Journal of Elementology ISSN 1644-2296

Żarczyńska K, Babińska I., Snarska A., Sobiech P. 2019.
Levels of selenium and vitamin E in the blood and morphological changes in the biceps femoris muscle during nutritional muscular dystrophy of calves.
J. Elem., 24(4): 1363-1370. DOI: 10.5601/jelem.2019.24.1.1768

RECEIVED: 10 December 2018
ACCEPTED: 22 May 2019

ORIGINAL PAPER

LEVELS OF SELENIUM AND VITAMIN E IN THE BLOOD AND MORPHOLOGICAL CHANGES IN THE BICEPS FEMORIS MUSCLE DURING NUTRITIONAL MUSCULAR DYSTROPHY OF CALVES*

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Abstract

In calves, selenium and vitamin E deficiency leads to a disease known as nutritional muscular dystrophy (NMD), which involves hyaline degeneration of muscle fibers and contributes to mobility disorders. The aim of this study was to describe morphological changes in the biceps femoris muscle and to analyse changes in the selenium and vitamin E levels and glutathione peroxidase activity in calves with nutritional muscular dystrophy. The study was conducted on 20 Holstein Friesian calves of both sexes, aged approximately 1 month, divided into two groups: a control group of 10 healthy animals and an experimental group of 10 calves with symptoms of nutritional muscular dystrophy. Calves of the control group were administered a single injection of a selenium (0.5 mg of sodium selenite per ml) and vitamin E (50 mg of tocopherol acetate per ml) preparation at 8 ml per animal on the second day after birth. Blood samples were collected from all animals to determine selenium and vitamin E concentrations, and the activity of glutathione peroxidase (GSH-Px). Sections of the biceps femoris muscle were collected from all calves for histopathological analyses (staining with hematoxylin and eosin-HE and hematoxylin-basic fuchsin-picric acid – HBFP). Hyposelenemia ($p \leq 0.05$), acute vitamin E deficiency ($p \leq 0.05$) and a decrease in glutathione peroxidase activity ($p \leq 0.01$) were observed in the blood of calves with nutritional muscular dystrophy. Changes characteristic of Zenker's degeneration were observed in numerous muscle fibers in the analyzed sections of the biceps femoris muscle of calves with symptoms of nutritional muscular dystrophy.

Keywords: calves, selenium, Nutritional Muscular Dystrophy, Zenker's necrosis.

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* This study was financed by a grant from the Polish Ministry of Science and Higher Education (N N308 584740).

INTRODUCTION

Nutritional muscular dystrophy (NMD), also known as white muscle disease, affects mostly lambs, goatlings, foals, calves and poultry. The disease is caused by selenium and vitamin E deficiency. In calves, NMD symptoms usually appear at up to 6 months of age. Stilted growth and stiff and unsteady gait are observed in the first stage of NMD. As the disease progresses, animals become recumbent and unable to rise. Even when assisted, calves are unable to maintain a standing position, and they may exhibit muscle tremors, in particular in the hind limbs. Some animals have incorrect posture with widely spread limbs, neck extended forward and excessive convex curvature of the spine. Calves have stilted gait, and they walk on the sides of their hooves (Żarczyńska et al. 2012).

In anatomopathological examinations of calves with symptoms of NMD, degenerative changes are observed mainly in limb muscles, in particular in the biceps femoris muscle, and less commonly in spine muscles. Pathologically changed white or gray muscle fragments resemble cooked fish meat, they are usually symmetrical and strongly contrast with healthy red-brown muscle tissue (Ghany-Hefnawy, Tortora-Perez 2010). Selenium and vitamin E deficiency induces changes in the ultrastructure of muscles, manifested by mitochondrial swelling, fragmentation of mitochondrial cristae, chromatin thickening around the nucleus, loss of sarcomere continuity and a decrease in glycogen concentrations below the limit of detection. In microscopic analyses, advanced hyaline degeneration (*degeneratio hyalinea*), also known as Zenker's necrosis (*necrosis Zenkeri*) or waxy degeneration (*necrosis cerea)*, is observed in muscle fibers, and it can lead to extensive muscle damage, necrosis and proliferation of connective tissue (Bostedt, Schramel 1990, Osame et al. 1994).

The objective of this study was to describe changes in the selenium and vitamin E levels and activity of glutathione peroxidase in the blood during nutritional muscular dystrophy of calves. Additionally, morphological changes in the biceps femoris muscles of calves affected with NMD were analyzed.

MATERIALS AND METHODS

The study was conducted on 20 Holstein-Friesian (HF) calves of both sexes, raised on two farms in the region of Warmia (farm I – 100 dairy cows, farm II – 30 dairy cows). The control group comprised 10 calves (7 females, 3 males) aged approximately 1 month (28-30 days) which did not show symptoms of NMD in a clinical examination. Control group animals were raised on farm I. The cows on Farm I did not receive selenium or vitamin E supplements, but calves were administered a single injection of a selenium (0.5 mg

of sodium selenite per ml) and vitamin E (50 mg of tocopherol acetate per ml) preparation (Eurovet Animal Health BV, Netherlands) at 8 ml per animal on the second day after birth. The experimental group consisted of 10 calves (5 females and 5 males) aged approximately 1 month (27-30 days), raised on farm II, with clinical symptoms of NMD (muscle tremor, stiff gait, recumbency). Experimental group animals and their mothers did not receive selenium or vitamin E supplements. The study was carried out between October 2014 and February 2015. Blood samples were collected from calves on a single occasion to determine selenium and vitamin E concentrations, and the activity of glutathione peroxidase (GSH-Px). Blood was sampled from the external jugular vein into test tubes containing lithium heparin (3 ml) for the determination of GSH-Px activity, and to test tubes with a coagulant (9 ml) for the determination of selenium and vitamin E concentrations.

Sections of the biceps femoris muscle were collected from all animals for histopathological analysis. The site of incision was shaved, disinfected and anesthetized by infiltration with 5 ml of polocaine (Biowet Drwalew, Poland). Muscle samples were obtained by scalpel incision of 0.8 x 0.8 cm with a depth of approximately 0.7 cm.

Serum selenium levels were measured by flame atomic absorption spectroscopy in a Unicam 939 Solar spectrometer coupled to a hydride generation system. Vitamin E concentrations were determined by high performance liquid chromatography (HPLC) in a Hewlett Packard HP-1050 chromatograph with the use of Recipe Chemical ClinRep kits (Recipe, Munich, Germany). The activity of GSH-Px was measured in whole blood by the kinetic method with the use of cumene hydroxide and phosphate buffer in the Epoll 20 analyzer using the Ransel diagnostic kit.

Muscle sections for histopathological analysis were immersed in saline solution (Natrium chloratum 0.9%, Baxter, Warsaw, Poland) for 10 min, neutralized with 10% formalin (Chempur, Piekary Śląskie, Poland) and embedded in paraffin. Microtome sections from longitudinal and cross sections of the examined muscles were stained with hematoxylin and eosin (HE) and hematoxylin-basic fuchsin-picric acid (HBFP) to detect necrotized fibers. Analyses of vitamin E concentrations, GSH-Px activity and histopathological evaluations were performed in the laboratory of the Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn, Poland. Selenium concentrations were analyzed in the laboratory of the Faculty of Veterinary Medicine of the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic.

The results of laboratory tests were presented in SI units and processed in the Statistica 9.0 application. The significance of differences between groups was determined by Student's *t*-test for two independent samples at $p \leq 0.05$ and $p \leq 0.01$. The study obtained an approval of the Local Ethics Committee for Experiments on Animals (49/2017).

RESULTS

Selenium and vitamin E concentrations in the blood serum and GSH-Px activity in whole blood of healthy calves and calves with symptoms of NMD are presented in Table 1.

Table 1

Concentration of selenium and vitamin E in serum and activity of glutathione peroxidase in blood in healthy calves and in calves with symptoms of nutritional muscular dystrophy

Se ($\mu g\ l^{-1}$)		Vitamin E ($\mu g\ ml^{-1}$)		GSH-Px ($U\ g^{-1}$ Hb)	
Healthy calves	calves with NMD	healthy calves	calves with NMD	healthy calves	calves with NMD
50.08 ± 0.77	36.12* ± 0.51	3.88 ± 0.07	1.27* ± 0.06	115.69 ± 335	24.98** ± 0.86

Results are expressed as a mean of ten determinations ± SEM.
NMD – Nutritional Muscular Dystrophy, GSH-Px – glutathione peroxidase,
* significant difference between groups at a confidence level of $p \leq 0.05$,
** significant difference between groups at a confidence level of $p \leq 0.01$.

In healthy animals, normal muscle fibers with visible cross striations were observed in the analyzed samples of the biceps femoris muscle stained with HE (Figure 1). Necrotic fibers were not found in muscle sections stained with HBFP (Figure 2).

In calves with symptoms of NMD, numerous muscle fibers with signs of sarcoplasmic hyalinization, sarcoplasmic degeneration and loss of cross striation were observed in HE-stained specimens of the biceps femoris muscle (Figures 3, 4, 5). In selected samples, muscle fibers differed in diameter (small diameter was indicative of fiber damage) and the presence of hypercontracted fibers was noted (Figures 3, 5). Giant muscle cells were also present (Figure 6). Phagocytic infiltration was observed in the area of damaged fibers (Figure 4). Satellite cells, numerous myogenic cells, myoblasts fused with myotubes and newly formed fibers were also noted in the area of damaged fibers (Figure 7). Extensive calcification, stimulation and proliferation of connective tissue cells, fibroblasts and myofibroblasts were observed in necrotized areas. The accompanying morphological changes included swelling and minor infiltration with mononuclear cells in the endomysium, perimysium and epimysium (Figure 3).

Focally extensive polyphasic necrosis of muscle fibers (Figure 8) with sarcoplasmic degeneration, loss of cross striations, hypercontraction of fibers and individual giant fibers were noted in HBFP-stained specimens. Infiltration with phagocytic cells and stimulated myogenic cells was observed in and around foci of necrosis.

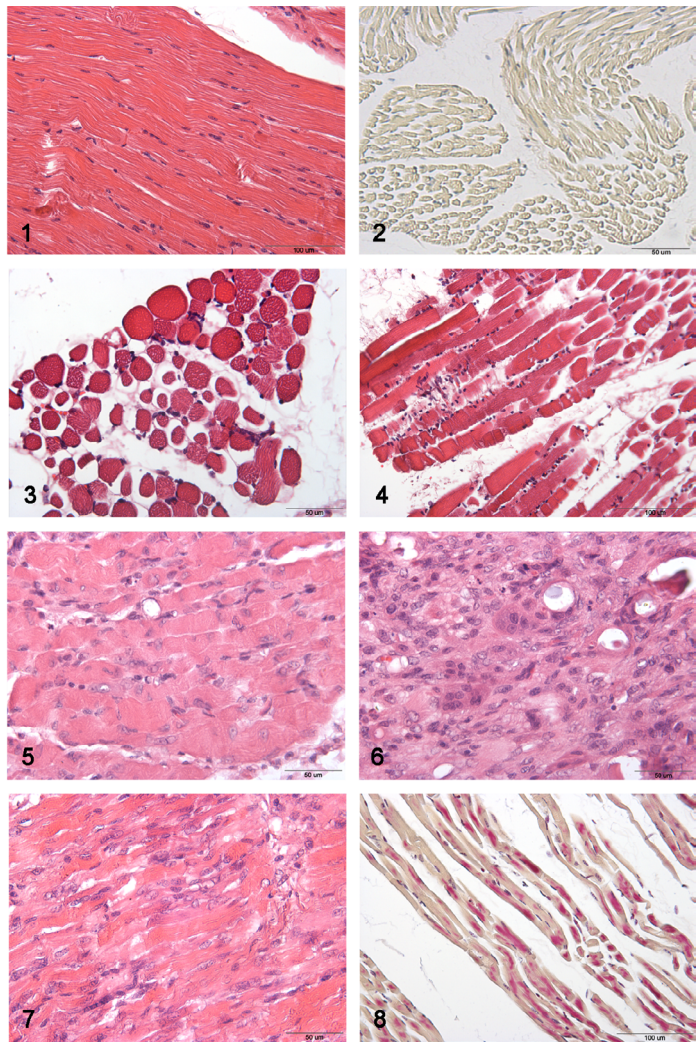

Fig. 1. The proper structure of the calf muscle of the control group.
Biceps femoris. HE staining
Fig. 2. Negative HBFP reaction (no fibers under necrosis)
in the calf muscle of the control group. Biceps femoris. HBFP staining
Fig. 3. Muscle fibers with different diameter, present big, round fibers with sarcoplasm
hyalization and hypercontracted fibers.
Endo- and perimysium oedema. Biceps femoris, calf. HE staining
Fig. 4. Segmental degeneration and hyalinization of sarcoplasm, loss of cross striation,
phagocytic infiltration. Biceps femoris, calf. HE staining
Fig. 5. Hypercontraction of fibers, loss of cross striation, activated satellite cells.
Biceps femoris, calf. HE staining
Fig. 6. Extensive muscle necrosis with giant muscle cells. Biceps femoris, calf. HE staining
Fig. 7. Regenerative focal point of muscle fibers with myoblasts and myotubes.
Biceps femoris, calf. HE staining
Fig. 8. Segmental positive reaction in muscle filaments. Biceps femoris, calf. HBFP staining

DISCUSSION

In the literature, considerable variations are noted in physiological serum concentrations of selenium in calves. According to PAVLATA et al. (2000), values below 70 μg l^{-1} are indicative of selenium deficiency. STOWE and HERDT (1992) determined marginal selenium levels at 40-70 μl, whereas GERLOFF (1992) observed that values below 40 μg l^{-1} were indicative of severe selenium deficiency. Considerable differences are also noted in published data relating to serum vitamin E levels. In the work of ADAMS (1982) and MCDOWELL et al. (1996), vitamin E concentrations in the blood serum of calves with NMD ranged from 0.6 to 1.6 μg ml^{-1}. According to MAAS et al. (2008), vitamin E deficiency in calves occurs at levels below 2 μg ml^{-1}. In studies on adult dairy cows, the reference intervals for serum concentrations of α-tocopherol were determined at 4 μg ml^{-1} by BAYFIELD and MYLREA (1969) and at 3-6 μl ml^{-1} by PULS (1994). In our study, selenium and vitamin E concentrations in healthy calves were within the lower limit of the above reference intervals. In calves with symptoms of NMD, selenium and vitamin E levels were significantly lower than in healthy calves and were indicative of hyposelenemia and hypovitaminosis.

According to ENJALBERT et al. (1999), the reference range for GSH-Px activity in cattle is 45-85 U g^{-1} Hb, and values below 40 U g^{-1} Hb point to selenium deficiency. In our study, GSH-Px activity in healthy calves exceeded the above range due to selenium and vitamin E supplementation on the second day after birth. In animals with NMD, GSH-Px activity was significantly lower than in healthy calves and significantly below the safe margin (PAVLATA et al. 2000). A decrease in GSH-Px activity activates mechanisms that lead to the defragmentation of cell membranes, calcium accumulation in cells and mitochondrial damage. Damaged mitochondria are unable to maintain homeostasis, which leads to cell death or segmental necrosis (ABUTARBUSH, RADOSTITS 2003). The usefulness of GSH-Px activity determination for detecting and monitoring NMD cases has been investigated by numerous authors, including EL-NEWEEHY et al. (2000), PAVLATA et al. (2001) and OR et al. (2003). According to the cited authors, a decrease in GSH-Px activity contributes to the severity of NMD symptoms. DAUN et al. (2001) investigated peroxidase activity in various organs of cattle after selenium supplementation and observed the highest levels of GSH-Px activity in muscles, which confirms that GSH-Px plays a very important role in the protection of muscle cells.

In the present study, morphological changes were frequently noted in samples of the biceps femoris muscle collected from calves with NMD. Segmental necrosis of muscle fibers and sarcoplasmic hyalinization were observed. The loss of cross striation and the presence of polygon-shaped fiber bundles with a small diameter were indicative of fiber necrosis. Hypercontracted fibers point to early stages of fiber necrosis, and the presence of hya-

linized and calcified fibers suggests a chronic process. Infiltration of connective tissue with adipocytes provides additional evidence of chronic degenerative myopathy. The presence of giant cells in muscle samples points to extensive foci of necrosis which are a characteristic symptom of NMD in calves (Radwińska, Żarczyńska 2013). Similar morphological changes, which are indicative of Zenker's degeneration, were described in the muscles of ruminants with NMD by Hafner et al. (1996), Beytut et al. (2002) and Tunca et al. (2009).

In our study, retrograde changes as well as signs of regeneration were noted in muscle tissues of calves with NMD. Regeneration is preceded by infiltration with phagocytic cells which remove necrotized segments of muscle fibers. Satellite cells, which are precursors to skeletal muscle cells, play a key role in the regeneration process. Satellite cells are capable of self-renewal and transformation into myoblasts. In initial stages of formation, oval-shaped myoblasts proliferate rapidly. In successive stages, myoblasts are elongated and tightly arranged along a straight line. Subsarcolemmal vacuoles appear at the place of contact in the adjoining myoblasts, and they lead to the loss of cell membrane continuity and the formation of an elongated multinucleated myotube (Jansen, Pavlath 2008). Similar observations were made by other authors (Beytut et al. 2002), who noted that both necrotic and regenerative processes are characteristic of NMD. Regenerative processes can also exert negative effects by stimulating connective tissue cells (fibroblasts, myofibroblasts) and promoting muscle recovery through the proliferation of fibrous connective tissue.

CONCLUSION

The data presented in this study indicates that during the course of NMD in calves decrease of selenium level and vitamin E in the blood is observed and is accompanied by a decrease in the blood glutathione peroxidase activity. The disease in calves also proceeds with significant morphological changes in the muscle tissue characteristic of Zenker's necrosis.

ACKNOWLEDGEMENTS

The authors would like to thank the Department and Clinic of Internal Diseases of the Faculty of Veterinary Medicine at the University of Warmia and Mazury in Olsztyn, the National Veterinary Research Institute in Puławy and the University of Pharmaceutical and Veterinary Sciences in Brno for their help with performing laboratory analyses.

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