Original article

Coagulology, biochemical profile and muscle pathology in calves diagnosed with nutritional muscular dystrophy

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Abstract

The aim of this study was to explain the correlations between selenium deficiency, hemostatic and biochemical disorders, and the progression of pathological changes in calves diagnosed with nutritional muscular dystrophy (NMD). The study was performed on 20 calves with supplementation of 8 ml selenium and vitamin E preparation and 20 calves with symptoms of NMD. Blood was sampled from calves aged 5, 12 and 19 days. On day 19, samples of the biceps femoris muscle were collected from 6 animals in each group for histopathological analysis. The following blood parameters were determined: PLT, PT, TT, APTT, fibrinogen and D-dimer concentrations, antithrombin III activity, glucose, selenium and vitamin E concentrations, activity of CK, LDH and GSH-Px. Muscle sections were stained with H&E and HBFP. Platelet counts were significantly lower in calves with symptoms of NMD. No significant differences in coagulation parameters were observed between the groups. Sick calves were diagnosed with hyperglycemia and elevation of CK and LDH activity. Selenium and vitamin E concentrations in the blood serum were significantly lower in the experimental group together with significant drop in GSH-Px activity. Changes characteristic of Zenker's necrosis were observed in a muscle of the sick animals. To our best knowledge this is the first study in which the attempt was made to explain the relationship between selenium deficiency and changes in the coagulation system in ruminants.

Key words: calves, coagulation profile, histopathology, Nutritional Muscular Dystrophy, selenium

Introduction

Negative consequences of selenium deficiency were first observed in 1958 when low levels of selenium and vitamin E were found to cause nutritional muscular dystrophy (NMD) (Muth et al. 1958).

Symptoms of NMD are most often observed in calves aged three days to six months. The first symptoms are stilted growth and abnormal gait. With time, the affected animals remain recumbent. Pathological changes in the diaphragm and the heart muscle are manifested by arrhythmia, cyanosis, dyspnea at rest,

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increased respiratory rate, intensified murmur over the pulmonary artery and cough. The animals with the cardiac form of NMD die in the first week of life (Katz et al. 2009). Pathologically changed white or gray muscle fragments with a hyaline appearance resemble cooked fish meat, and they strongly contrast with the healthy red-brown muscle tissue. In most cases, the changes in limb muscles are symmetrical, and muscles may be covered with white calcium deposit (Beytut et al. 2002). In microscopic analyses, highly advanced hyaline degeneration, referred to as waxy degeneration or Zenker's necrosis, is observed in muscle fibers (Bostedt and Schramel 1990).

In the mid-1970s, Fontaine et al. (1977) attempted to determine the effect of selenium and vitamin E deficiency on the porcine coagulation system, but they were not able to describe all mechanisms involved in the process. Interactions between the coagulation system and selenium concentrations in animals remain weakly explored. Probably selenium deficiency can affect the increase in homocysteine and consequently a response of the coagulation system (Undas et al. 2007). For this reason, the present study attempts to explain correlations between selenium deficiency, hemostatic disorders and the progression of pathological changes in calves diagnosed with nutritional muscular dystrophy.

Materials and Methods

Animals

The study was performed on one farm (about 350 dairy cows) located at Warmia (north part of Poland) in 40 Holstein-Friesian calves during closed system of breeding from October 2011 to March 2013. In the past every year symptoms of NMD were observed in calves on this farm. There was no treatment with selenium and vitamin E preparations on the farm. The calves were divided randomly into two equal groups (20 animals in each group). The control group comprised the healthy animals (12 females and 8 males), whereas calves showing symptoms of NMD constituted the experimental group (11 females, 9 males). On the second day of life, control group animals were administered a preparation containing vitamin E (50 mg of tocopherol acetate/ml) and selenium (0.5 mg of sodium selenite/ml; Eurovet Animal Health B.V., Bladel, Netherlands) in a single dose of 8 ml by intramuscular injection. All calves (experimental and control groups) were kept in a confinement housing system, and they were fed individually only mother's milk (in the first week of life three times per day 4-6 liters, in the second and

third week 2 times per day 6-8 liters). The mothers were fed a total mixed ration composed of 8 kg of grass haylage, 28 kg of maize, 1 kg of rapeseed cake, 1 kg of soybeans and 0.5 kg of protein concentrate (38% protein content; Agrocentrum, Grajewo, Poland).

Samples

Blood for laboratory analysis was sampled from the external jugular vein from calves aged 5, 12 and 19 days. All the animals each day over the study period (starting from the second day of life) underwent general clinical examination made by the same person.

Blood platelet counts (PLT) were determined in the Siemens ADVIA 2120 hematology analyzer (flow cytometry based on laser light scatter) (Simens Healthcare Diagnostic, Tarrytown, USA). Coagulation parameters were measured with the Bio-Ksel Coag Chrom 3003 analyzer and Bio-Ksel reagent (Bio-Ksel, Grudziądz, Poland). The following coagulation parameters were determined: prothrombin time (PT) – with the use of recombinant thromboplastin containing calcium chloride, prothrombin time (TT) – with the use of thrombin, activated partial thromboplastin time (APTT) – with the use of reagents containing synthetic phospholipids and calcium chloride, fibrinogen concentrations – with the use of recombinant thromboplastin containing calcium chloride, D-dimer concentrations – by the latex method with sodium azide, activity of antithrombin III – with the use of a thrombin reagent. The following biochemical parameters were determined: glucose concentrations – by the oxidase test, creatine kinase activity (CK) – by International Federation of Clinical Chemistry (IFCC) kinetic method, lactate dehydrogenase activity (LDH) – by the German Society of Clinical Chemistry (GSCC) kinetic method. The above parameters were determined with the use of the Cormay ACCENT 200 automatic biochemical analyzer and Cormay diagnostic kits (Cormay, Łomianki, Poland). Serum selenium concentration were measured in 10 calves (always the same animals, 6 female, 4 males) from each group by flame atomic absorption spectroscopy in the Unicam 939 Solar spectrometer coupled to a hydride generation system. Serum vitamin E concentrations were determined in 10 calves (the same which selenium was obtained) from each group by high performance liquid chromatography in the Hewlett Packard HP-1050 chromatograph with the use of Recipe ClinRep Complete Kits (Recipe, Munich, Germany). The activity of glutathione peroxidase (GSH-Px) was measured in whole blood in all the animals by the kinetic method with the use of cumene hydroxide and

Table 1. Values of coagulation parameters and number of platelets in calves.

Parameter	Examination					
	I		II		III	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
PLT (10^9/l)	524.35^Y±233.72	684.70±312.21	895.05^{YX}±247.62	1155.40^X±265.35	713.85^{YX}±278.33	1133.10^X±274.31
PT (s)	28.14±2.71	28.66±2.68	27.41±2.18	28.16±2.94	26.70±2.65	27.31±2.04
APTT (s)	34.07±3.89	33.45±4.13	33.42±4.21	33.36±3.56	33.67±3.87	32.93±4.28
TT (s)	23.97±2.03	24.85±1.55	24.26±2.19	25.01±1.91	23.96±2.10	24.71±2.23
Fibrynogen (g/l)	3.21±0.54	3.14±0.39	3.15±0.68	3.03±0.24	3.19±0.54	3.08±0.41
Antithrombin III (%)	120.90±7.19	117.45±8.27	113.65±8.20	120.35±8.44	111.95±7.61	112.00±10.19
D-Dimer (µg/l)	291.65±36.78	299.80±33.52	285.10±33.60	294.05±36.63	289.30±24.35	298.45±35.83

X – statistically significant difference at $p \leq 0.01$ between examinations

Y – statistically significant difference at $p \leq 0.01$ between groups

Table 2. Concentration of glucose and activity of creatine kinase and lactate dehydrogenase in calves.

Parameter	Examination					
	I		II		III	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
Glucose (mmol/l)	5.83^Y±0.70	3.99±0.89	5.44^Y±1.17	3.85±1.00	5.56^Y±0.69	3.92±0.94
CK (U/l)	254.62^Y±110.22	181.60±45.83	374.48^{YX}±102.62	183.24±55.99	464.61^{YX}±75.72	198.30±65.30
LDH (U/l)	4775.60±210.52	3464.15±396.78	4947.20^Y±377.52	3462.00±264.36	5568.90^{YX}±268.96	3519.05±379.83

X – statistically significant difference at $p \leq 0.01$ between examinations

Y – statistically significant difference at $p \leq 0.01$ between group

phosphate buffer in the Epoll 20 analyzer using the Ransel diagnostic kit (Randox Laboratories, Crumlin, UK).

On day 19, samples of the biceps femoris muscle were collected from six animals (randomly chosen) in each group for histopathological analysis. The biopsy site of the biceps femoris muscle was shaved, disinfected and anesthetized by infiltration with 5 ml polocainum chydrochloricum 5% (Biowet Drwalew, Drwalew, Poland). Muscle samples were obtained by scalpel incision of 0.8 x 0.8 cm with the depth of 0.7 cm. Muscle sections were immersed in saline solution (Natrium chloratum 0.9%, Baxter Poland, Warsaw, Poland) for 10 minutes, neutralized with 10% formalin (Chempur, Piekary Śląskie, Poland) and embedded in paraffin. Microtome sections were stained with hematoxylin and eosin (H&E) and hematoxylin-basic fuchsin-picric acid (HBFP) to expose necrotic muscle fibers.

The study was performed with the approval of the Local Ethics Committee for Experiments on Animals (Olsztyn, Poland, Number: 49/2010).

Statistical analysis

The significance of differences between sampling dates were determined for control and experimental groups at p≤0.01. The differences between the groups (factor or sampling date) were determined by ANOVA. The use of ANOVA was justified by the Brown-Forsythe test for the equality of group variances. Data were processed statistically using the STATISTICA 9.0 application (StatSoft inc., Tulsa, Oklahoma, USA).

Results

Clinical examination

In the first days of life (day 3 to 7) 7 calves from the experimental group showed decreased appetite, but symptoms of NMD were not observed. Symptoms of the discussed disease involving problems with

Table 3. Concentration of selenium and vitamin E in serum and activity of glutathione peroxidase in blood in calves..

Parameter	Examination					
	I		II		III	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
Selenium (µg/l)	43.76^Y±5.11	58.28±5.94	41.07^Y±4.15	55.60±4.43	37.20^Y±3.96	52.71±4.47
Vitamin E (µg/ml)	1.62^Y±0.56	4.43±0.93	1.41^Y±0.53	4.24±1.04	1.32^Y±0.36	4.08±0.73
GSH-Px (U/gHb)	40.55±17.08	44.30±15.67	31.55^Y±9.79	79.24^X±37.76	27.03^{YX}±7.10	131.15^X±49.47

X – statistically significant difference at $p \leq 0.01$ between examinations
Y – statistically significant difference at $p \leq 0.01$ between group

standing up and maintaining a standing position began to appear in all the animals between days 10 and 19. Muscle tremor was observed in 14 animals, in particular in pelvic limbs. The 10 affected calves had incorrect posture with widely spread limbs and stilted gait. Three calves became recumbent towards the end of the experiment. Clinical symptoms of NMD were not observed in control group calves which remained healthy throughout the study.

Coagulation parameters

Platelet counts increased significantly in both the groups, and on the last two sampling dates, they were significantly higher in the control group than in the experimental group. No changes in PT, TT and APTT values were observed in the control or experimental animals throughout the experiment. Plasma fibrinogen levels in calves with NMD symptoms and in healthy calves fluctuated insignificantly and were maintained within the lower reference range for cattle. No differences between D-dimer concentrations were reported between the control and experimental animals. Minor plasma fluctuations in the activity of antithrombin III were observed both animal groups throughout the experiment (Table 1).

Biochemical parameters

Glucose levels were significantly higher in the experimental than in the control animals throughout the study. Serum creatine kinase activity in experimental animals increased throughout the experiment, and the highest CK values were noted on the last sampling date. Serum CK activity remained stable in the control calves throughout the entire study. A significant increase in LDH activity in animals showing symptoms of NMD was observed during the experiment. LDH activity was higher in diseased calves than in the control animals on all sampling dates (Table 2).

Selenium and vitamin E concentrations and glutathione peroxidase activity

Serum concentrations of selenium in experimental animals were significantly lower than in the control calves, and they decreased with age and severity of NMD symptoms. Selenium concentrations in the serum of control animals remained significantly higher throughout the experiment. The highest selenium concentrations were found on the first sampling date, i.e. three days after the administration of the Se supplement. A progressive drop in selenium levels was observed on successive sampling dates. Serum vitamin E concentrations in the experimental animals were significantly lower in comparison with those found in the control calves and continued to decrease throughout the experiment. GSH-Px activity in the blood samples of diseased animals decreased considerably throughout the experiment and was significantly lower than that determined in the healthy calves. An increase in GSH-Px activity in the control animals was observed on the second and third sampling date, i.e. 10 and 17 days after the administration of the selenium supplement, respectively (Table 3).

Histopathological parameters

Numerous muscle fibers showing signs of sarcoplasmic hyalinization and loss of cross striations were observed in H&E-stained specimens of the biceps femoris muscle in calves with NMD. Retrograde changes as well as signs of regeneration were noted in the muscle tissues. Focally extensive polyphasic necrosis of muscle fibers with sarcoplasmic degeneration and loss of cross striations was noted in HBFP-stained specimens (Figs. 1, 2, 3).

Normal muscle fibers with clear cross striations were found in H&E-stained samples of the biceps femoris muscle from the control animals. The HBFP stain response was negative, and the presence of necrotic muscle fibers was not reported in the control calves.

Fig. 1. Muscle of calf with symptoms of NMD. Loss of cross striations. H&E staining, x1200; Fig. 2. Muscle of calf with symptoms of NMD. Hyaline degeneration (short arrow) and loss of cross striations (long arrow). H&E staining, x240; Fig. 3. Necrotic muscle fibers of the calf with symptoms of NMD. HBFP staining, x240

Discussion

Coagulation profile

In calves with symptoms of NMD, thrombocyte counts on the 2nd and 3rd sampling dates were significantly lower than in the healthy animals. The changes in platelet counts were similar to those reported by Fontaine et al. (1977) in pigs suffering from selenium and vitamin E deficiency where a significant drop in thrombocyte levels was also noted. A decrease in PLT values could be triggered by one of three mechanisms: decrease in or ineffective production of blood platelets, increased aggregation or reduced platelet life span. In calves with selenium deficiency, thrombocytopenia can also result from changes in the relationship between blood levels of homocysteine

and platelet aggregation (Undas et al. 2007). Homocysteine and homocysteine thiolactone modify platelet proteins to change the concentrations of carbonyl groups, 3-nitrotyrosine and thiol groups. The proteins which transduce signals induced by homocysteine in blood platelets are kinases (Src, p72syk, p38MAPK), phospholipase PLC2-γ and phospholipase A_2 which controls the synthesis of arachidonic acid that is metabolized to thromboxane A_2. Enhanced synthesis of thromboxane A_2 and disrupted production of anti-aggregatory substances, such as nitrogen oxide and PGI_2, intensify platelet aggregation and activation. The above mechanisms were observed when increased homocysteine levels in the cell medium intensified platelet adhesion to endothelial cells. The effect of selenium on the synthesis of thromboxane A_2 and PGI_2 was also described by Abdulah et al. (2007) who observed that an increase in peroxide concentrations led to changes in arachidonic acid metabolism and cyclooxygenase activity. Intensified platelet aggregation in calves with NMD can also result in vitamin E deficiency. Vitamin E inhibits the activity of protein kinase C which is responsible for enhanced thrombocyte aggregation. The mechanism of α-tocopherol's inhibitory effect on PKC has been proven in both *in vitro* and *in vivo* studies (Azzi 2007). The activity of phosphatase 2A is stimulated, which intensifies dephosphorization of protein kinase C and leads to its inactivation (Schneider 2005). The above mechanisms indicate that reduced platelet counts in calves with NMD could be related to excessive platelet aggregation.

PT, TT and APTT values found in experimental and control group calves in this study are indicative of normal coagulation. In a study of pigs with vitamin E and/or selenium deficiency, Fontaine et al. (1977) observed shorter prothrombin times in piglets with selenium deficiency, but the above authors were unable to explain the relevant mechanism of action. Recent studies (Undas et al. 2007) demonstrated that the activation of procoagulant mechanisms, manifested by shorter prothrombin times, takes place with an increase in homocysteine levels in the blood. Homocysteine decreases the availability of thrombomodulin on endothelial cells, which activates protein C and factor V. Lentz et al. (2002) observed that the activation of protein C by thrombin and the activation of factor Va by activated protein C was not inhibited in an *in vivo* study of monkeys with moderate hyperhomocysteinemia.

In a study of foals with NMD, Katz et al. (2009) did not observe changes in fibrinogen concentrations, and their findings are consistent with the results of this study. No changes in fibrinogen levels were reported by Fontaine et al. (1977) in piglets with

selenium and vitamin E deficiency. Studies in humans with Duchenne muscular dystrophy did not reveal changes in fibrinogen concentrations (Saito et al. 2001). Other authors (Sauls et al. 2006) observed that homocysteine thiolactone (HTL) can modify the properties of fibrinogen and disrupt its biological functions through N-homocysteinylation. The results of an *in vitro* study demonstrated that fibrinogen is easily modified by HTL. HTL-induced modification of fibrinogen was also observed *in vivo* where the presence of homocysteine linked by amine bonds was observed in fibrinogen. HTL changes the structure of produced fibers, and the resulting clot comprises thinner and more densely packed fibers. Dense fibrin scaffolds become less available for fibrinolytic factors because the gaps between packed fibrils are significantly smaller than in loose clots. Modified clots are less susceptible to lysis by the t-PA/plasmin complex. Plasminogen activation induced by t-PA takes place more rapidly in the presence of thick rather than thin fibrils, which facilitates plasmin generation (Undas et al. 2007).

D-dimers are the most sensitive marker of stabilized fibrin and a specific product of fibrin degradation. An increase in plasma D-dimer levels is indicative of the activation of the fibrinolysis system and plasmin generation. Elevated FDP and D-dimer concentrations in blood serum and plasma were observed by Saito et al. (2001) who studied coagulation and fibrinolytic systems in human muscular dystrophy. An increase in FDP and D-dimer levels points to enhanced fibrinolysis after coagulation cascade activation, and it is linked with progressing muscle degeneration. Plasma D-dimer concentrations in calves with symptoms of NMD and in the healthy animals were similar to those found in healthy cows by Sobiech et al. (2008).

In this study, AT III activity was somewhat lower than that described by Sobiech et al. (2005) in Holstein-Friesian cattle. A similar trendency was reported by Gentry et al. (1994). Nishinaga et al. (1993) suggested that homocysteine lowers the activity of AT III and protease bonds, which could lead to severe thromboembolism. Bienvenu et al. (1991) did not observe any correlations between homocysteine concentrations and levels of blood coagulation inhibitors.

Biochemical profile – glucose and the activity of aspartate aminotransferase, creatine kinase and lactate dehydrogenase

Recent studies (Sheng et al. 2004) in humans with type 2 diabetes seem to confirm that dietary supplementation with sodium selenite induces glucose trans-

formations. Insulin and insulin mimetics are required to stimulate glucose incorporation into tissues, to convert glucose into energy or to store glucose for future use. Stapleton (2000) demonstrated that selenium participates in a series of insulin-line activities both *in vivo* and *in vitro*, including stimulation of glucose uptake and regulation of metabolic processes such as glycolysis, gluconeogenesis, fatty acid synthesis and the pentose phosphate pathway. The mechanism responsible for insulin-like activity of selenium has not been fully explained. It is believed that selenium enhances the activity of key proteins involved in the insulin signaling pathways. In this study, a significant drop in glucose concentrations was not observed throughout the experiment, i.e. until the 19th day of life. Similar results were reported by Knowles et al. (2000) who observed a significant drop in glucose levels only in 56-day-old calves.

Creatine kinase is the most muscle-specific enzyme and the most sensitive diagnostic indicator of muscle damage. In this study, serum CK activity in the experimental calves increased throughout the experiment, and the highest increase was found on the last sampling date. In the animals with symptoms of NMD, CK activity exceeded 460 U/l, and similar results were reported by other authors (Pavlata et al. 2001), which points to similar rates of disease progression.

Lactate dehydrogenase is not a muscle-specific enzyme, and its activity can also increase as the result of liver, kidney, skeletal and digestive system disorders. A significant increase in LDH activity in various species of animals diagnosed with NMD was reported by other authors (Pavlata et al. 2001, Tunca et al. 2009), indicating that this enzyme is an effective indicator of the discussed disease.

Selenium and vitamin E concentrations and glutathione peroxidase activity

Serum concentrations of selenium were significantly higher throughout the experiment in the control animals. Selenium supplementation comprising I/M injections of sodium selenite increased Se levels in the control animals. The highest concentrations of the analyzed element were found on the first sampling date, i.e. three days after supplementation, whereas an insignificant drop in selenium concentrations was observed on successive sampling dates. Similar results were reported by Abutarbush and Radostits (2003) in whose study, selenium supplements were administered parenterally to calves with symptoms of NMD.

In this study, lower serum concentrations of vitamin E were correlated with intensified symptoms

of NMD. The results of other studies (Abutarbush and Radostits 2003, Katz et al. 2009) demonstrate that α-tocopherol deficiency is one of the main causes of the discussed disease.

In the control calves, serum levels of vitamin E increased after administration of a supplement containing selenium and α-tocopherol. Eichler et al. (1997) demonstrated that serum vitamin E concentrations in the calves increased significantly already 14-32 hours after supplementation. In this study, the highest serum levels of vitamin E were observed on the first sampling date (three days after supplementation). Vitamin E concentrations decreased gradually towards the end of the experiment, but remained higher than those found in the experimental animals.

In our experiment, higher levels of GSH-Px activity were observed in the control calves on the 2nd and 3rd sampling dates, i.e. 10 and 19 days after administration of the selenium supplement, respectively. In other studies (Andres et al. 1996, Pavlata et al. 2011), an increase in GSH-Px activity was observed 10-12 days after selenium supplementation. According to Philipoo et al. (1987), GSH-Px activity is not enhanced directly the administration of selenium because selenium is first used to supplement tissue reserves, and it becomes involved in peroxidase synthesis only later. According to Arthur (2000), the above can be explained by the deposition of selenium in erythrocytes during erythropoiesis and the time required to synthesize the enzyme. According to Gerloff (1992) GSH-Px activity is a robust indicator of the effectiveness of long-term selenium supplementation, whereas short-term fluctuations in selenium levels can be evaluated based on the serum concentrations of this microelement.

Histopathological profile

Histopathological changes observed in specimens of the biceps femoris muscle from calves with NMD are characteristic of Zenker's necrosis. Similar morphological changes in muscles of ruminants with symptoms of the discussed disease were described by Beytut et al. (2002) and Żarczyńska et al. (2013).

In this study, no significant coagulation disorders were observed in calves with symptoms of nutritional muscular dystrophy. A correlation was observed between decreased selenium concentrations and intensified platelet aggregation. The experimental calves were diagnosed with hyperglycemia. The analyzed disease is accompanied by a significant increase in the activity of creatine kinase and lactate dehydrogenase. Determination of the above enzyme levels supports early diagnosis of NMD. Nutritional muscular dystro-phy is also accompanied by significant selenium and vitamin E deficiency and decreased activity of glutathione peroxidase, an indirect indicator of selenium status. Changes characteristic of Zenker's necrosis are observed in histopathology specimens of muscles from calves with NMD.

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