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SPONTANEOUSLY ARISING DISEASE

Morphometric Properties of the Thoracic Aorta of Warmblood and Friesian Horses with and without Aortic Rupture

V. Saey[*], M. Ploeg[†], C. Delesalle[‡], G. van Loon[§], A. Gröne[†], R. Ducatelle[*], L. Duchateau[‡] and K. Chiers[*]

[*] *Laboratory of Veterinary Pathology, Department of Pathology, Bacteriology and Poultry Diseases, Ghent University, Merelbeke, Belgium,* [†] *Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands,* [‡] *Department of Comparative Physiology and Biometrics and* [§] *Department of Large Animal Internal Medicine, Ghent University, Merelbeke, Belgium*

Summary

Rupture of the aorta is much more common in Friesians compared with other breeds of horse. Rupture always occurs adjacent to the scar of the ligamentum arteriosum. Previous histological examination of ruptured aortic walls suggested the presence of an underlying connective tissue disorder. Therefore, the aim of the present study was to compare the structural characteristics of the tunica media of the mid-thoracic aorta, distant to the lesion, in warmblood and Friesian horses with and without thoracic aortic rupture. In unaffected Friesian horses, the thickness of the tunica media, as well as the percentage area comprised of collagen type I, were significantly higher compared with the warmblood horses, supporting the hypothesis of a primary collagen disorder in the Friesian horse breed. However, in the tunica media of the affected Friesian horses there was no significant wall thickening. Moreover, the percentage area comprised of elastin was significantly lower, while the percentage area comprised of smooth muscle was higher, compared with unaffected Friesian and warmblood horses. These lesions are suggestive of an additional mild elastin deficiency with compensatory smooth muscle cell hypertrophy in affected Friesians.

Keywords: aorta; horse; morphometry; rupture

Introduction

The aorta is a crucial dynamic functional unit in the cardiovascular system (Watanabe *et al.*, 1993). It is composed of multiple constituents that ensure the proper structure and function of the wall. The tunica media plays a major role in aortic stability (Dingemans *et al.*, 2000) and is characterized by lamellar units, consisting of elastin, smooth muscle cells and collagen (Clark and Glagov, 1985). Elastin and collagen impart the aortic elastic properties and tensile strength, respectively (Holzapfel *et al.*, 2000). Elastin is the main component of the thoracic aorta (McCloskey and Gleary, 1974). The aortic collagen consists predominantly of types I and III collagen, which account for 80–90% of the total collagen in the aortic media (Dingemans *et al.*, 2000; Silver *et al.*, 2001). Type I collagen is the major structural component of the vessel wall and type III collagen is mainly a reparative component (Raman *et al.*, 2011).

Rupture of the aorta is extremely rare in horses. When it occurs, it is typically located near the junction with the heart (Sleeper *et al.*, 2001). In Friesian horses, aortic rupture is much more common and occurs as a transverse tear near the ligamentum

Correspondence to: V. Saey (e-mail: veronique.saey@ugent.be).

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arteriosum. The rupture is often associated with a dissection, periaortic haematoma or pseudoaneurysm that ruptures into the pulmonary artery (Ploeg *et al.*, 2013). Abnormalities in both elastin and collagen amount and structure could cause a weakening of the aortic wall, resulting in rupture (Tsamis *et al.*, 2013). Details of the composition and structure of the aortic wall in different breeds of horses have not been reported.

The aim of the present study was to analyze the morphological characteristics of the tunica media of the equine thoracic aorta in order to gain insight into the possible role of different structural components in the pathogenesis of aortic rupture in Friesian horses. Therefore, the tunica media structure of the mid-thoracic aorta was compared in Friesian horses with aortic rupture, healthy Friesian horses and warmblood horses.

Materials and Methods

Animals

The animals investigated were divided into three groups. Group WB comprised of 17 warmblood (WB) horses (0–10 years old, median age 4 years). Twelve of these horses were presented for post-mortem examination for reasons unrelated to the cardiovascular system at either Utrecht University ($n = 5$) or Ghent University ($n = 7$). The other horses were sampled at a Belgian slaughterhouse ($n = 5$).

Group NAF consisted of 18 non-affected Friesian (NAF) horses (0–10 years old, median age 4 years). All but one of these horses were presented for post-mortem examination for reasons unrelated to the cardiovascular system at either Utrecht University ($n = 3$) or Ghent University ($n = 14$). One horse was sampled at a Dutch slaughterhouse.

Group AF consisted of 20 Friesian horses with an aortic rupture (affected Friesian horses, AF; 1–10 years old, median age 5 years). All were diagnosed with aortic rupture during post-mortem examination at either Utrecht University ($n = 8$) or Ghent University ($n = 12$).

All horses that were admitted alive to the University hospital were treated following the institutional guidelines. Formal ethical approval was waived by the chairperson of the ethical committee, based on Belgian and European legislation (EU directive 2010/63/EU), as all tissues were derived *post mortem*.

Sampling and Sample Preparation

The complete thoracic aorta was removed from the heart base to the diaphragm. The surrounding connective tissue was removed. Samples were taken from the middle of the thoracic aorta and fixed in 4% neutral buffered formalin for at least 24 h and then processed routinely and embedded in paraffin wax. Sections were stained with haematoxylin and eosin (HE). For demonstration of elastin and smooth muscle, sections (5 μm) were labelled with monoclonal mouse anti-human elastin antibody BA-4 (Leica Biosystems, Diegem, Belgium; 1 in 600 dilution) or monoclonal mouse anti-human smooth muscle actin (Dako, Brussels, Belgium; dilution 1 in 200), respectively. Immunolabelling was achieved with a highly sensitive horseradish peroxidase diaminobenzidine kit (Envision DAB + kit, Dako) in an automated immunostainer (Dako).

For collagen I and III labelling, sections (3 μm) were pretreated with normal horse serum (1 in 10 dilution) and then incubated with monoclonal mouse anti-collagen type I (Sigma, St. Louis, Missouri, USA) or monoclonal mouse anti-collagen type III (Abcam, Cambridge, UK). 'Visualization' was with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, California, USA), ABC/PO complex solution elite (Vector) and diaminobenzidine chromogen.

Morphometry

Tissue sections were randomized and examined in blinded fashion. HE-stained sections were used to evaluate lesions of the aortic media. The thickness of the media (defined as the perpendicular distance between the innermost and outermost elastic lamella of the aortic media) was measured on sections labelled to show elastin under low-power magnification (×25). Three digital image frames were taken for each specimen. One measurement was performed for each image and the averages of the three images were calculated.

The percentage areas comprised of elastin, collagens type I and III and smooth muscle actin were determined by image analysis. The measurements were made with a light microscope to visualize the tunica media at a magnification of ×400 using a Leica DFC320 camera (Leica Microsystems, Wetzlar, Germany) coupled to a computer-based image analysis system LAS v.3.8. (Leica Microsystems). Three (collagen) or four (elastin and smooth muscle actin) image frames were taken per slide.

Fragmentation of the elastic fibres in the tunica media was scored from 0 (no fragmentation) to 4 (severe fragmentation) according to the system described by Carr-White *et al.* (2000). Fragmentation of collagen I and III fibres was scored using a scale from 0 (no fragmentation) to 3 (severe fragmentation).

Statistical Analysis

The differences between groups were analyzed using a mixed model with horse as random effect and group and their interaction as categorical fixed effects. Testing was done based on F-tests at the 5% global significance level, but for the pairwise comparisons the *P* value was adjusted by Tukey's multiple comparisons technique.

Results

Histopathology

The tunica media showed a regular lamellar organization of smooth muscle cells, elastin and collagen fibres in all three groups. Prominent smooth muscle cell hypertrophy was seen in fourteen of the group AF horses. Patchy medial necrosis was present in one group AF horse (a 5-year-old gelding), two group NAF horses (a 5-year-old mare and <1-year-old mare) and two group WB horses (a 6-year-old mare and a 3-year-old gelding). Multifocal medial mineralization was observed in one group WB horse (a 6-year-old mare). Inflammation of the tunica media was absent in all horses.

Medial Thickness

The tunica media of the mid-thoracic aorta of group NAF horses was significantly thicker than that of group WB horses ($P < 0.001$; NAF: 4,773 ± 369 μm; WB: 3,546 ± 319 μm) (Fig. 1).

Elastin

Elastin fibres occupied a significantly smaller percentage area of the aortic media in group AF horses compared with group NAF and group WB horses (AF versus NAF, $P = 0.0004$; AF versus WB, $P = 0.0054$; AF 42 ± 2%; NAF 51 ± 2%; WB 49 ± 2%; Figs. 2 and 3). The median score for elastin fragmentation was not significantly different between the groups (AF 3; NAF 2.75; WB 3). The range of the scores was smaller in the group AF horses (2–4) compared with the group NAF (1–4) and group WB horses (0–4).

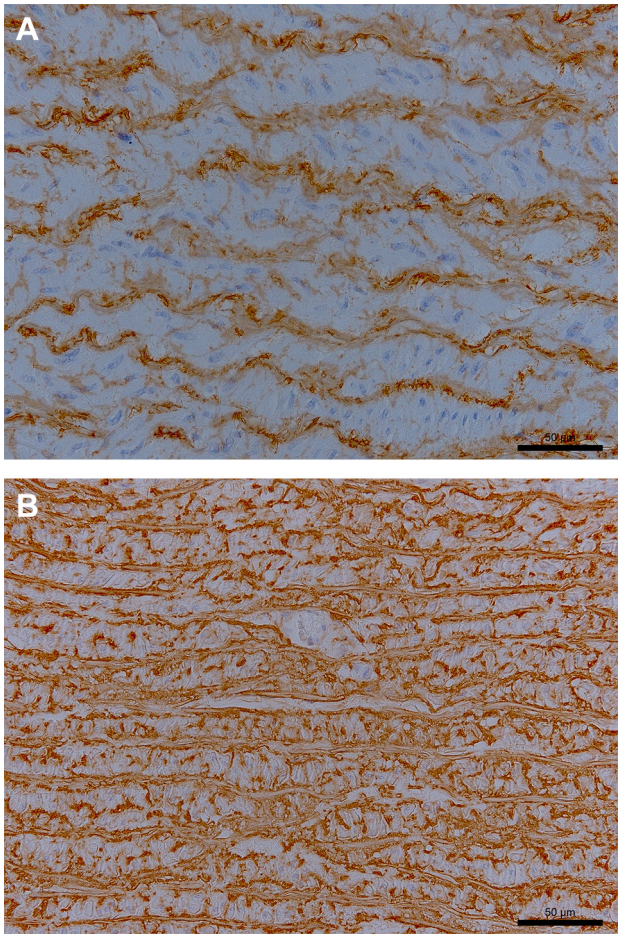

Fig. 2. (A) Mid-thoracic aorta. Loss of elastin in an affected Friesian horse shown by elastin immunohistochemistry. (B) Mid-thoracic aorta. Lamellar pattern of elastin in an unaffected warmblood horse using elastin immunohistochemistry.

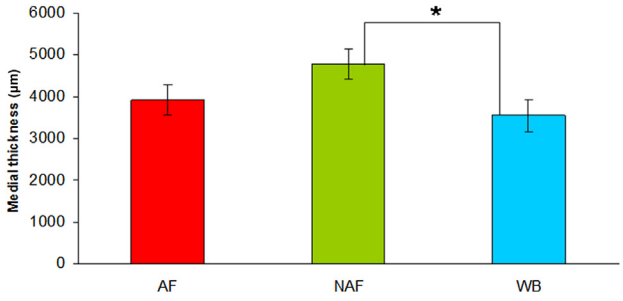

Fig. 1. Thickness of the tunica media of the mid-thoracic aorta of Friesian horses with aortic rupture (AF), non-affected Friesians (NAF) and warmblood horses (WB). $^{*}P < 0.001$.

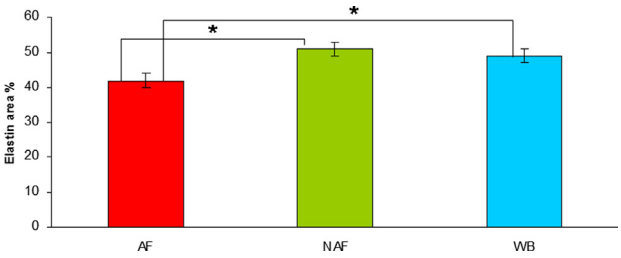

Fig. 3. Percentage area of elastin in the tunica media of the mid-thoracic aorta of Friesian horses with aortic rupture (AF), non-affected Friesians (NAF) and warmblood horses (WB). $^{*}P < 0.01$.

Collagen

Collagen type I occupied a significantly higher percentage of the aortic media in the two groups of Friesian horses compared with the group WB horses (AF 37 ± 4%; NAF 46 ± 4%; WB 26 ± 4%; AF versus NAF: $P = 0.0974$; AF versus WB: $P = 0.0486$; NAF versus WB: $P < 0.0001$; Fig. 4). There were no differences in collagen type III percentage area between the different groups (AF 33 ± 3%; NAF: 30 ± 3%; WB: 35 ± 3%). The median score for collagen type I and III fragmentation was not different between the groups and was the same for both collagen types (AF 1; NAF 1; WB 1).

Smooth Muscle

The percentage area comprised of smooth muscle was higher in group AF horses compared with horses of the other two groups (AF versus NAF: $P = 0.0024$; AF versus WB: $P = 0.0025$; AF 52 ± 2%; NAF 41 ± 2%; WB 41 ± 2%; Figs. 5 and 6).

Discussion

Aortic rupture in Friesian horses can result in sudden death or, alternatively, lesions can be present for several weeks to months, with many horses developing an aortopulmonary fistula (Ploeg *et al.*, 2013). Recently, samples taken at the site of aortic rupture (1–2 cm proximal to the ligamentum arteriosum) from Friesian horses have been examined microscopically (Ploeg *et al.*, 2015). Lesions included accumulation of mucoid material, disorganization and fragmentation of the elastic laminae, aortic smooth muscle cell hypertrophy and medial necrosis. Inflammation ranged from predominantly neutrophilic infiltration of the media and peri adventitial tissue in acute cases to the presence of mainly haemosiderophages in the periadventitial tissue in chronic cases. Medial fibrosis with aberrant collagen morphology was present in the subacute and chronic cases. A primary connective tissue disorder was suggested based on the presence of mucoid accumulation combined with elastin fragmentation (Ploeg *et al.*, 2015).

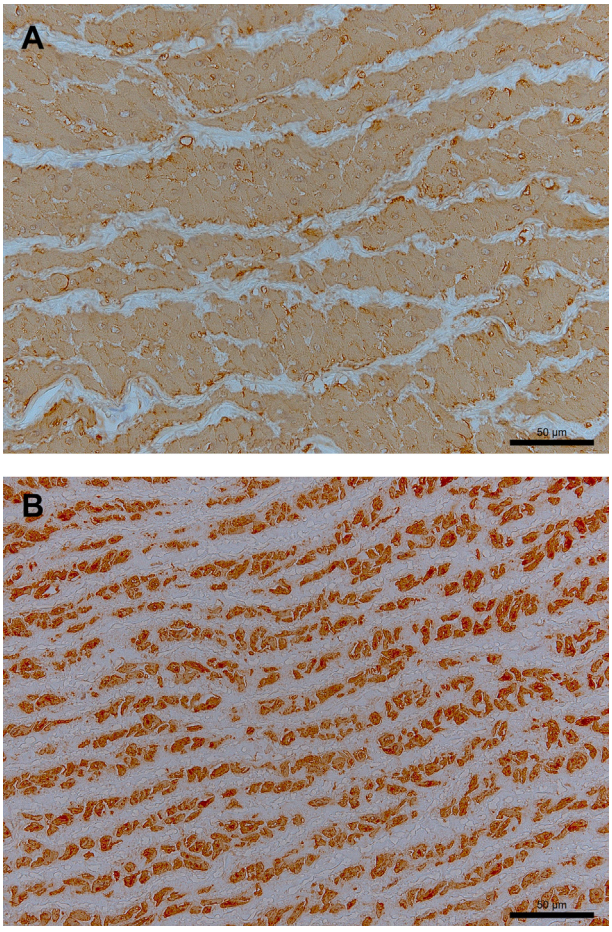

Fig. 5. (A) Mid-thoracic aorta. Smooth muscle hypertrophy in an affected Friesian horse using smooth muscle actin (SMA) immunohistochemistry. (B) Mid-thoracic aorta. Typical morphology of smooth muscle cells in an unaffected warmblood horse using SMA immunohistochemistry.

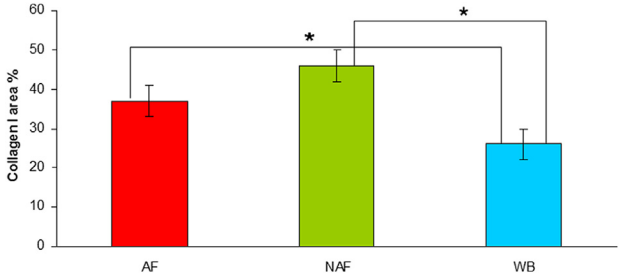

Fig. 4. Percentage area of collagen I in the tunica media of the mid-thoracic aorta of Friesian horses with aortic rupture (AF), non-affected Friesians (NAF) and warmblood horses (WB). $^{*}P < 0.0001$.

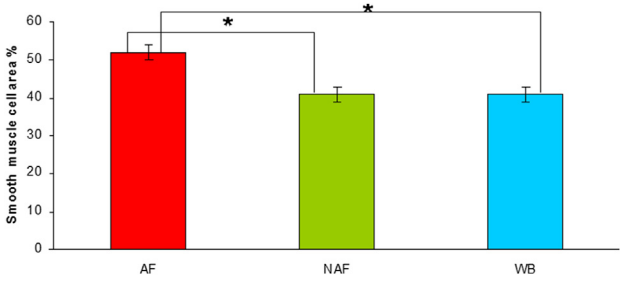

Fig. 6. Percentage area of smooth muscle in the tunica media of the mid-thoracic aorta of Friesian horses with aortic rupture (AF), non-affected Friesians (NAF) and warmblood horses (WB). $^{*}P < 0.005$.

In order to demonstrate a possible underlying connective tissue defect, the present study has examined samples of mid-thoracic aortic media. Since this location is distant from the predilection site, secondary changes, as observed adjacent to the aortic rupture, will be absent or minimal. Since the warmblood horse is not considered at risk of aortic rupture, comparison of aortic wall samples from Friesian horses (affected and non-affected) with warmblood horses could provide a first step in elucidating a possible underlying mechanism for aortic rupture in Friesian horses.

In the present study, two important differences were observed in the thoracic aortic wall tunica media between warmbloods and Friesians. Firstly, both non-affected and affected Friesians had a higher percentage area of collagen type I compared with the warmblood horses. This could reflect secondary fibrosis caused by hypertension (Repova-Bednarova *et al.*, 2013). However, in hypertension-induced aortic fibrotic remodelling, both collagens type I and III are expected to increase (Repova-Bednarova *et al.*, 2013), while only collagen type I was increased in the Friesian horses in the present study. Alternatively, the higher percentage area of collagen type I may indicate a primary collagen defect. The existence of a general collagen disorder in the Friesian horse has been suggested before, as tendon and ligament stretch properties of Friesians differ from that of other breeds (Gussekloo *et al.*, 2002) and collagen I is a primary component of tendons (Wang *et al.*, 2006).

The second breed-specific difference was a larger mean aortic medial wall thickness in Friesians compared with warmbloods. Racial/ethnic differences in mean aortic wall thickness have also been described in man (Rosero *et al.*, 2011). Increased aortic medial thickness, together with the above-mentioned increased density of collagen I fibres, may compensate for any reductions in tensile strength. This may explain why, for in-vitro tensile tests, no significant differences were observed between Friesians and warmbloods (Saey *et al.*, 2015).

Friesian horses with aortic rupture, however, for an unknown reason, did not have a significant increase in aortic medial thickness when compared with the warmbloods. This may be one factor related to why they are prone to develop aortic rupture. Decreased aortic wall thickness has been mentioned as a potential risk factor for aortic dissection in man (Shiran *et al.*, 2014).

In the affected Friesians, the percentage area of elastin in the tunica media of the mid-thoracic aorta was significantly lower. Since elastin is deposited mostly early in life and has poor regenerating potential (Pezet *et al.*, 2008), reduced elastin may indicate either a primary deficiency in elastin synthesis or an increased susceptibility of elastin to elastolytic breakdown. In either case, a mild primary elastin defect can be proposed. Indeed, in the case of severe elastin defects, as for example observed in Marfan syndrome, aneurysm formation is typical (Milewicz *et al.*, 2005), but this is not observed in Friesians. A mild defect in elastin could result in increased load bearing on the smooth muscle cells, leading to hypertrophy. This has also been demonstrated in bovine and human Marfan syndrome (Scheck *et al.*, 1979; Potter and Besser, 1994). In the affected Friesian horses, the percentage area of smooth muscle actin in the mid-thoracic aorta was increased compared with the unaffected Friesian and warmblood horses. This can be explained by smooth muscle hypertrophy, as observed in the HE-stained tissue sections. This phenomenon could be induced by haemodynamic changes. Smooth muscle hypertrophy of the aorta has been associated with cyclic mechanical stress (Chiu *et al.*, 2013), as, for example, seen in induced hypertension (Owens, 1987). Interestingly, blood pressure values in horses vary between breeds. For example, Thoroughbreds generally have higher values compared with Standardbreds (Parry *et al.*, 1984). Therefore, the smooth muscle hypertrophy observed in the aorta of the affected Friesian horses could be secondary to increased blood pressure. Medial collagen deposits, as observed in the present study in non-affected and affected Friesians, have also been associated with hypertension (Rossi *et al.*, 2002). A sustained increase in blood pressure can lead to arterial wall thickening (Ghiadoni *et al.*, 1998). Affected Friesians, however, lack the typical collagen type III increase as well as the aortic wall thickening.

In conclusion, the results of the present study suggest that a breed-specific collagen defect with a mild elastin defect underlies the pathogenesis of aortic rupture in Friesian horses. These results will help to focus further genetic studies to unravel the pathogenesis of this intriguing disease.

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Conflict of Interest Statement

The authors have no affiliations with any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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