group of normal rabbits (P<0.01).

Total average thickness of muscle fibres in *m. longissimus lumborum* (table 1) in healthy rabbits achieved the value 42.6±2.49 μm and in luxant rabbits the thickness of fibres was lower (39.36±3.29 μm) but without statistically significant differences (P>0.05). Thinner red fibres were noticed in luxant rabbits (32.60±0.99 μm) and thicker - in normal individuals (36.13±1.24 μm); this difference was statistically significant (P<0.05). α White fibres reached the largest thickness and their values are balanced in healthy as well as luxant rabbits (47.92±0.87 μm : 46.12±2.19 μm). In luxant rabbits there was a great variability in total thickness of muscle fibres (26.44 %) determined by marked differences in thickness of white and red fibres.

Thickness of muscle fibres in *m. triceps brachii* (MTB) was in normal and luxant rabbits (table 2) almost on the same level (42.11±2.28 μm and/or 42.16±3.04 μm) and without statistical differences (P>0.05). We found considerable balance and coincident tendency in both groups of rabbits in thickness of individual types of muscle fibres. In healthy rabbits, an average of red fibres and white fibres were 35.97±0.83 μm and 48.25±0.97 μm respectively. Thickness of red muscle fibres in luxant rabbits was 36.06±0.86 μm and of white ones 48.25±1.97 μm. Statistical differences in size of red and α white muscle fibres between groups were not confirmed (P>0.05), however, there were moderate statistically significant differences (P<0.01) between red and white fibres in both groups. Greater variability of total thickness in muscle fibres of luxant animals was in this case conditioned by greater variability of white fibres (20.5 %).

Thickness of muscle fibres in *m. biceps femoris* (MBF) of hind leg (table 3) is characterized by the most marked differences between normal and luxant rabbits. Average thickness of muscle fibres in this muscle was 42.10±2.14 μm in normal rabbits and 37.66±2.13 μm in

Table 1: Thickness of muscle fibres MLL (μm)

Rabbits	Muscle	Fiber type	n	$\bar{x}$	s_x	v %	P*	$\bar{x}$	s_x	v %	P*
Normal	MLL	β + α Red	6	36.13	1.24	17.90	N:L+	42.06	2.49	18.88	N:L-
		α White	4	47.92	0.87	9.10	N:L-				
Luxant	MLL	β + α Red	5	32.60	0.99	15.20	N:L+	39.36	3.29	26.44	
		α White	11	46.12	2.19	23.80	N:L-				

MLL – musculus longissimus lumborum, P*- significance, N:L- normal:luxant, - (P≥0.05), + (P≤0.05)

Table 2: Thickness of muscle fibres MTB (μm)

Rabbits	Muscle	Fiber type	n	$\bar{x}$	s_x	v%	P*	$\bar{x}$	s_x	v %	P*
Normal	MTB	β + α Red	4	35.97	0.83	11.50	N:L-	42.11	2.28	17.13	N:L-
		α White	5	48.25	0.97	10.00	N:L-				
Luxant	MTB	β + α Red	4	36.60	0.86	11.90	N:L-	42.16	3.04	22.84	
		α White	10	48.25	1.97	20.50	N:L-				

MTB – musculus triceps brachii, P*- significance, N:L- normal:luxant, - (P≥0.05), ++ (P≤0.01), β:α – (β Red, α White

Table 3: Thickness of muscle fibres MBF (μm)

Rabbits	Muscle	Fiber type	n	$\bar{x}$	s_x	v %	P*	$\bar{x}$	s_x	v %	P*
Normal	MBF	β + α Red	4	37.53	0.78	10.4	N:L+	42.10	2.14	16.42	N:L+
		α White	5	46.67	0.91	9.78	N:L+				
Luxant	MBF	β + α Red	2	32.41	0.50	6.96	N:L+	37.66	2.13	15.98	
		α White	4	42.91	0.89	9.30	N:L+				

MBF – musculus biceps femoris, , P*- significance, N:L- normal:luxant, + (P≤0.05),

luxant ones. From the statistical viewpoint significant differences (P<0.05) in this parameter were found. Thickness of red muscle fibres was in normal rabbits (37.53±0.78 μm) statistically significantly higher (P<0.05) than in luxant rabbits (32.41±0.50 μm). We noticed similar tendency in thickness of α white muscle fibres that was statistically significantly higher (P<0.05) in normal rabbits (46.67±0.91 μm) than in luxant ones (42.91±0.89 μm).

These results document that the average thickness of muscle fibres of the three studied muscles (MTB, MLL, MBF) in normal rabbits is very balanced; this is true also with thickness of red and α white fibres. α White fibres were statistically significantly thicker (P<0.05) than the red ones.

However, the studied muscles of luxant rabbits showed marked differences. The greatest thickness of muscle fibres was in MTB and the lowest one in MBF; this recorded difference was also statistically significant (P<0.05). Medium values of muscle fibre thickness were found in MML; they were not statistically significant (P<0.05) compared with the MTB and MBF muscles. Also, in this case α white fibres in luxant rabbits, had significantly greater thickness (P<0.05) than the red ones in the studied muscles.

The percentual area representation of the interstitial connective tissue and individual types of muscle fibres gives a more detailed picture of the inner structure of muscles. Total percentual area representation of interstitial connective tissue in studied MTB, MLL and MBF muscles was 6.82 % in healthy rabbits and

Table 4: Representation of muscle fibre types and of interstitial connective tissue (%)

Rabbits	Fiber type	Muscles	$\bar{x}$	s_x	v %
NORMAL	α+β Red	MTB	11.77	0.96	18.32
		MLL	11.79	1.18	22.41
		MBF	13.76	0.51	7.43
	α White	MTB	81.78	1.12	3.07
		MLL	83.05	0.91	2.44
		MBF	79.07	2.07	5.24
	Inv	MTB	6.49	0.29	9.85
		MLL	5.67	0.28	10.95
		MBF	8.43	0.40	9.43
LUXANT	α+β Red	MTB	12.58	1.00	17.91
		MLL	14.54	0.97	14.91
		MBF	16.66	0.96	11.43
	α White	MTB	81.42	0.40	1.09
		MLL	79.47	1.40	3.93
		MBF	75.90	1.00	2.61
	Inv	MTB	5.90	0.49	18.71
		MLL	6.36	0.38	13.7
		MBF	7.32	0.50	13.62

MLL – musculus longissimus lumborum; MTB – musculus triceps brachii; MBF – musculus biceps femoris; Inv – intersticial tissues

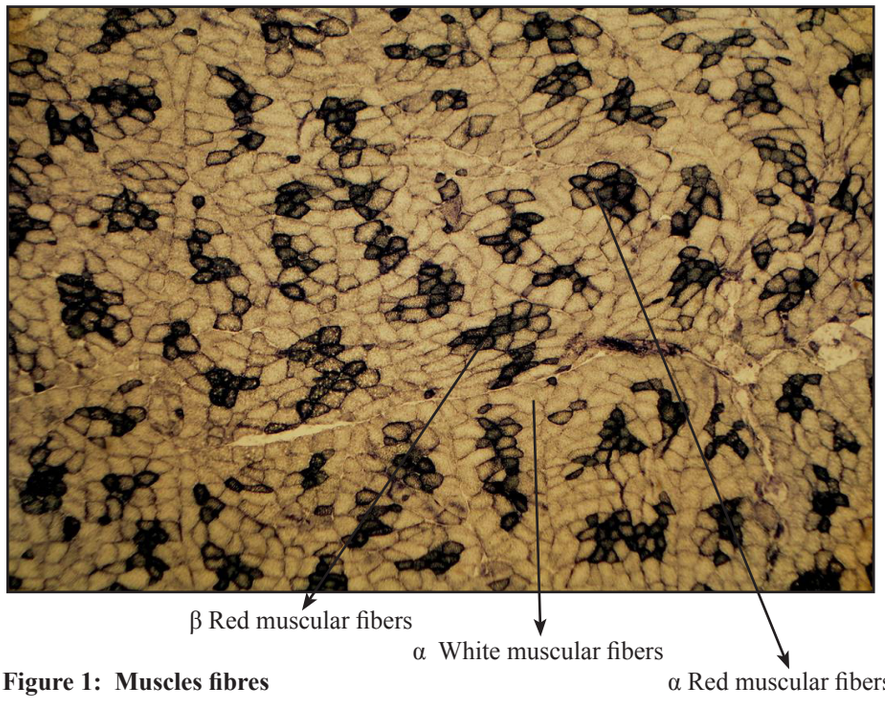

Figure 1: Muscles fibres

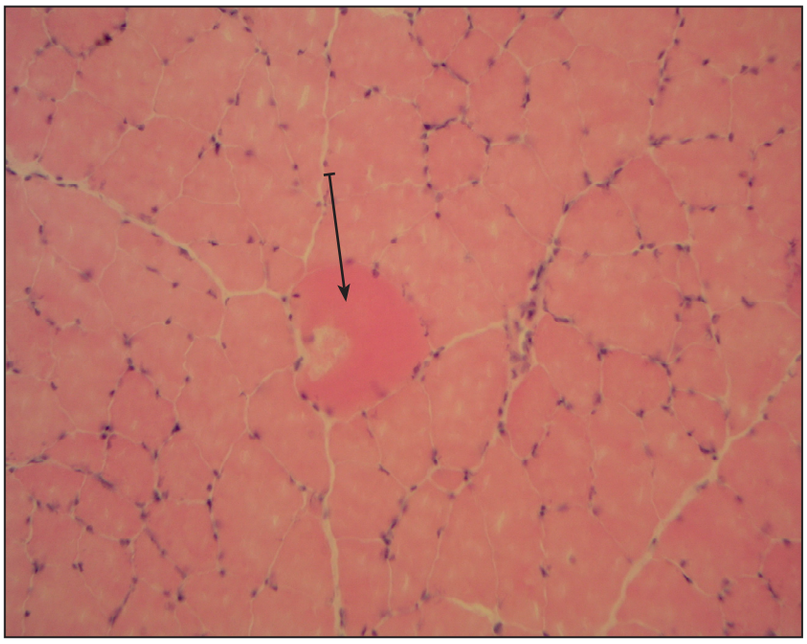

Figure 2: Muscle giant myofibers

6.52 % in luxant ones; this difference was not statistically significant (P>0.05). The largest area representation of interstitial connective tissue was in MBF (8.43 : 7.32) in both groups of rabbits. Percentual area representation of red fibres in normal rabbits was balanced in MTB, MLL and MBF and it varied from 11.77 % (MTB) up to 13.76 % (MBF). Similar relation was detected also with α white fibres, where the values varied from 79.07 % (MBF) to 83.05 % (MLL). High statistical differences (P<0.001) were observed only when red and α white fibres in all studied muscles were compared.

Percentual representation of red fibres in luxant rabbits was the lowest in MTB (12.58 %) and the highest in MBF (16.66 %); the difference was statistically significant (P<0.05). Percentual representation of α white fibres in luxant rabbits in MBF was 75.90 %; it was statistically significantly lower (P<0.05) representation than in MTB with the value 81.42 %. From the above mentioned follows that in studied muscles of healthy, as well as luxant rabbits, the area representation of α white fibres are dominating. Luxant rabbits had the greatest representation of red muscle fibres in MBF (16.66 %) compared with other muscles, and the lowest representation of α white fibres.

During subjective evaluation of giant fibres we paid our attention at their form, thickness, localization, frequency in bundles and occurrence of fat cells in their close neighbourhood. Giant fibres were present also in muscles of healthy rabbits, in which the consequences of luxation were not phenotypically manifested. It is necessary to emphasize that they occurred in primary bundles, rarely, without specific location.

Giant fibres were characteristic in histological preparations and they differed markedly from normal muscle fibres. They were mostly of oval form, sometimes slightly elliptical. Around these fibres thicker layer of endomysium occurred, so they were distinctly separated from the rest of fibres. On the cross section of giant fibres the multiplication of nuclei was clearly seen, the location of which varied quite a lot. The nuclei were partly tightly under the sarcolemma but also deeper inside the fibres. Nuclei inside the fibres were located randomly, but more often they were arranged into a concentric spiral directed from periphery to the centre of the fibre. In places where the nuclei were localized into the concentric spiral, fissures can be seen, probably indicating fibre splitting. The line of splitting can be observed in the place of nuclei aggregation, disruption being created either in the centre of fibre or they penetrate upright from the periphery to their centre.

Reaction of giant fibres on SDH varied quite a lot. The thicker these fibres were the weaker the reaction was, and it was highly positive on the periphery of the fibre, whilst the centre showed very weak reaction. In some fibres diformasan granules were concentrated into centrally convergent spiral similarly to nuclei.

Thickness of giant fibres in muscles of healthy rabbits varied from 58.50 μm to 80.00 μm, and their average thickness was 69.10 μm. Comparison of these fibres with total average value of normal fibres showed

that they were approximately 1.7 times thicker. In muscles of luxant rabbits the thickness of giant fibres within the span fluctuated from 63.14 μm up to 95.6 μm and their average thickness was 76.00 μm. Giant fibres of luxant rabbits are approximately two times thicker than total average of normal muscle fibres. Average thickness of giant fibres in muscles of luxant rabbits was by 6.9 μm greater than in muscles of healthy rabbits.

Location of giant fibres is not specific. Giant fibres that occurred in bundle rarely were located on the periphery as well as inside. They formed at high frequency clusters of fibres that were on the surface of the bundle as a rule, and sometimes they formed bands directed from the periphery into the bundle. In these places always amplification of endomysium took place. In two cases we noticed that in giant fibres myelin degeneration took place.

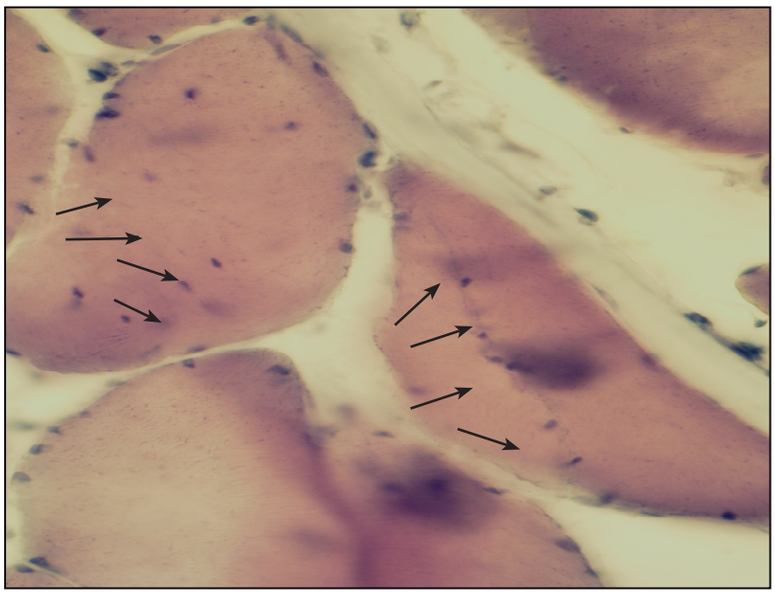

Figure 3: Muscle giant myofibers

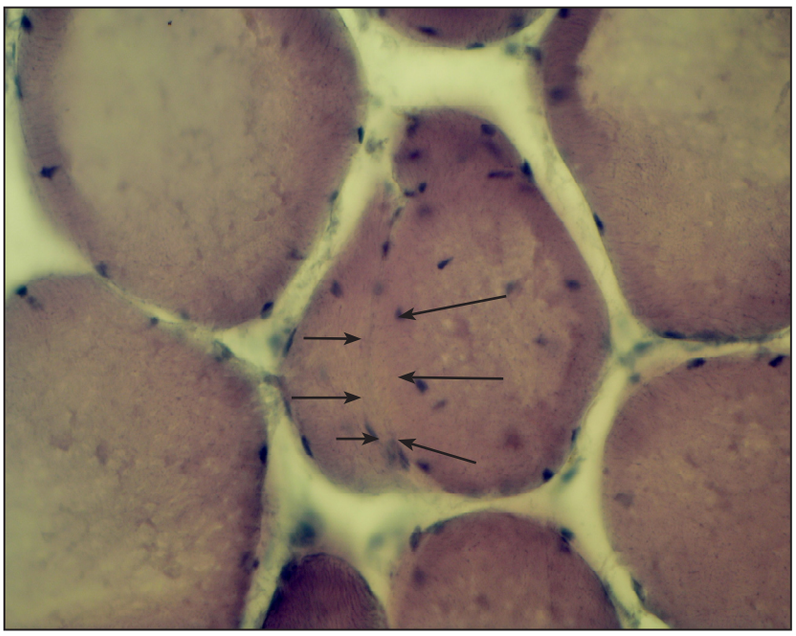

Figure 4: Muscle giant myofibers

Frequency of giant fibres in muscles of normal rabbits was rare (1:100). In luxant rabbits the highest frequency in m. biceps femoris was observed, where the ratio of normal fibres to giant ones was from 1:10 up to 1:25. Frequency of giant fibres was lower in other studied muscles. We noticed the least number of giant fibres (basing on the frequency of occurrence) in m. longissimus lumborum (1:100 up to 1:120).

Total average thickness of fat cells in muscles of healthy rabbits was 38.15 μm and in luxant rabbits 37.50 μm.

DISCUSSION

It is a problem to confront the results of our experiment with knowledges of other authors. Only Zelník et al. (1980) studied the influence of this defect (luxation) on growth and live weight in rabbits. They found out that in luxant rabbits an unfavourable influence of the defect on live weight growth begins at the age of 48 to 84 days, and the difference between healthy and sick rabbits was approximately 600 g, which corresponds also to the results of our experiment. Whilst the mentioned authors observed also macroscopic changes in hip joints, we concentrated our attention to microscopic build of muscle. We can state, in agreement with other authors, that thickness of α white fibres is greater than the thickness of red fibres and that there differences exist in thickness of muscle fibres also among different muscles in the body.

Giant fibres are described more or less only theoretically (Bednář, 1984). Therefore we set criteria for description of these fibres, which are interpreted in results of our experiment. Our morphological knowledge about the form, size and location of giant fibres is in concert with data of Cabello and Ricoy-Campo (2003) and Fazarinc et al. (2002). Process of giant fibre splitting was in our experiment similar to those described by Jeannet et al. (2004). The frequency of giant fibres found in luxant rabbits, studied in our experiment, should also, according to Gayathri et al. (2000), represent chronic muscular dystrophies up to centronuclear myopathies.

CONCLUSION

These results document that the average thickness of muscle fibres of three studied muscles (MTB, MLL, MBF) in normal rabbits is very balanced; this is true also with thickness of red and α white fibres. The evaluation of fat cell location showed that their occurrence among the primary muscle bundles was sporadic. Among tertiary bundles a fat cells lobules were formed, as a rule, in the neighbourhood of blood vessels. There were basically no differences in the thickness of fat cells among the studied groups and muscles.

We found the greatest thickness and highest frequencies of giant fibres in *m. biceps femoris* of luxant rabbits, which indicates a certain relation with luxation. However, we cannot say with certainty if direct impact of muscle structure (content of giant fibres) on manifestation of this defect is under question, because other endogenous factors can also affect this state.

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