Measurement of plasma endothelin-1 concentration in healthy horses and horses with cardiac disease during rest and after exercise

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ABSTRACT. Cardiac biomarkers are important tools for monitoring disease progress and can monitor progression of therapy. Endothelin-1 (ET-1) has been studied for its use as a cardiac biomarker in human and small animal medicine while in horses with cardiac disease it has not been evaluated yet. The objective of the present study was to determine the concentration of plasma ET-1 in healthy horses and compare it with ET-1 concentration in horses with cardiac disease during rest and after exercise. Fifty four horses admitted to the Equine Clinic of Free University of Berlin were used in the present study, of which 15 horses were clinically healthy with no evidence of cardiac disease (Group 1), 22 horses suffered from cardiac disease with normal heart dimensions (Group 2) and 17 horses with cardiac disease and enlarged heart diameters (Group 3). Clinical examination, electrocardiography and echocardiography were performed. Endothelin-1 concentration was determined using ET-1 ELISA kit. The concentration of plasma ET-1 was significantly increased in horses with cardiac disease and normal cardiac dimensions (Group 2) and in horses with cardiac disease and enlargement of the left atrium (Group 3) compared to its concentration in clinically healthy horses (Group 1). In addition, the concentration of plasma ET-1 after exercise was significantly increased in diseased horses compared to its concentration at rest. Detection of ET-1 plasma concentration in horses at rest may be useful for detecting horses with changes in left atrial cardiac dimensions.

KEY WORDS: cardiac disease, endothelin-1, exercise, horses
exercise. Thus, the objective of the present study was to determine whether plasma ET-1 concentration in horses with cardiac disease was high compared to those of healthy horses and also to compare plasma ET-1 concentration in horses during rest and after exercise.

MATERIALS AND METHODS

Horses and clinical examination

Fifty four horses admitted to the Equine Clinic of Free University of Berlin, Germany were included in the present study. Sampling of horses affected by cardiac disease was not classified as animal experiments by the State Office of Health and Social Affairs Berlin (LaGeSo), sampling of control horses was approved (Reference No. L0294/13). The owners gave permission to involve their horses in the study. The horses included in the present study were presented with signs of poor exercise performance and severe respiratory distress after exercise.

On admission, the clinical examination was performed for all horses including history, animal description and identification, exercise performance level and animal usage. The breed, sex, age, body weight, lung and cardiac examination were recorded for each animal. Special examination of the cardiovascular system included inspection of mucous membrane, arterial and venous pulse, capillary refill time, auscultation of the heart and evaluation of abnormal heart sounds were performed. The lungs were examined by auscultation. Exercise tolerance test was performed for all horses during clinical evaluation in a special lunging yard. The test includes walk for 10 min, trot for 10 min and gallop for 5 min then followed by monitoring of respiratory and heart rates until returns to its normal level. The clinically healthy horse (Group 1, n=15) showed no signs of disease. The diseased horses showed signs of poor exercise performance with suspected cardiovascular disease. The diseased horses were classified according to further clinical examination of the heart into horses with heart valve insufficiencies and normal cardiac dimensions (Group 2, n=22) and horses with heart valve insufficiencies and enlarged cardiac dimensions (Group 3, n=17). Both group 2 and 3 have no signs of respiratory disease evidenced by normal lung sound on auscultation.

Echocardiographic and electrocardiographic examinations

Echocardiographic examination was performed in all horses and included standardized right and left parasternal B-Mode and colour flow Doppler images [30] and right parasternal short axis M-Mode images [14] using ultrasound unit (Vivid 7, GE Medical Systems, Horton, Norway) equipped with a 3.5 MHz annular phased array probe, maximal depth of 30 cm. The images were recorded digitally as cineloops. In addition, the cardiac cycles of echocardiographic examination were stored for later analysis. Offline analysis was performed by the same experienced veterinarian using Clinical Workstation Software (EchoPAC Software, Version 7.0, GE Healthcare, Horton, Norway) including measurements of cardiac dimensions and calculation of fractional shortening percent (FS%) in 3 consecutive heart cycles [31]. Mean ± SD for each variable was calculated from the 3 values. Measurements were performed at the end of diastole with beginning of QRS complex [21]. According to previous studies by Gehlen, Sundermann, Rohn and Stadler [12], Stadler and Robine [31] a left atrium and left ventricle end diastolic diameter ≤135 mm was considered normal. In all horses, the electrocardiographic examination was performed by ECG integrated into the ultrasound machine parallel with echocardiographic examination. In horses with cardiac arrhythmias, a Holter ECG (Televet 100, ECG & Holter) was performed. Horses were excluded, if complete examination revealed only arrhythmia.

Blood sample collection and processing

Blood samples were collected at rest and immediately after exercise by jugular venipuncture with pre-cooled polypropylene syringes (B-Braun, Melsungen AG, Germany). Four millilitres of blood was transferred to pre-cooled EDTA tubes (Sarstedt, Nürnberg, Germany, 1.6 mg EDTA/ml blood) with 0.2 ml aprotinin to improve sample stability and cooled on ice until centrifugation. Blood samples were centrifuged in a Table Top Refrigerated Centrifuge Hermle Z326K (Hermle Labortechnik GmbH, Germany) at 1,500 rpm for 15 min under 4°C. Plasma samples were stored at −80°C until assayed. Plasma samples were used for determination of endothelin-1 concentration.

Determination of ET-1 concentration in plasma

The concentration of plasma ET-1 was determined using a commercially available sandwich ELISA (BI-20052, Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria), which has been validated for use in the horse by Costa, Eades, Venugopal and Moore [6] and, the validation included inter- and intrassay variability. All materials and buffers were supplied with the kit, and the assay was performed following the manufacturer's instructions. The concentration of the plasma ET-1 was expressed as pg/ml.

Data analysis

Data were analyzed by the use of commercially available software (SPSS 20.00 for windows). The level of significance was set at P<0.05 and data was expressed as mean ± SD. Data was examined for normal distribution by Shapiro Wilks test. As normal distribution was lacking, non-parametric tests were used for further analyses. Comparison between ET-1 plasma concentrations at rest and after exercise in clinically healthy and diseased horses was performed by the use of Wilcoxon signed rank test for paired sample. Furthermore the comparison of ET-1 plasma concentration between the clinically healthy and diseased horses was performed by Kruskal-Wallis test. A post-hoc analysis (Dunn-Bonferroni) was performed for pairwise comparisons following significant Kruskal-Wallis test.
RESULTS

The present study was performed using 54 horses, of which 15 horses were clinically healthy (Group 1), without signs of disease. Thirty nine horses showed signs of poor exercise performance with suspected cardiovascular disease. The diseased horses were classified according to further clinical examination of the heart into horses with heart valve insufficiencies and normal cardiac dimensions (Group 2, n=22) and horses with heart valve insufficiencies and enlarged cardiac dimensions (Group 3, n=17). Both group 2 and 3 have no signs of respiratory disease evidenced by normal lung sound on auscultation. The study population were comprised 31 geldings, 20 mares and 3 stallions of various breeds including warm bloods (n=34), trotters (n=11), thoroughbreds (n=4), Arabian horses (n=3), quarter horse (n=1) and Criollo (n=1). The average age was 16 years and mean weight was 511 ± 75 kg (Table 1). Horses were used for leisure riding (n=27), show horses (n=9), racehorses (n=2) or were not ridden at all (n=16). According to the owners´ declarations, 20 horses were considered as trained and 32 as untrained. In 2 cases owner did not specify fitness level of their horse.

Cardiovascular examination

The clinically healthy horses showed normal heart rates (Table 1) and normal heart auscultation. Cardiovascular abnormalities were detected in diseased horses (Group 2 and 3) included pallor of mucous membranes, prolonged refill time, irregular arterial pulse and jugular distension. Auscultation of the heart revealed cardiac murmurs (median intensity, 3/5).

Echocardiographic and electrocardiographic examination

In clinically healthy horses (Group 1), the echocardiographic examination revealed no evidence of cardiac disease. Heart valve insufficiencies were diagnosed in 39 out of 54 horses. Most frequently aortic valve insufficiency (AVI) (n=13) or combined AVI and mitral valve insufficiency (MVI) was found (n=7). MVI was diagnosed in 4 horses, tricuspid valves insufficiency (TVI) in 2 horses and pulmonary valve insufficiency (PVI) in one horse. In 9 horses MVI in combination with TVI or PVI was diagnosed. Animal grouping was based mainly on the characteristics of echocardiographic findings. Twenty-two horses with heart valve insufficiencies had normal cardiac dimensions (Group 2) while enlarged cardiac dimensions were found in 17 horses (Group 3). In detail, enlargement of the left ventricle (LV) was found in 9 horses, dilation of the left atrium (LA) in 4 horses, and a combination of both in 1 horse. One horse with LA dilation additionally showed enlarged RA and RV dimensions. In 3 cases solely RA and/or RV dilation was found (Table 2). ECG revealed atrial fibrillation (AF) in 5 horses diagnosed with cardiac valve insufficiencies.

Table 1. Age, sex, body weight and heart rates of horses under study

|                    | Group 1 | Group 2 | Group 3 |
|--------------------|---------|---------|---------|
| Number of horses   | 15      | 22      | 17      |
| Gender             | Mare    | 9       | 7       | 4       |
|                    | Stallion| 1       | -       | 2       |
|                    | Gelding | 5       | 15      | 11      |
| Age (years)        | 14 ± 7  | 16 ± 8  | 18 ± 8  |
| Body weight (kg)   | 516 ± 89| 507 ± 69| 545 ± 77|
| Heart rate (bpm)   | 39 ± 6  | 37 ± 6  | 41 ± 12 |

Data was expressed as mean ± SD.

Table 2. Two-dimensional and M-Mode echocardiographic measurements of right and left atrial and ventricular dimensions

|                              | Group 1 | Group 2 | Group 3 |
|------------------------------|---------|---------|---------|
| B-Mode right-parasternal four-chamber long-axis view |         |         |         |
| RADd (cm)                    | 6.9 ± 0.1| 10 ± 0.1| 14.7 ± 0.1|
| RVdd (cm)                    | 7.9 ± 0.8| 7.9 ± 1.1| 8.4 ± 0.9|
| LADd (cm)                    | 9.7 ± 1.3| 9.7 ± 1.3| 10.9 ± 1.5|
| LVDd (cm)                    | 11.6 ± 1.2| 12.0 ± 0.6| 13.6 ± 1.7|
| B-Mode left-parasternal long-axis view |         |         |         |
| LADd (cm)                    | 8.4 ± 1.1| 11.2 ± 1.4| 12.9 ± 1.6|
| LVDD (cm)                    | 10.3 ± 1.0| 12.1 ± 0.9| 13.5 ± 1.9|
| M-Mode echocardiography of the left ventricle (right-parasternal long axis) |         |         |         |
| LVDd (cm)                    | 11.1 ± 1.3| 11.5 ± 0.9| 13.8 ± 2.6|
| M-Mode echocardiography of the left ventricle (right-parasternal short axis) |         |         |         |
| LVDd (cm)                    | 11.6 ± 1.3| 12.2 ± 0.8| 14.0 ± 2.9|

Data was presented as mean ± SD. RADd, right atrial diameter measured at end-diastole; RVdd, right ventricular diameter measured at end-diastole; LADd, left atrial diameter measured at end-diastole; LVDd, left ventricular diameter measured at end-diastole.
**Endothelin-1 plasma concentration**

The results of the plasma concentration of ET-1 were presented in Table 3. At rest, the plasma concentration of ET-1 was significantly increased in horses with cardiac disease and normal cardiac dimensions (Group 2) (4.23 ± 3.45 pg/ml, P value 0.009) and in horses with cardiac disease associated with enlargement of the left atrium (Group 3) (8.54 ± 2.82 pg/ml, P value 0.012) compared to its concentration in clinically healthy horses (Group 1) (0.94 ± 0.45 pg/ml). On the other hand, the results of paired samples comparison using Wilcoxon signed rank test showed significant increase in the plasma ET-1 concentration after exercise compared to its concentration at rest in diseased horses (Table 3), although the results of ET-1 after exercise in healthy horses showed no significant difference from that before exercise.

**DISCUSSION**

The concentration of endothelin-1 in plasma of healthy horses has been studied during rest [2, 6], after exercise [15, 16] and in horses with recurrent airway obstruction [2, 6]. To our knowledge, this was the first study to evaluate ET-1 plasma concentration in horses with cardiac disease during rest and after exercise.

The results of the present study indicate that horses with cardiac disease and normal cardiac dimensions and horses with cardiac disease associated with enlargement of the left atrium showed higher plasma ET-1 concentration compared to its concentration in clinically healthy horses. In addition, the ET-1 concentration was significantly increased in diseased horses after exercise testing compared to its concentration at rest. This was in agreement with previous study by McKeever, Antas and Kearns [15] who found an increased plasma ET-1 concentration in healthy horses after standardized submaximal treadmill exercise test.

ET-1 is regarded as an important factor in the pathophysiology of chronic heart disease [3]. It is largely synthesized in vascular smooth muscle cells, cardiac myocytes and fibroblasts [13], and ET-1 receptors are numerously distributed in myocardial and endocardial cells and in cardiac conduction system [26]. Endothelin contributes to cardiac remodeling [18], which leads to deterioration of cardiac function and clinically apparent cardiac insufficiency [5].

The mean plasma ET-1 concentration in clinically healthy horses was within the range of the previously reported results by other authors [2, 6, 15, 16]. Radioimmunoassay was used by Benamou, Art, Marlin, Roberts and Lekeux [2], Nagy, Solti, Kulcsar, Reiczigel, Huszenicza, Abavary and Wolfing [19], McKeever and Malinowski [16] and McKeever, Antas and Kearns [15]. While ELISA technique that reported in the present study was reported by Costa, Eades, Venugopal and Moore [6]. The ET-1 is unstable at room temperature [27], continuous cooling of blood samples until freezing at −80°C seems to be of great importance [20, 32].

In humans with severe CHF increased ET-1 plasma concentration was found [38] and ET-1 plasma concentration was found to be correlated with cardiac functional parameters like LV ejection fraction, LV end diastolic volume or mitral E/A ratio [38]. Also, it is believed that ET-1 is an independent prognostic marker for mortality [35, 38] and predictor for survival [35]. Similar findings in dogs and cats indicate a relation between ET-1 plasma concentration and severity of cardiac disease in these species. In dogs and cats with cardiac disease ET-1 plasma concentration is correlated with disease severity and atrial size [24, 32]. In dogs as well as in cats with CHF plasma ET-1 concentration is increased compared to healthy animals and animals with heart disease but without CHF [24, 25]. In dogs with dilated cardiomyopathy, plasma ET-1 concentration seems to have prognostic value, as chronically increased ET-1 goes along with shorter survival time [20].

In accordance with human studies [7, 23], the body weight, gender and fitness level might influence the results of plasma ET-1 concentration. Great difference in body weight might complicate the comparison of echocardiographic measurements because of linear relation between body weight and heart dimensions measured echocardiographically [28]. Also breed and fitness level are known to influence echocardiographic measurements and complicate comparability [10, 12]. This might limit the results of the present study and might be interpreted with caution. It is concluded that, plasma ET-1 concentration in horses at rest may be useful for detecting horses with changes in left atrial cardiac dimensions. Further investigations are required to determine the value of plasma ET-1 as a biomarker for cardiac diseases in horses.

**Table 3.** Concentration of plasma endothelin-1 pre and post exercise (pg/ml)

| Group         | Pre-exercise     | Post-exercise    | Wilcoxon rank test significance |
|---------------|------------------|------------------|---------------------------------|
| Group 1       | 0.94 ± 0.45<sup>a</sup> (0.1–1.7) | 1.07 ± 0.52<sup>a</sup> (0.32–2.1) | 0.092 |
| Group 2       | 4.23 ± 3.45<sup>b</sup> (1–9.01) | 5.42 ± 3.74<sup>b</sup> (0.95–9.1) | 0.008 |
| Group 3       | 8.54 ± 2.82<sup>c</sup> (2.56–10.1) | 9.95 ± 2.72<sup>c</sup> (2.17–10.1) | 0.005 |

Kruskal-Wallis test significance 0.000

Data is expressed as mean ± SD (Min.–Max.). Means with different superscripts within column are different (P<0.05).

ACKNOWLEDGMENTS. We would like to acknowledge all staff members of Equine Internal Medicine, Equine Clinic, Free University of Berlin, Berlin, Germany.
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