Examination of sheep in which injection of Yeldif® induced sudden death*)

AHMET UYAR, ZABIT YENER, YILDIRAY BASBUGAN*,
OMER FARUK KELEŞ, TURAN YAMAN

Department of Pathology, Yuzuncu Yil University, Faculty of Veterinary Medicine, 65080, Van, Turkey

*Department of Internal Medicine, Yuzuncu Yil University, Faculty of Veterinary Medicine, 65080, Van, Turkey

Received 06.04.2017

Accepted 05.06.2017

Uyar A., Yener Z., Basbugan Y., Keleş O. F., Yaman T.

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Summary

Two breeders from the Başkale district of the Van province and the Hizan district of the Bitlis province reported deaths of their lambs one day after injection of a commercial preparation containing a combination of Se and Vit E (Yeldif®). Purulent-hemorrhagic-necrotic myositis was detected at the injection site during necropsy of the lambs (2 animals) brought to the Pathology Department of our Faculty by these breeders. This study was conducted to determine whether these post-injection deaths occurred as a result of the drug used or due to a faulty application of the medicine by the breeders. The trial period for this study was 1 week. Eighteen 2-month-old lambs were used in this study. The lambs were divided into three groups (control and 2 study groups) of 6 lambs each. An injection of 1 ml (recommended dose) of the product purchased in the market from the same batch as the one that had caused the deaths was administered I.M. to lambs in the control group. The medicine reported to have caused the deaths by the breeders was administered to the first study group (A) at 1 ml I.M. and to the second study group (B) at 3 ml I.M. (three times the recommended dose). The lambs were bled for biochemical analysis before the administration, on the first day after the administration, and at the end of the trial. Necropsies were performed on dead animals during the trial and on all animals at the end of the trial. At the end of the trial, no clinical-pathological findings were noted in lambs of the control group and group A. However, in all lambs of group B, emphysematous gangrene was detected at the injection site. *Cl. chauvoei* was isolated and identified in bacteriological and cultural examinations. It was concluded that bacterial contamination occurred as a result of incorrect drug administration by the breeders and injection of a high dose of the contaminated drug.

Keywords: hemorrhagic purulent necrotic myositis, lamb, Yeldif®

Black disease, also known as blackleg, gangrene myositis, emphysematous gangrene or clostridial myositis, causes serious economic losses and can be seen in almost every country of the world (1, 19) in cattle, sheep and other ruminants, as well as in humans (6, 11). The etiology of this disease is *Clostridium chauvoei* (*Cl. chauvoei*) (10). The incubation period of this agent is fairly low (1-3 days), and the mortality rate is almost always 100% (3, 17).

As with other clostridial agents, infection occurs as a result of the introduction of bacterial spores into the body through cutaneous wounds caused by ear-tagging, dog bites, shearing injuries, contaminated needles and injection equipment, tail amputations under non-hygienic conditions (17, 19), or posterior reproductive canal traumas associated with dystocia (3,

10, 19). The bacteria release necrotic and hemolytic toxins at the point of entry into the body. These toxins spread in a hematogenous manner, causing destruction in muscle tissues, which is shown by black color and gas formation in the muscles. As a result, death occurs due to severe edema and toxemia (4).

Typical clinical signs in sick animals are high fever, loss of appetite, depression (12, 17), lameness, muscle tremors, and crepitation in palpation of muscle groups (4, 19).

In necropsies of animals killed by black disease, infection sites are dark red to black. In these areas, muscle groups are characteristically emphysematous with an edematous circumference and are hemorrhagic (12). Skin over infected muscle groups is either dark red or purple. These areas have an edematous structure with pastry-like texture with crepitation when compressed with a thumb. When edematous areas are

*) We would like to thank Yuzuncu Yil University, Department of Scientific Research Projects [Project Code: 2015-HIZ-VF194 (ID Number: 1490)].

dissected, there is liquid with gas bubbles giving off a strong acidic odor like that of rancid butter (10, 12). Microscopically, characteristic lesions are present in skeletal muscles, which contain multi-focal hemorrhagic and emphysematous foci (12). Necrotic muscle groups are encircled by neutrophil leukocytes (4). Vaccination is the most effective method of protecting animals from this disease (12). Black disease is among notifiable diseases; slaughtering of such animals is forbidden, and cadavers should be disposed of (10, 17).

In this study, two breeders from the Başkale district of the Van province and the Hizan district of the Bitlis province in Turkey reported deaths of two lambs one day after Yeldif® injection. In the necropsy of these animals, purulent hemorrhagic-necrotic myositis was noted at the injection site. On the basis of necropsy findings, indicative of bacterial contamination, it was assumed that the mortality could have resulted from faulty injection. Therefore the aim of the study was to inform breeders of the results of an experimental trial carried out with the remainder of the medicine.

Material and methods

Experimental animals. Experiments on animals in this study were approved by Yuzuncu Yil University, Animal Experiments Local Ethics Committee (YUHADYEK), and the Ministry of Food, Agriculture and Livestock. A total of 18 lambs (1-3 month old) were used in the study.

Experimental design. Lambs were divided into three groups (control, group A, and group B) of 6 animals each. A combination of selenium (Se) and vitamin E (Vit E) (Yeldif®) was administered to the lambs as follows: 1 ml (dose recommended by the manufacturer) of the medicine from the same batch as the one that had caused mortality was administered I.M. to the control group, whereas 1 ml

and 3 ml (3 times the recommended dose) of the medicine that had caused the deaths were given to group A and group B, respectively. The trial period was 1 week.

Biochemical analysis. Blood samples were taken for biochemical analysis at the end of the trial to determine alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), calcium (Ca), magnesium (Mg), total protein, albumin, and creatine kinase (CK) levels.

Histopathological and immunohistochemical examination. Necropsies were performed on animals which died or almost died during the trial and on the rest of the animals from all groups. Samples were taken from tissues and organs, such as muscle tissue at the injection site, liver, lung, kidney, stomach, and intestines. These samples were fixed in 10% buffered formalin, embedded into paraffin blocks, and sectioned with a microtome (Leica RM 2135) at 4 µm. Some of the cross-sections were stained with hematoxylin-eosin and examined routinely. The rest of the cross-sections were transferred to poly-lysine-coated slides and examined immunohistochemically under a light microscope (Nikon 80i-DS-Ri2) using the streptavidin-biotin-peroxidase technique by the method reported by Pires et al. (15).

Microbiological examination. Slides prepared for bacteriological evaluation were stained by Gram's stain technique to detect Gram-positive agents, indicative of a possible anaerobic contamination (8). The samples taken for culture were incubated in Reinforced Clostridial Medium (RCM) broth at 37°C in a GasPak anaerobic jar. The suspension was later inoculated to RCM agar and re-incubated in the same atmosphere. Passages were performed from suspected colonies to blood agar (18).

Statistical analysis. Biochemical analysis results, the mean and standard deviation ($X \pm SD$) were obtained by standard methods with a ready-to-use program (Minitab for Windows). Differences between group mean values were calculated by One-way ANOVA test.

Fig. 1. Control group: Normal appearance of an injected left leg

Fig. 2. Group B: Dark red and edematous skin on a left leg

Results and discussion

Evaluation performed on the first day after the injection revealed no symptom in the control group (Fig. 1), whereas only one animal from group A showed lameness. In group B, 2 animal died within 48 hours post-injection. Clinically affected lambs showed signs of severe depression and intoxication, anorexia, fatigue, teeth grinding, lameness, indigestion, fever of 40.5-41°C, tachycardia, and an increased respiratory rate. Skin between the upper part of the injected leg down to the ankle joint and muscles close to the injection site turned dark red/purple (Fig. 2). The affected areas were characterized by hot, painful, edematous and emphysematous lumps. A strong putrid odor was detected during necropsy. Muscles with lesions lost their normal color and turned black. Accumulation of bloody and

foamy serous fluid was notable in the inflamed area (Fig. 3). Muscle lesions occurred mostly in the upper regions of legs. In addition, a rancid odor (rancid butter odor) was noted during dissections performed in lesion areas (Fig. 4). There were no pathologies in visceral organs.

All animals in group B showed intense edema and hemorrhagic exudation within muscle fibers. Transversal lines in muscle fibers disappeared (loss of cross striations). Bands of muscle fibers had separated in patches due to exudate and gas accumulation. Pyknosis and karyorrhexis in addition to karyolysis were marked in the nucleus of muscle fiber cells. In animals with very severe lesions, degenerated muscle threads were disrupted, vasculitis and myolysis occurred, and inflammatory cell infiltration was significant (Fig. 5 and 6).

Fig. 3. Group B: Dark red/blackish muscles of a left leg

Fig. 4. Group B: Hemorrhagic necrotic myositis in a cross-section of muscles of a left leg

Fig. 5. Group B: Severe fibrino-prulent exudation, gas bubbles, degeneration and necrosis of muscle fibers. H.E.

Fig. 6. Group B: Vasculitis, perivascular cell infiltration, degeneration and necrosis of muscle fibers. H.E.

In addition, immunohistochemical examination showed positive results for *Cl. chauvoei* within muscle fibers (tissues), when preparations were stained by the streptavidin-biotin-peroxidase technique (Fig. 9).

Clinical, gross and microscopic findings are presented in Tab. 1. Findings related to biochemical parameters are presented in Tab. 2.

In bacteriological and cultural examinations, carried out to determine the possibility of anaerobic contamination, *Cl. chauvoei* was isolated and identified (Fig. 7 and 8).

According to the manufacturer, administration of Yeldif® at 8-10 times the recommended dose may cause

Fig. 7. Group B: Gram-positive stained *Cl. chauvoei* among muscle fibrils

Fig. 8. Group B: Appearance of the Gram-positive stained *Cl. chauvoei* among muscle fibrils

Tab. 1. Clinical, gross and microscopic findings

Findings		Control group	Group A	Group B
Clinical findings (fever, severe depression, anorexia, fatigue, teeth grinding, lameness, indigestion)		-/6	1/6 ^a	6/6 ^{ab}
	Slight	-	1	-
	Moderate	-	-	1
	Severe	-	-	5
Gross findings (hot, painful, edematous and emphysematous lumps, crepitation, cyanosis, bloody and foamy serosity, stench)		-/6	-/6	6/6 ^{ab}
	Slight	-	-	-
	Moderate	-	-	1
	Severe	-	-	5
Microscopic findings (edema and hemorrhagic exudation, loss of cross striations, gas accumulation, inflammatory cell infiltrations, degeneration, necrosis, myolysis)		-/6	-/6	6/6 ^{ab}
	Slight	-	-	-
	Moderate	-	-	1
	Severe	-	-	5

Explanations: values in a row with different superscript letters differ significantly at $p \leq 0.01$, ^a significantly different from the control group, ^b significantly different from group A

intoxication. In addition, although rarely, parenteral use of Thiamine may trigger anaphylactic shock (16). This experimental study, involving histopathological, immunochemical, microbiological and biochemical evaluation, was aimed at determining whether the mortality had been caused by the drug itself or by its faulty administration.

Tab. 2. Biochemical parameters

Parameters	Control group	Group A	Group B
ALT (U/L)	19.00 ± 1.73	22.00 ± 5.51	39.00 ± 4.36*
AST (U/L)	92.00 ± 10.66	92.00 ± 20.88	221.00 ± 37.29*
ALP (U/L)	185.00 ± 93.04	220.00 ± 98.00	207.00 ± 60.50
CA (mg/dL)	10.40 ± 0.25	10.90 ± 1.49	9.10 ± 1.55
Mg (mg/dL)	1.82 ± 0.12	1.95 ± 0.44	1.90 ± 0.03
TP (g/dL)	6.90 ± 1.46	7.30 ± 0.86	7.50 ± 0.7
ALB (g/dL)	2.47 ± 0.11	2.51 ± 0.32	2.44 ± 0.22
CK (U/L)	120.00 ± 39.60	180.00 ± 40.86	516 ± 132.29***

Explanations: differences between values in the same row are statistically significant (* $p < 0.05$; *** $p < 0.001$), * significantly different from the control group, *** significantly different from group A

Fig. 9. Group B: *Cl. chauvoei* stained positive between muscle fibrils by the streptavidin-biotin peroxidase technique

Researchers report the following criteria of black disease: the 1-3 days incubation period (10), clinically detected high fever, loss of appetite, depression (12, 17), lameness, crepitation, difficulty to stand up in lying lambs (3, 19), mortality due to septicemia within 1-2 days (12), tremors, gas accumulation in muscle groups of the extremities or the neck region, red/dark red color changes on the skin, edema and a strong acidic odor like that of rancid butter following dissection of edematous sites (9, 10, 12). In this study, two animals in group B died within 48 hours post-injection. In other animals of group B similar clinical symptoms were seen determined by researchers (4, 10, 12, 19).

Necropsies of animals killed by black disease reveal delayed putrefaction of cadavers, severely dry, swollen, dark red to black colored hemorrhagic and emphysematous muscle groups (10, 12). In this study, all animals in group B showed findings similar to those specified by researchers. This confirms that the group B animals contracted black disease.

Microscopically, disruption of fascias in infected muscle groups, emphysema and edema formation within myofibrils (10), necrotic muscle groups surrounded by neutrophil leukocytes, and hyaline degeneration in myofibrils are reported (4, 12). In addition, muscle fibers are disrupted, and myolysis occurs, associated with pycnosis, karyorrhexis, and karyolysis in the cell nucleus in sarcolemma (12). In group B, all lambs showed histopathological findings specified by researchers (4, 10, 12).

Pires et al. (15), in studies of pigs, and Casagrande et al. (5), in studies of cattle, using the streptavidin-biotin-peroxidase technique, reported positive staining in immunohistochemical analysis. In this study, performed by similar methods, positive immunohistochemical results were obtained that supported gross, microscopical, and microbiological findings, similar to those of the researchers.

In this study, Ca, Mg, TP, ALB and ALP levels were similar to those in healthy sheep, as specified by researchers (2, 3, 13, 17, 20). The results of statistical analysis show that the findings are statistically insignificant in comparison to the control group ($p > 0.05$). This situation, is considered that damage has not yet occurred in the liver and kidneys of the animals enough to change in laboratory parameters.

AST, ALT, CK enzyme levels indicative of skeletal muscle damage are elevated (1, 4, 7, 17). The ALT level in healthy sheep is around 22-84 (U/L) (3, 20). ALT levels detected in all groups in this study were similar to those in healthy animals as specified by researchers (3, 20). The difference between ALT levels in group A and the control group was statistically insignificant ($p > 0.05$), but ALT levels in group B, compared with those in the control group and group A, were significantly elevated ($p < 0.05$). This finding may be indicative of muscle degeneration in animals

(Tab. 2). It supports pathological findings and points to the possibility of muscle damage in group B. The researchers (3, 20) suggest a possible increase in serum ALT levels associated with muscle damage.

The level of AST, an indicator of the degree of skeletal muscle damage, is reported as 30-280 (U/L) in healthy sheep (1, 2, 7, 13, 17, 20). Differences in AST levels between the control group and group A in this study were statistically insignificant. These levels corresponded to those found by other researchers in healthy animals (2, 13, 17, 20), which indicates little to none muscle damage in group A. In group B, on the other hand, the AST level was statistically significantly ($p < 0.05$) higher than it was in the other groups (Tab. 2). This condition, which according to researchers (13, 20), points to possible muscle damage, is indicative of muscle damage in group B animals. Again, this result supports pathological findings.

Creatine kinase levels in healthy sheep are reported as 8.0-13.0 (U/L) (14, 17, 20), but according to Basbugan et al. (2) the CK level in 0-6 month old lambs is 96.4 ± 11.3 (U/L) and decreases with age. Yüksek and Basbugan (21) report CK levels of 213.52 (IU/L) in control group animals, and this value may increase with muscle damage.

Control group findings in this study were similar to those published by other researchers (2, 21). This can be regarded as an indicator of the good health of the lambs. Even though CK levels in group A were elevated compared with those in the control group, the difference was not statistically significant. In group B, however, CK levels were significantly increased compared with those in both the control group and group A ($p < 0.001$). This is consistent with other authors' (2, 20, 21) findings of elevated CK levels in muscle damage.

The clinical, gross, microscopic, immunohistochemical, microbiological, and biochemical findings of the experimental trial indicate that black disease, characterized by hemorrhagic necrotic myositis, was caused by the breeders. All these results suggest that the breeders administered the medicine incorrectly, contaminated it and used the same contaminated medicine to inject other animals, thereby spreading the contamination and finally causing mortality as a result of black disease.

In conclusion, the results of our study showed that an education is of great importance on legislations of animal health for the official and private veterinarians, veterinarians of slaughterhouses, officials of animal warehouses, breeders, butchers, cattle-dealers and transporters in order to provide a more efficient and productive of animal health services. These educational sessions should be held by the Ministry of Food, Agriculture and Livestock, university faculties of veterinary medicine, and the Chamber of Veterinarians.

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Corresponding author: Assist. Prof. Dr. Ahmet UYAR, Department of Pathology, Veterinary Faculty, Yuzuncu Yil University, 65080 Van, Turkey; e-mail: uyarahmet@hotmail.com, ahmetuyar@yyu.edu.tr