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## Evaluation of Cardiac Troponin I and Inducible Nitric Oxide Synthase Expressions in Lambs with White Muscle Disease

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## Evaluation of Cardiac Troponin I and Inducible Nitric Oxide Synthase Expressions in Lambs with White Muscle Disease

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**Abstract:** White muscle disease (WMD) is a world-wide nutritional disease of all animal species characterised by degeneration of skeletal and cardiac muscles. In the present study we determined the cardiac troponin I (cTnI) and inducible nitric oxide synthase (iNOS) expression in hearts of the lambs with WMD (n = 15). Eight clinically healthy lambs served as control. Creatine kinase (CK), creatine kinase MB (CK-MB), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were analysed for both groups. cTnI levels were measured by a commercially available ELISA kit. Mean cTnI (10.49 ± 0.25 ng/ml), CK (261.16 ± 38.61 U/l), CK-MB (214.16 ± 12.71 U/l), AST (125.83 ± 1.05), and LDH (228.77 ± 14.86 U/l) concentrations were higher in WMD cases. Immunohistochemistry revealed definitive loss of cTnI expression in all cases. None of the 8 controls showed loss of the cTnI expression. However, iNOS immunoreactivity was augmented in degenerated cardiac myocytes and macrophages infiltrating to the interstitium compared to controls. The results of the study suggested that cTnI antibody might successfully be used as a sensitive test in the diagnosis of myocardial injury. iNOS expression augments in cardiac myocytes in lambs with WMD, most likely through influencing the signalling pathways to iNOS induction.

**Key Words:** White muscle disease, lamb, immunohistochemistry, cTnI, iNOS

### Kuzulardaki Beyaz Kas Hastalığında Kardiyak Troponin I ve İndüklenebilir Nitrik Oksit Sentetaz Ekspresyonunun Değerlendirilmesi

**Özet:** Hayvanlardaki beyaz kas hastalığı (WMD) iskelet ve kalp kasında dejenerasyonla karakterize oldukça yaygın nutrisyonel bir hastalıktır. Çalışmada WMD tanısı konulan (n = 15) ve klinik olarak sağlıklı (n = 8) kuzuların kalp kasındaki kardiyak troponin I (cTnI) ve indüklenebilir nitrik oksit sentetaz (iNOS) ekspresyonları araştırıldı. Kreatin kinaz (CK), kreatin kinaz MB (CK-MB), aspartat aminotransferaz (AST) ve laktat dehidrogenaz (LDH) aktiviteleri de her iki grupta değerlendirildi. Ayrıca serum cTnI seviyeleri de ticari ELISA kiti ile belirlendi. WMD olgularında ortalama serum cTnI (10,49 ± 0,25 ng/ml), CK (261,16 ± 38,61 U/l), CK-MB (214,16 ± 12,71 U/l), AST (125,83 ± 1,05) ve LDH (228,77 ± 14,86 U/l) konsantrasyonu yüksekti. İmmunohistokimyasal veriler tüm WMD olgularında, dokudaki cTnI ekspresyonunda belirgin azalmanın şekillendiğine işaret ederken kontrol olarak kullanılan 8 kuzunun hiçbirinde cTnI ekspresyonunda azalmaya rastlanmadı. Bununla beraber iNOS immunoreaktivitesi dejenerasyonlu kardiyak miyositlerde ve interstitiyumdaki makrofajlarda kontrol olgularına göre oldukça yoğundu. Sonuçlar cTnI'nin miyokardiyal hasarın ortaya konulmasında oldukça duyarlı olarak kullanılabileceğini gösterdi. WMD'nin kardiyak miyositlerde iNOS ekspresyonunu ise arttırdığını ortaya koydu.

**Anahtar Sözcükler:** Beyaz kas hastalığı, kuzu, immunohistokimya, cTnI, iNOS

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## Introduction

White muscle disease (WMD) or nutritional muscular dystrophy is most common in neonatal and fast growing young animals and is caused by deficiency of selenium (Se) and vitamin E (Vit E) or both (1,2). Diagnosis of WMD relies on necropsy and clinical pathology especially enzymes [creatinase kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH)] indicative of muscular dystrophy (3,4). In recent years, use of troponin, especially cardiac troponin I (cTnI), has gained popularity in early diagnosis of myocardial damage (5,6). cTnI has been shown to be highly sensitive and specific marker of myocardial damage (7). Because of its high sensitivity and specificity to cardiac tissue, cTnI has thus emerged as the preferred diagnostic tool and gold standard for assessing myocardial necrosis (8). cTnI elevates within 4-6 h after a myocardial injury in circulation, and is superior to conventional measurement of creatine kinase MB (CK-MB), CK, AST, and LDH for the detection of myocardial injury as a biochemical marker (5,6). Human cTnI assay has been validated in the primate, pig, rat, calves, and sheep (9-11).

Nitric oxide (NO) is synthesized from L-arginine by the action of enzyme inducible nitric oxide synthase (iNOS). Nitric oxide synthase (NOS) is expressed either constitutively (neuronal, type I nNOS; endothelial, type III eNOS) or after stimulation by cytokines (inducible, type II iNOS) (12). Inducible pathway of NO synthesis has a biological role in the mammalian heart (12,13). Studies in rats have demonstrated that the myocardium expressed iNOS after treatment with cytokines. This suggests that induction of iNOS expression in the heart may cause a specific cardiac dysfunction observed in endotoxin shock and certain immunological and inflammatory conditions, including post-cardiac transplantation, myocarditis, and ischemia-reperfusion injury, in which various cytokines have been implicated (14,15).

In the present study, serum concentration of cTnI and immunohistology of the cTnI and iNOS expression were investigated to assess the significance and extend of myocardial injury in WMD in lambs.

## Materials and Methods

### Animals

The study involved 15 WMD cases admitted to the clinics of the Faculty of Veterinary Medicine between

2005 and 2007 and 8 healthy lambs. Clinically suspected WMD cases were blood sampled and necropsied. Blood samples were also collected from healthy lambs. Sera were collected after centrifugation at 3000 rpm for 10 min and stored at -20 °C until analyses.

### Serum biochemistry and cTnI analyses

Serum CK, CK-MB, AST and LDH concentrations were determined calorimetrically using commercial kits (IBL-Germany). cTnI concentration was determined using a commercial ELISA kit (CARD-I-KIT ELISA Troponin I, Labmaster, Finland) on an ELISA reader (Tecan-spectra, Austria) and calculated (ng/ml) as instructed by the manufacturer.

### Necropsy and histopathology

A complete necropsy was performed on all animals. Following the macroscopical evaluation, tissue samples were taken from heart, diaphragm, tongue, and skeletal muscle, and fixed in 10% buffered formalin. Selected blocks were processed and embedded in paraffin wax. Sections (4-6 µm) were then cut from each block for histological examination and from only heart for immunohistochemical analyses.

### Immunohistochemistry

Sections from all heart tissue samples were cut (4-6 µm) and processed for immunohistochemical examination by a streptavidin-biotin-peroxidase method. Tissue sections were placed on 3-amino-propyltriethoxysilane (Sigma-Aldrich, St. Louis, Montana, USA) coated slides, dewaxed, and hydrated. Antigen retrieval was facilitated by heating in citrate buffer (pH 6.0) for 10 min in a microwave oven with a power of 800 watts. The slides were then dipped in freshly prepared absolute methanol containing hydrogen peroxide 3% v/v for 15 min to block the endogenous peroxidase activity. Next, polyclonal antibodies were utilised: goat anti-cTnI (1:100; C-19: sc-8118, Santa Cruz Biotechnology, Santa Cruz, California, USA) and rabbit anti-iNOS (1:100; N-20: sc-651, Santa Cruz Biotechnology). After washing with PBS, the slides were incubated with biotinylated goat anti-rabbit immunoglobulin G (Dako Corporation, Carpinteria, USA) for anti-iNOS and rabbit anti-goat immunoglobulin G for cTnI diluted at 1:300 in PBS for 30 min at room temperature. Sections then were incubated with streptavidin peroxidase complex (ABC; Dako Corporation, Carpinteria, USA) diluted at 1:300 in tris-buffered solution for 30 min at room temperature. After washing with PBS, the slides were treated for 5 min at room

temperature with 3,3-diaminobenzidinetetrahydrochloride (DAB, Dako Corporation) in PBS (0.5 mg DAB/ml) containing hydrogen peroxide 30% v/v. Finally, sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted. Negative control tissue sections were incubated with normal goat or rabbit serum depending on the primary antibody utilised.

#### Scoring of cTnI and iNOS immunostaining results

The percentages of the total area of the cTnI and iNOS positive cells were assessed semi-quantitatively under a light microscope with  $\times 10$  ocular grids and  $\times 40$  objective. Positively stained cells were counted in 10 random microscopic areas and their mean was accepted as staining score for each animal. The mean staining count determined for each animal was also explored in statistical analyses.

#### Statistical analysis

For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and Duncan test using SPSS for Windows version 10.0. Data were presented as mean  $\pm$  standard errors, and P values less than 0.05 were considered significant.

## Results

#### Clinical findings

The clinical signs varied from case to case. Some were in lateral or sternal recumbency and unable to sit up with sudden onset of dullness and respiratory distress. In some cases the signs were stiffness, weakness, arched back, muscle tremor when stand up, swollen muscle in gluteal, and dorsolumbar and shoulder area on palpation. No temperature rise was noted but most cases had elevated heart and respiratory rates.

#### Biochemical and cTnI findings

Concentrations of cTnI, CK, CK-MB, AST, and LDH are given in the Table. Serum cTnI concentration in healthy lambs ( $0.32 \pm 0.06$  ng/ml) were significantly lower compared to WMD cases ( $10.49 \pm 0.25$  ng/ml) ( $P < 0.001$ ). Concentrations of CK, CK-MB, AST, and LDH in WMD cases were  $261.16 \pm 38.61$  U/l,  $214.16 \pm 12.71$  U/l,  $125.83 \pm 1.05$  U/l, and  $228.77 \pm 14.86$  U/l while the values of controls were  $17.81 \pm 2.27$  U/l,  $23.11 \pm 7.05$  U/l,  $15.71 \pm 0.94$  U/l, and  $125.47 \pm 9.82$  U/l, respectively. The differences between the groups were also statistically significant ( $P < 0.001$ ).

Table. Serum cTnI and biochemical changes in WMD cases and healthy lambs. Values are presented as mean  $\pm$  SE.

Parameters	WMD (n = 15)	Control (n = 8)	Significance
cTnI (ng/ml)	$10.49 \pm 0.25^a$	$0.32 \pm 0.06^b$	$P < 0.001$
CK-MB (U/l)	$214.16 \pm 12.71^a$	$23.11 \pm 7.05^b$	$P < 0.001$
CK (U/l)	$261.16 \pm 38.61^a$	$17.81 \pm 2.27^b$	$P < 0.001$
AST (U/l)	$125.83 \pm 1.05^a$	$15.71 \pm 0.94^b$	$P < 0.001$
LDH (U/l)	$228.77 \pm 14.86^a$	$125.47 \pm 9.82^b$	$P < 0.001$

Different letters in rows refer to statistical difference.

#### Macroscopic findings

Ventricle muscles were the most severely affected part of myocardium. Widespread lesions characterized by the chalky-white necrosis and mineralization in the heart muscles were seen on the interventricular septum and particularly on the endocardial surfaces. Scattered longitudinal striations or a pronounced chalky whiteness like fish flesh were seen frequently on the skeletal muscle, neck, back, gluteal muscles, tongue, and diaphragm. Necropsy of control lambs revealed no pathological changes in heart and skeletal muscle referring to WMD.

#### Microscopic findings

Microscopically, Zenker's degeneration and necrosis were evident in the subepicardial, intramural, and subendocardial regions of the myocardium (Figure 1a). In some areas, the sarcoplasm exhibited dense basophilic granules indicating presence of calcium deposition. Bizarre and multinucleated giant cells were common and thought to be attempts of regeneration by myocytes (Figure 1b). Necrotic segments contained numerous macrophages. In some areas necrotic segments were infiltrated by proliferating fibroblasts among which newly developing capillaries were present. Endothelial cells located in the medial layer of some myocardial arterioles were swelled with presence of amorphous hyaline bodies. Zenker's degeneration and fibrosis are common findings for all the samples of skeletal muscles examined. Affected fibres of the sarcoplasm with evidence of necrosis was infiltrated with homogenous eosinophilic granules, and many degenerated fibres were invaded by macrophages. Other common findings were degenerated vessels with presence of endothelial proliferation and mild fibrinoid

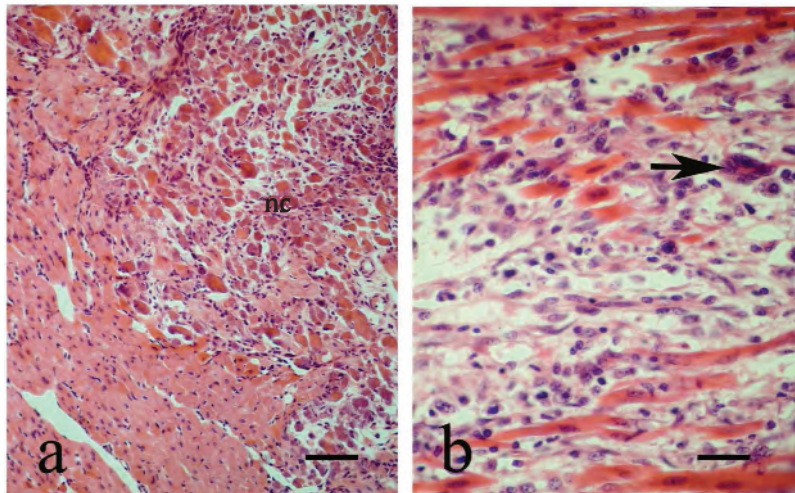


Figure 1. Nutritional muscular dystrophy in lambs illustrated (a) as Zenker's degeneration and severe necrosis (nc) in the subepicardial regions of the myocardium and (b) bizarre and multinucleated giant cells (arrow) thought to be attempts at regeneration by myocytes are common. H&E. (Bar: a, 80  $\mu$ m; b, 25  $\mu$ m).

necrosis. Adventitia of large arteries were also infiltrated by mononuclear cells.

#### Immunohistochemistry

The histological investigation of the samples revealed a depletion of the cTnI in all the lamb hearts with WMD. On the other hand, iNOS immunoreactivity was exclusively augmented in the same samples. Figures 2 and 3 show typical examples of the presence of iNOS and depletion of cTnI, respectively. In normal myocardium, strong cytoplasmic cTnI immunoreactivity was observed in the cardiac myocytes. The semi-quantitative assessment of cTnI and iNOS immunostaining and statistical differences in the cardiac muscle of lambs with WMD and control lambs are summarised in Figure 4.

Loss of cTnI immunoreactivity occurred in the cardiac muscle cells particularly in the myocytes showing high degree of degeneration and necrosis. Loss of cTnI tended to be greater at the periphery of the regions rather than at their centres.

Prominent immunostaining for iNOS was present in a variety of cell types, including cardiac myocytes, endocardial and endothelial cells, vascular smooth muscle cells, and fibroblasts. Staining intensity for the iNOS antibody was low for the myocytes exhibiting signs of necrosis. However, iNOS immunoreactivity was augmented in the degenerated myocytes. iNOS

immunoreactivity was not detected in the immature, regenerating myocytes and myocytes with calcification. A strong, granular type cytoplasmic iNOS reaction was observed in the macrophages infiltrating to the interstitium. Weak iNOS immunoreactivity was occasionally seen in some myocytes, endocardial and endothelial cells of control hearts.

#### Discussion

In the present study, elevated serum CK, CK-MB, AST, and LDH concentrations were elevated in lambs diagnosed with WMD following clinical findings and complete necropsy and histopathology results. The histopathology of affected lambs in the present study was similar to what has previously been described in lambs with WMD (16). Our study also demonstrated loss of tissue cTnI and increased serum cTnI concentration in damaged myocardial cells. cTnI was recently used as a histological marker for myocardial injuries in human (17) and dog (18). It has been reported that, compared to the cTnT and cTnC, cTnI is the most specific marker for myocardial injuries (17).

Present study indicates that immunohistochemical staining for cTnI is more sensitive than routine H&E staining for the recognition of myocardial necrosis. Whenever we did observe loss of staining, there was



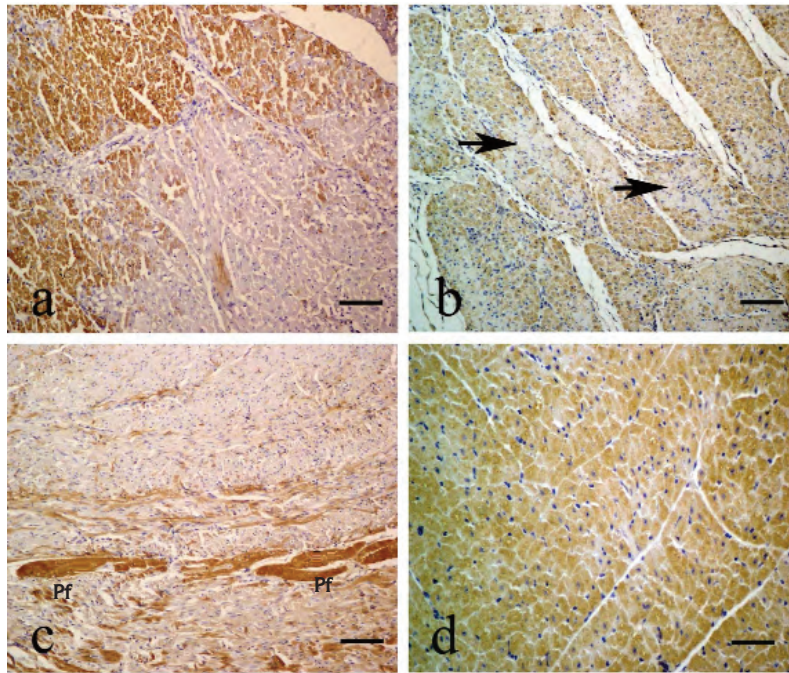


Figure 2. The cTnI immunoreactivity of the lamb heart with nutritional muscular dystrophy showing depletion (a, b, and c) of the cTnI compared with control lambs (d). Avidin biotin peroxidase complex. (a) Normal cTnI expression in the upper left. Lower right reveals almost exclusively loss of staining. (b) Apparent loss of cTnI immunoreactivity in the regions with severe degeneration and necrosis (arrows). (c) cTnI reaction in Purkinje fibres (Pf). Apparent loss of cTnI in the cardiac myocytes is seen in the upper part of the figure. (d) Typical immunohistochemical staining patterns for cTnI in normal myocardium. (Bar: a, b, c, 80  $\mu$ m; d, 40  $\mu$ m).

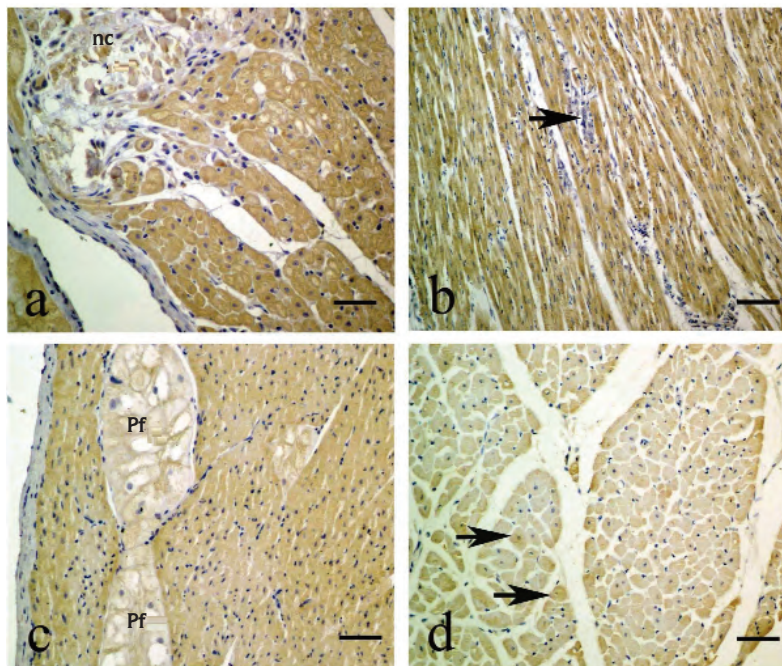


Figure 3. The iNOS immunoreactivity of the lamb heart with nutritional muscular dystrophy. Prominent immunostaining for iNOS is seen in the lamb heart with nutritional muscular dystrophy (a, b and c) compared to control (d). Avidin biotin peroxidase complex. (a) Prominent iNOS expression is present in myocytes surrounding areas with necrosis (nc). (b) Affected myocytes reveal intense iNOS immunoreactivity. Young, regenerating myocytes (arrow) are negative for iNOS immunostaining. (c) Weak iNOS immunoreactivity in Purkinje fibres (Pf). (d) Weak iNOS immunoreactivity is seen in some myocytes (arrow) of control hearts. (Bar: a, b, c, d; 80  $\mu$ m).

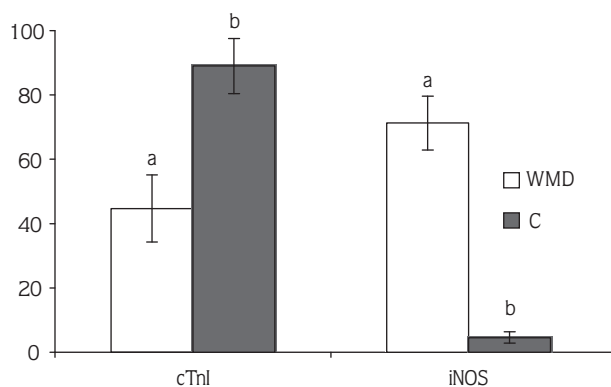


Figure 4. Semiquantitative analysis of cTnI and iNOS immunostaining results. Values are presented as mean  $\pm$  SE.

histological evidence of irreversibly injured myocytes in the tissue. We also observed greater loss of cTnI at the periphery of necrotic area, which likely reflects greater antegrade and retrograde flow at the periphery with “washout” of proteins from the necrotic myocardium (19). Accentuation of this phenomenon with reperfusion was previously shown in an experimental model of myocardial ischemia in dogs. The greater loss of cTnI seen at the periphery, especially with reperfusion, was attributed to manifestation of this washout (18).

We observed heterogeneous loss of cTnI in necrotic myocardium. There was a great deal of variability of intensity of residual staining for cTnI within the necrotic region. It is known that cardiac troponins are cleared from the blood faster than CK-MB, but levels stay elevated longer (20). These observations suggest continued and/or delayed release of cTnI from the necrotic myocytes. These findings indicate that the

elevation of cTnI in the blood is not the result of slow elimination from the circulation, but rather, continuous liberation from disintegrating myofibers (18).

In the present study the antibody generated against human cTnI stained lamb myocardium and were diminished in necrotic myocardium. Thus, this antibody should be useful in studies of myocardial injury in a variety of animal models including ruminants.

In the present study, we found an increase in iNOS expression in lambs with WMD. In a previous study on heart transplantation model, iNOS was induced both in macrophages infiltrating the myocardium and in cardiac myocytes, in response to cytokines released during the immune response to the grafted tissue (21). Pinsky et al. (22) showed that NO released by macrophages could kill adjacent myocytes by an NO-dependent mechanism. They also showed that the induction of iNOS by cytokines in isolated purified cardiac myocytes was auto destructive to the cardiac myocytes by an NO-dependent mechanism. It was suggested that NO is capable of triggering apoptosis in cardiac myocytes (23). In these studies, iNOS induction in the myocytes suggests that NO produced by iNOS in infiltrating macrophages and cardiac myocytes may be a trigger for the apoptotic response of cardiac myocytes. These results clearly suggest that iNOS expression augments in cardiac myocytes in lambs with WMD, most likely through influencing the signalling pathways to iNOS induction.

In conclusion determination of serum cTnI and immunohistological demonstration of cTnI depletion and iNOS expression may be of valuable diagnostic markers in nutritional myocardial degeneration.

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