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Original article

Expression of ADAMTS-7 in myocardial dystrophy associated with white muscle disease in lambs

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Abstract

The aim of the present study was to investigate the role of ADAMTS-7 gene in the pathogenesis of myocardial dystrophy associated with white muscle disease (WMD) in lambs. A total of 217 cardiac tissue samples from lambs with WMD were used in the study. Histopathological sections of the samples were stained with hematoxylin-eosin (HE) and examined using Western-blot, real-time PCR (RT-PCR) and immunohistochemistry for ADAMTS-7 gene expression, and the findings were statistically evaluated. Histopathological examinations revealed fibrosis associated with hyalinization, necrosis and granular calcifications in cardiomyocytes. Western blot and RT-PCR showed a statistically significant upregulation of ADAMTS-7 ($p < 0.05$) ($p < 0.05$). Immunohistochemical analyses showed that immunopositive cell numbers significantly high for ADAMTS-7 ($p < 0.05$). The study has revealed that ADAMTS-7 gene is significantly expressed in myocardial dystrophy associated with WMD in addition to its role in the pathogenesis of this disease.

Key words: ADAMTS-7, myocardial dystrophy, lamb, white muscle disease

Introduction

White muscle disease (WMD), or nutritional muscular dystrophy, is an acute disease that results in degeneration and necrosis in cardiac muscle. It generally leads to death associated with heart failure in fast-growing young animals. Paleness, degeneration, fibrosis, necrosis and calcification develop in the cardiac muscle (Abutarbush et al. 2003). Free oxidative radicals have a significant role in the disease

pathogenesis. Unsaturated fatty acids in the cell membrane are converted into a radical form in a chain reaction: a fatty acid next to the fatty acid radical can be converted into another free oxygen radical (ROS) (Van Winden and Kuiper 2002). ROS induces degradation of the cellular membrane, proteins and integrins and causes damage in the extracellular matrix (ECM). Oxidative damage in healthy animals is prevented by an antioxidant mechanism (Chuang et al. 2014). These antioxidants protect membrane integrins

in cardiomyocytes and ECM by activating enzymes, such as glutathione peroxidase. ECM is an adhesion glycoprotein secreted by thrombocytes (Hughes and Jacobs 2017). ECM surrounding myocytes, fibroblasts, capillaries and other veins is a multi-functional complex that ensures structural and functional integrity of the heart (Shahid et al. 2017). And also generates the required environment for cell migration, growth and proliferation, and is responsible for intercellular communication (Brown 2005). The amount and structure of the collagen in ECM depends on the balance between synthesis and degradation. The synthesis and degradation of ECM in normal hearts is a perennial and firmly controlled process. ECM is composed of structural components including collagen and elastin, and specialized proteins including fibrillin, fibronectin, proteoglycan and matricellular proteins (Hegarova et al. 2015). Interstitial collagen fibers provide an extensive supportive network, and protect the thickness and architecture of the myocardium (Chan et al. 2012). The degradation of collagen is regulated by the balance between metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). It has been reported that the elevated production and secretion of MMPs and TIMPs have a role in the pathogenesis of cardiovascular diseases (Shiomi et al. 2010).

A-Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) were initially defined in 1997 and it is an ECM gene consisting of 19 subunits that is responsible for various physiological and pathological conditions, and it has recently been used by researchers for diagnosis and treatment of diseases (Porter et al. 2005). ADAMTSs play a role in pathological processes, such as cell proliferation, apoptosis, tumor formation, inhibition of angiogenesis and degradation of ECM and basal membrane by binding to cellular matrix molecules including collagen, versican and aggrecan, and degrading structural proteins of cell-to-cell and cell-to-matrix (Wagstaff et al. 2011). ADAMTS-7 is known as COMP-ADAMTSs as it degrades cartilage oligomeric matrix protein (COMP). It has been found that ADAMTS-7 levels are significantly elevated in diseases, such as osteoarthritis and rheumatoid arthritis (Liu et al. 2006). Moreover, the expression of ADAMTS-7 is significantly upregulated in cardiomyopathies, arterial ruptures, neointimal transformation and atherosclerotic calcification (Wang et al. 2010).

In the present study, the role of ADAMTS-7 gene, a novel issue in research of the pathogenesis of cardiovascular disease, was investigated in the pathogenesis of WMD which often results in severe myocardial dystrophy in lambs.

Materials and Methods

Sample Collection

Cardiac tissue samples from 217 lambs diagnosed with WMD between the years of 2012 and 2017 were used in the present study. Following the necropsy, 200 mg of the each samples were stored at -80°C for Western blotting and RT-PCR examinations. Tissues were fixed in neutral (pH-7.0) formaldehyde for histopathological and immunohistochemical studies. Healthy cardiac tissues from a similar number of lambs taken in a slaughterhouse were examined as controls.

Tissue samples from living animals were not used. All stages of the study were performed with the approval of the National Ethics Committee and under the supervision of the Local Ethics Commission (IRB Approval No.: DOLLVET2016/027).

Histopathology

The tissues for histopathological analyses were fixed in 10% neutral buffered formalin, embedded in paraffin blocks after standard tissue processing, and 4- μ m-thick sections were cut from each of the blocks by Leica RM 2125 RT. The first 3 sections and every 10th section were placed on glass slides. And sections were stained with hematoxylin-eosin (HE) by passing through graded alcohol (50%, 75%, 96%, 100%) and xylol series. All samples were examined under high-resolution light microscope (Olympus BX-53, Tokyo, Japan) at 40x-100x magnification.

RNA isolation, cDNA synthesis and qRT-PCR analysis

Tissue samples were homogenized at 6500 rpm for 40 seconds and RNA isolation was performed using the High Pure FFPE RNA Isolation kit (Roche Applied Science, Germany) in accordance with the company protocol. cDNA synthesis was accomplished following the product catalog protocol using the First Strand cDNA Synthesis Kit (Roche Applied Science, Germany). Primer sequences were: ADAMTS-7; forward: 5'-GTCATCGACTTCCCTTCCATAC-3', reverse: 5'-TGTCCATGTCATCGCAGAAG-3' (product length: 159bp); and GAPDH: forward: 5'-ATGCCTCCTGCACCACCA-3', reverse: 5'-AGTCCCTCCACGATGCCAA-3' (product length: 269bp). RT-PCR master mix was prepared by the addition of 1 μ l (10 pmol) from each primer, 10 μ l of LightCycler® 480 SYBR Green I (Roche Applied

Science, Germany) kit and 3 µl of PCR Grade Water. The final volume was completed to 20 µl with the addition of 5 µl of cDNA for each sample. Qiagen Rotor-Gene Q systems were run in line with the recommendations of company protocol. The denaturation process was performed at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and elongation at 72°C for 5 sec. A single reading was taken. Melting analysis was performed at 95°C for 5 min, 50°C for 60 sec, continuously at 50°C -95°C for 60 sec. Readings were taken at 40°C for 60 sec. The findings obtained from RT-PCR were evaluated using Rotorgene Software. The concentration values were calculated based on the crossing point (Cp) values of the samples and these data were used for the calculation. Prior to the calculation of the sample results, the amplification curves and Cp value of each sample were checked to determine if they were at the intended level. The expression of ADAMTS-7 was normalized to GAPDH. The results were calculated using the concentration determination method in accordance with $2^{-\Delta\Delta CT}$ Cp values.

Western Blotting

Muscle tissues were homogenised on ice in mammalian cell lysis buffer (ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit, Fermentas) containing a final concentration of 1/1 protease inhibitor cocktail (Cell Signaling Technology, #5872, Danvers, USA) with a tissue homogeniser (Heidolph homogenizers, SilentCrusher, Germany). The lysates were cleared by centrifugation at 14,000 × rpm for 15 min. at 4°C, and the supernatant was transferred to a new tube. Protein concentrations were evaluated by Quick Start Bradford Protein Assay (Bio-Rad Laboratories). Proteins (25 µg each) were subjected to SDS-PAGE with 8% polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immun-Blot PVDF, Bio-Rad, USA) using Bio-Rad Trans-Blot Turbo for 40 minutes under a constant current of 25 mA. After blocking the membrane with 5% skimmed milk for 2 hours at room temperature, the membrane was incubated with a primary antibody (Rabbit polyclonal anti-ADAMTS7: Abcam, Cambridge, UK, ab28557), 1:500 dilution, Abcam; Rabbit polyclonal anti-GAPDH: sc-25778, 1:1000 dilution, Santa Cruz Bio Inc., Dallas, TX, USA) overnight at 4°C. After washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (sc-2004, 1:10000 dilution, Santa

Cruz Bio Inc., Dallas, TX, USA) for 2 hours at room temperature, and then washed with TBST. Signals were detected by chemiluminescence using Clarity™ Western ECL Substrate (#1705061, Bio-Rad). GAPDH was used as an internal control and band intensities were quantified using Image Studio™ Lite (LICOR).

Immunohistochemistry

Cardiac tissues were verified immunohistochemically for the presence of ADAMTS-7 using anti-ADAMTS-7 primer antibody. Four-µm-thick sections were obtained from previously prepared paraffin blocks, mounted on poly-L-lysine coated glass slides and labeled according to streptavidin-biotin-peroxidase complex (ABC) technique following deparaffinization and rehydration (Zymed, Histo-tain Plus Kit, California, USA). The thermal procedure was conducted in a microwave at 800 W for 20 min with citrate buffer (pH 6.0) for tissue antigen retrieval. Endogenous peroxidase activation was blocked with 0.3% hydrogen peroxide in methanol and sections were then washed in 0.01M phosphate-buffered saline (PBS) for 10 min. Protein blocking was performed by incubating tissue sections with 5% normal bovine serum for 30 min prior to primary antibody. Sections were incubated with ADAMTS-7 primary antibodies (Abcam, Cambridge, UK, ab28557, 1:200 dilution) at room temperature for 60 min. Sections were incubated in rabbit anti-mouse biotinylated secondary antibody for 30 min and in streptavidin-peroxidase conjugate for 30 min. Diaminobenzidine (DAB, Dako/Denmark) was dropped on the sections and left for 10 min to make the reaction visible. Finally, sections were stained with hematoxylin for background staining and washed in running water. Sections were passed through graded series of alcohol and xylol and coverslipped with Entellan. The staining procedure was performed at room temperature in moist cabinets. Phosphate buffered saline (PBS) solution was used to wash the sections during staining procedures. Data were observed under high-resolution microscope (Olympus DP73 camera, Olympus BX53-DIC microscope; Tokyo, Japan) at 40x and 100x magnifications for each sample. To assess the immunohistochemistry, ten fields were randomly chosen and the cytoplasmic staining in the cells was globally scored (Inanir et al. 2016) as 0: no staining, 1: mostly negative cells, 2: moderate numbers of positive cells, 3: apparent in positive cells and 4: mostly positive cells.

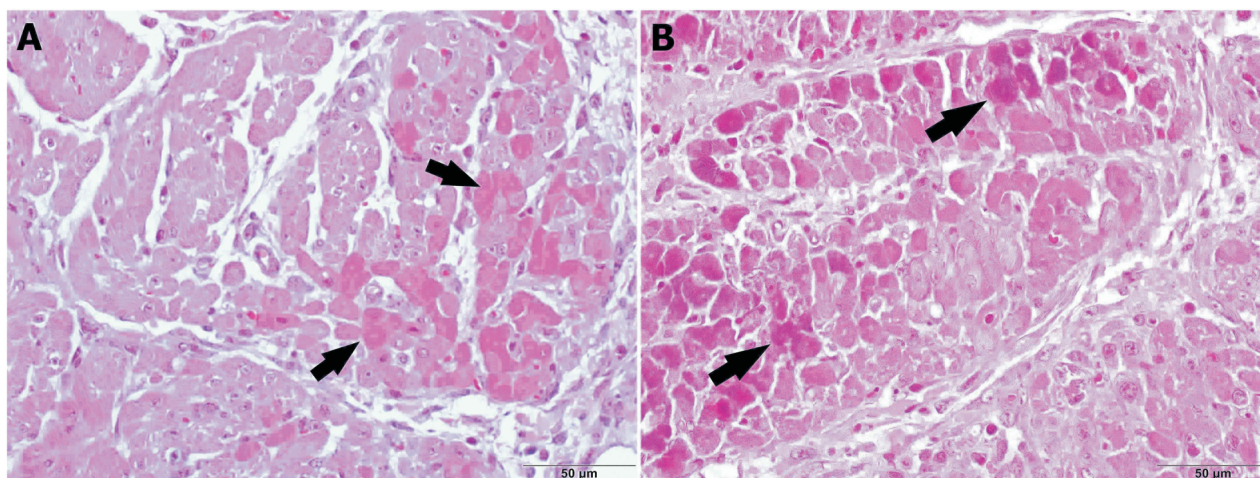


Fig. 1. Histopathological findings in cardiac tissues. A: Hyaline degeneration of cardiomyocytes (arrows), HE staining, x200. B: Mineralization in degenerative cells (arrows), HE staining, x200.

Statistical Analysis

Statistical analyses were applied using SPSS for Windows version 24.0 software. The normality of distribution of continuous variables was tested with the Shapiro Wilk test. The Mann-Whitney U test was used for the comparison of two independent groups of variables with non-normal distribution. Descriptive statistics were stated as median, 25% and 75% values. A value of $p < 0.05$ was accepted as statistically significant.

Results

Post-mortem Examination

The hearts of necropsied lambs were totally dilated, and the left ventricular myocardium in particular was very loose. The myocardium was pale in colour. Moreover, extensive and pale necrotic areas and extensive mineralization were observed in the outer layer of the heart and especially in the left ventricle.

Histopathology

Histopathological examination revealed myofibrils with irregular borders and significant swelling of myocytes. Diffuse hyaline degeneration was noticeable in myocytes (Fig. 1A). Basophilic calcium deposits were detected in sarcoplasm (Fig. 1B). Fibrosis was observed between the muscle cells and necrosis was detected in the cells. Macrophage deposits were also found in certain areas.

ADAMTS-7 Expression

RT-PCR revealed that the expression level of ADAMTS-7 gene was statistically significantly higher in cardiac tissue samples with diagnosed WMD compared to the control group ($p < 0.05$). A statistically significant difference was detected in the tissues when the C_p values of ADAMTS-7 genes were proportioned with the C_p values of housekeeping genes ($p < 0.05$). The C_p values and melt curves of ADAMTS were found to be significant variables as seen in the graph curves (Fig. 2).

To elucidate involvement of ADAMTS-7 in the heart tissues of sheep with WMD, protein expression was determined by Western blot analysis. GAPDH was used as a reference control. As it was the first evaluation of ADAMTS-7 in the sheep sample, human cell lysate was also used as a positive control sample. ADAMTS-7 protein level was examined in control and WMD heart groups. Western blot analysis confirmed significantly increased ADAMTS-7 protein level in the hearts of WMD group ($p < 0.05$) (Fig. 3).

In the immunohistochemical examinations, a statistically significant high density of immunopositive cells was found from the control tissues in myocytes in which degeneration and necrosis were detected by labeling with ADAMTS-7 ($p < 0.05$). In certain areas, pale and weakly stained cells were observed between immunopositive cells (Fig. 4). Whereas the sections of the control were immunohistochemically negative ($p > 0.05$) (Fig. 4A).

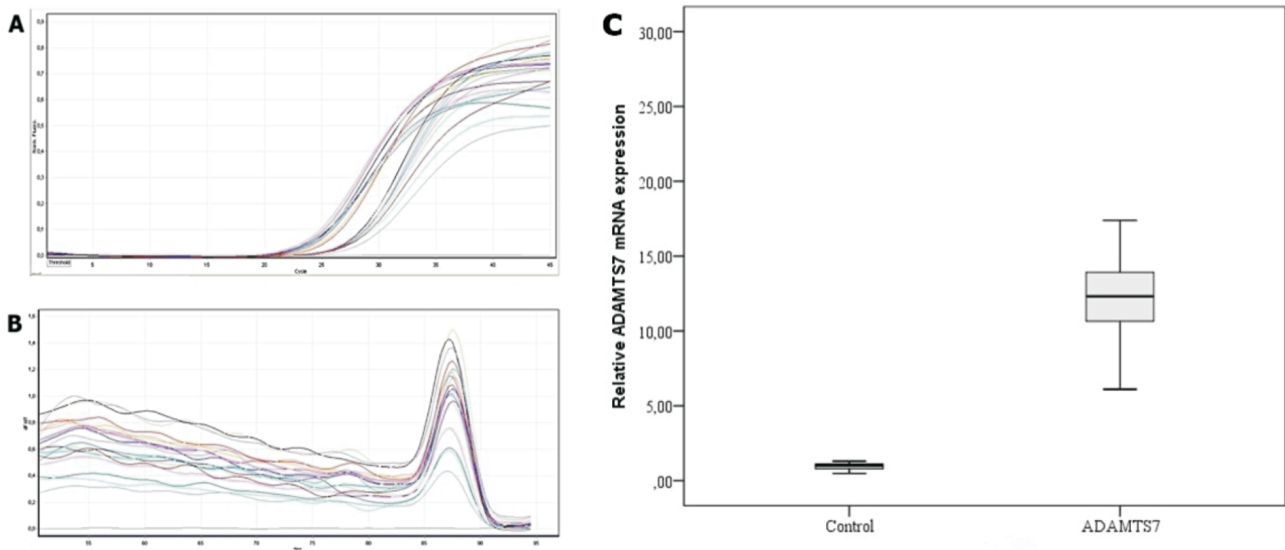


Fig. 2. mRNA expression was determined with real-time RT-PCR. A: ADAMTS-7 gene Cp curves. B: ADAMTS-7 gene melt curves. C: Graphical description of the statistical difference between the groups.

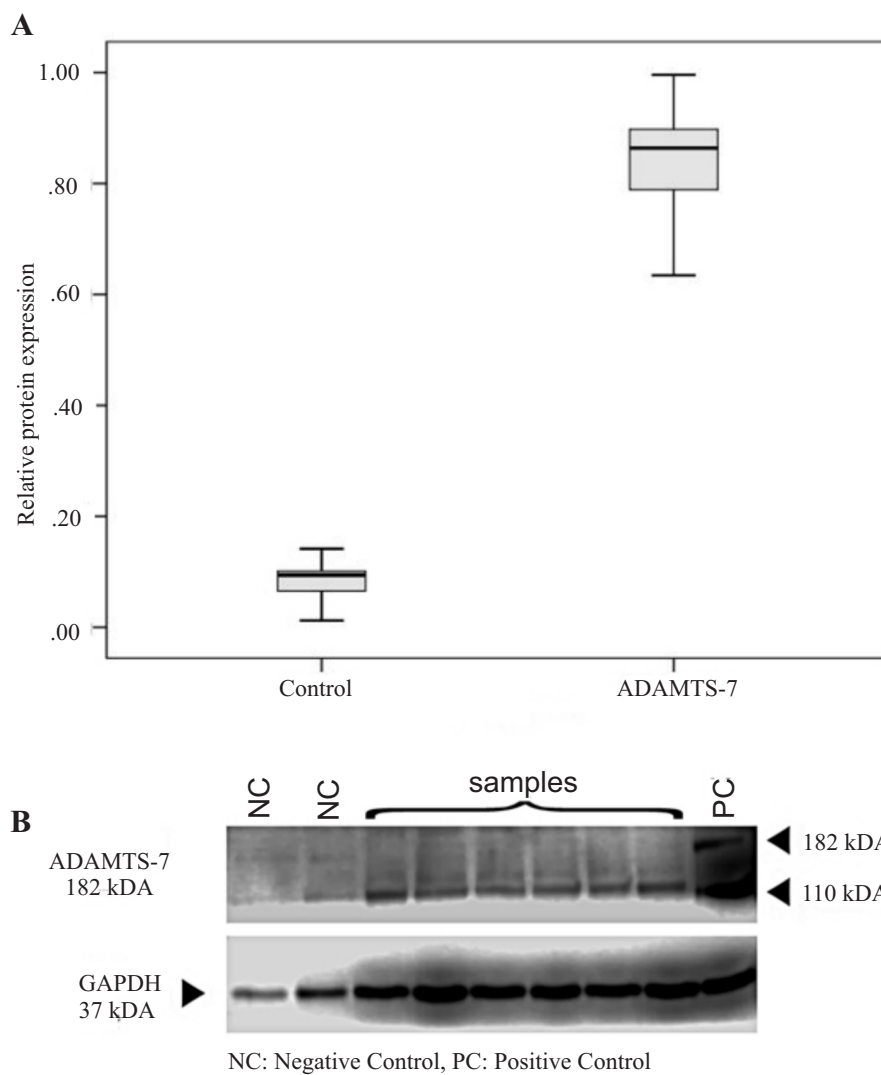


Fig. 3. A: Statistical difference in ADAMTS-7 protein expression between the groups. B: ADAMTS-7 protein expression determined by Western blot analysis.

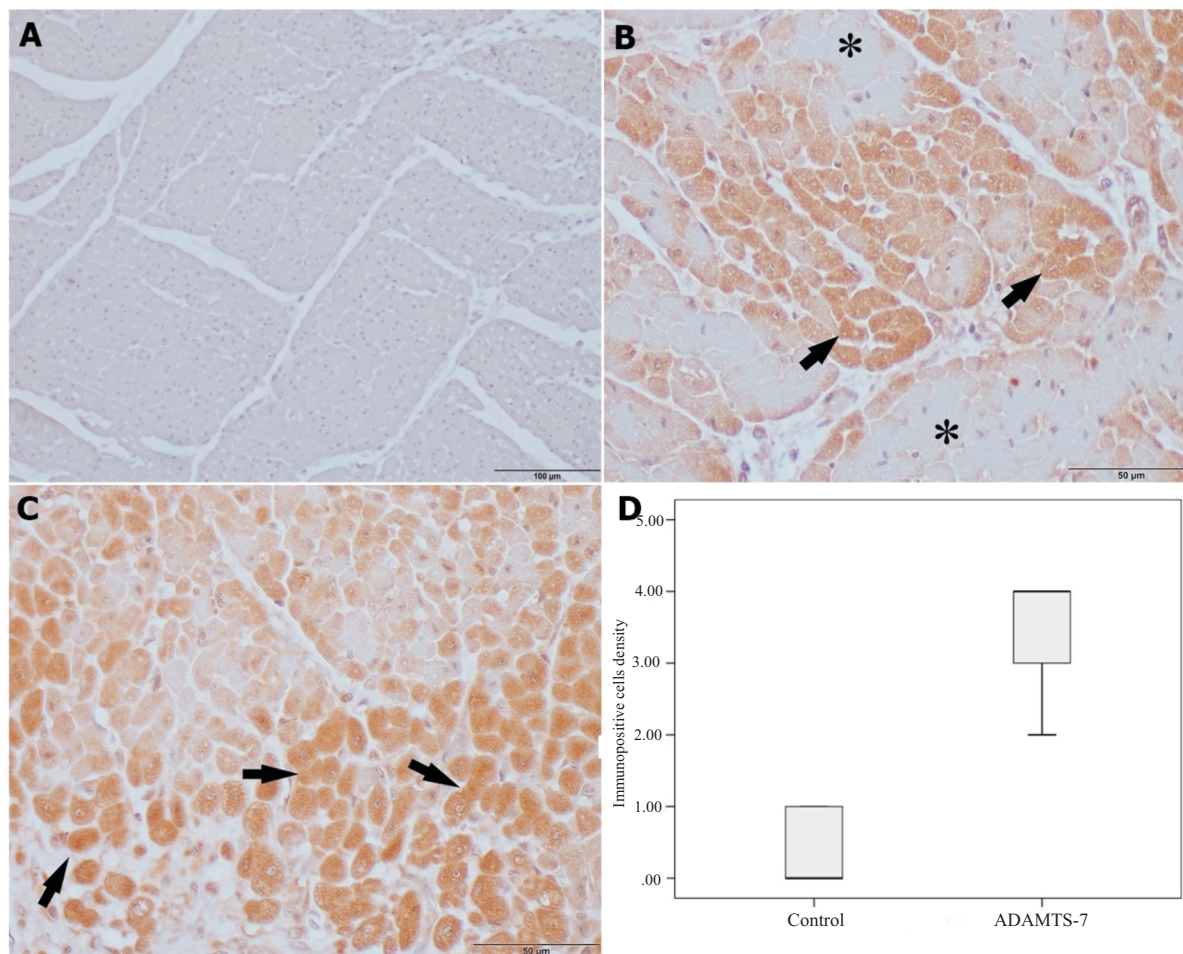


Fig. 4. Immunohistochemical expression of ADAMTS-7 in cardiac muscle. A: Cells from the control group showing immunonegative reaction, x40. B: Strongly immunopositive cells (arrows) and weakly immunoreactive cells (asterisk), x200. C: Numerous strongly immunopositive cells (arrows), x200. D: Statistical difference in ADAMTS-7 immunopositive cell number.

Discussion

WMD is a disease which commonly causes death in young ruminants as a consequence of acute heart failure resulting from hyaline degeneration, necrosis and calcification in the myocardium. Lipoperoxidation initiated by the attack of free radicals, such as elevated levels of hydrogen peroxide and superoxide anions, has a role in the pathogenesis (Van Winden and Kuiper 2002, Abutarbush et al. 2003). This elevated level of cellular free radicals induces damage and disintegration of the ECM and cells (Wang et al. 2009, Chuang et al. 2014, Bauer et al. 2015). It has recently been determined that ADAMTS genes play an important role in the pathophysiology of genetic and chronic inflammations in humans. Based on previous human and animal studies, it has been claimed that ADAMTS genes also play a role in cardiovascular diseases (Shiomi et al. 2017). In the present study, the role of ADAMTS-7 gene in myocardial dystrophy associated with WBD in lambs was examined using

RT-PCR, Western blot and immunohistochemical methods. The results showed that ADAMTS-7 was significantly upregulated in cardiac tissues with the disease. Study findings have shown that ADAMTS-7 gene can have a role in the pathogenesis of myocardial dystrophy associated with WMD.

Recent studies have specified the role of ADAMTS-7 gene in cardiovascular system disorders in humans and rats (Patel and Ye 2013). However, the exact mechanism of action has not yet been identified. In contrast, the importance of ECM for the structural preservation of cardiovascular tissues has been well known for many years (Brown 2005). When the function and structure of ADAMTS-7 gene is examined, it can be understood that ADAMTS-7 gene is ECM protein comprising a signal peptide, a prodomain and disintegrin-like domain. The prodomain in particular is cleaved-off during ADAMTS-7 maturation and activation (Somerville et al. 2004, Hanby and Zheng 2013). Active ADAMTS-7 is a proteolytic enzyme and its well-known substrate is COMP, which is an ECM

protein present in vessel wall and cartilage (Liu et al. 2006). Huang et al. (2013) determined the presence of COMP in the cardiomyocytes with immunohistochemical analysis. Wu et al. (2015) conducted a study on rats and found that COMP in the cardiomyocytes was suppressed by ADAMTS-7 in the dilatation associated with ventricular damage. While ventricular muscle damage is among the important causes of heart failure, ECM is responsible for the preservation and functioning of ventricular structure (Hughes and Jacobs 2017). In cases of structural disturbances in ECM, total ventricular dilatation, ventricular rupture and cardiac functional impairments might develop (Chan et al. 2012). In the present study, dilated ventricles were observed in the post-mortem examinations of hearts with WMD. Muscle tissues were thinner and soft. These findings suggested that this condition might be related to the degeneration and necrosis in the myocytes because of ECM degradation.

In an experimental study conducted on mice with acute myocardial infarction (AMI) by Wu et al. (2016), it was suggested that the expression of ADAMTS-7 was significantly elevated in the degenerative cells, especially in the heart muscle of the ischemic areas. In a similar study by Reilly et al. (2011), the expression of ADAMTS-7 protein was significantly upregulated in consequence of neointimal thickening associated with cardiac damage. Wang et al. (2009) reported that ADAMTS-7 was upregulated in cases of vascular damage correlated to the cell migration and neointimal formation in balloon-injured rat arteries. In human studies, ADAMTS-7 has been observed to be mostly accumulated at the intima-media border and fibrous cap in coronary and carotid atherosclerotic plaques produced by VSMCs (Pu et al. 2013, Bengtsson et al. 2017). Du et al. (2012) found that miR-29 stimulated VSMC calcification by upregulating ADAMTS-7 in patients with atherosclerosis. Similarly, the repression of miR-29a/b (an inhibitor of ADAMTS7) which in turn facilitated VSMC calcification was observed in patients with chronic renal failure (Wang et al. 2010, Du et al. 2012). In the present study, cells with positive immunohistochemical staining were generally intense in degenerated and necrotic areas. This finding indicated that ADAMTS-7 upregulation was correlated with damage in the myocytes associated with WMD. Moreover, extensive granular calcified areas were detected on the degenerative cardiomyocytes in the heart tissues. The intensity of immunopositive cells was increased in these areas and the surrounding region. These findings demonstrated that WMD had a mechanism similar to the pathophysiology of atherosclerotic calcification.

In addition to these findings, ADAMTS-7 has been shown to be upregulated corresponding to the

elevated levels of inflammatory cytokines, such as TNF- α , NF- κ B and interleukin, in patients with rheumatoid arthritis, femoral neck fracture and osteonecrosis of femoral head fracture (Buckland 2013, Lai et al. 2014). Pi et al. (2015) determined that ADAMTS-7 stimulated fibrosis by binding to connective tissue growth factor (CTGF) in liver injury. Moreover, the formation of fibrosis was noticeable among the degenerative cells. These findings are also accepted as an indicator of fibrosis for inducing the inflammation in the heart tissue depending on the elevated levels of ADAMTS-7.

In a diagnostic study conducted by Roy et al. (2008), it was stated that the level of ADAMTS-7 expression was high in the urine of patients with prostate, bladder and breast cancers. Wu et al. (2015) determined the presence of a substantial relationship between ADAMTS-7 expression and heart functions in patients with acute myocardial ischemia. It was reported that ADAMTS-7 can be used for diagnosis of tumor development, and the treatment of invasions and metastasis by designating the diagnostic and prognostic roles of ADAMTS-7. Bauer et al. (2015) suppressed ADAMTS-7 genes in mice on a high cholesterol diet and the formation of atherosclerotic lesions were significantly prevented in these animals. In recent studies performed on osteoarthritis in humans, the role of ADAMTS-7 gene has been determined in the disease pathogenesis and novel treatment approaches have been developed by inhibiting ADAMTSs (Tortorella et al. 2009, Patel and Ye 2013).

In conclusion, the expression of ADAMTS-7 gene was significantly upregulated in myocardial dystrophy associated with WMD. This finding revealed the role of ADAMTS-7 upregulation that was detected by RT-PCR, Western blot and immunohistochemical assays in the pathology of WMD. Despite to this limited study, if the potential biological mechanisms of ADAMTS-7 in WMD is understood more clearly, ADAMTS-7 can be considered a therapeutic target for ECM damage in WMD.

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