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Serum total antioxidant capacity and enzymatic defence of dogs with chronic heart failure and atrial fibrillation: a preliminary study

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Abstract

Introduction: Atrial fibrillation may potentially contribute to oxidative stress to a greater extent than chronic heart failure. The aim of the study was to compare the serum total antioxidant capacity and enzymatic antioxidant defence of dogs with chronic heart failure and atrial fibrillation with those of subjects with chronic heart failure and sinus rhythm and healthy controls. **Material and Methods:** A total of 33 dogs were divided into three groups: dogs with chronic heart failure and atrial fibrillation (CHF + AF; n = 12), chronic heart failure and sinus rhythm (CHF + SR; n = 9), and healthy controls (n = 12). Serum total antioxidant capacity (TAC), serum CuZn-superoxide dismutase (SOD) and catalase, and plasma glutathione peroxidase (GPx) activity were determined. **Results:** SOD activity and serum TAC were significantly lower in the study groups than in control animals. Catalase activity was significantly higher and plasma GPx activity significantly lower in dogs with CHF + AF compared with the CHF + SR and control dogs. **Conclusion:** The results suggest that chronic heart failure in dogs significantly impacts the serum TAC and the antioxidant enzymatic defence, while plasma GPx activity is markedly lower in dogs with chronic heart failure and atrial fibrillation. The role of that imbalance needs further investigation.

Keywords: dogs, cardiology, atrial fibrillation, chronic heart failure, oxidative stress.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia in dogs and is characterised by rapid and disordered atrial electrical activity leading to a fast ventricular response and, as a result, low cardiac output and heart failure (19, 20). It usually develops as a result of a left atrial enlargement secondary to structural heart disease. However, lone AF, *i.e.* atrial fibrillation without apparent heart disease, is also frequently noted. The condition mostly affects large and giant breeds due to their large atrial mass (13). Arrythmia is most often caused by the structural and electrical remodelling of the atria. Current studies focus on the role of other factors such as calcium overload, microRNAs, inflammation, and oxidative stress in the initiation and perpetuation of atrial fibrillation.

Oxidative stress is defined as a loss of balance between pro-oxidants and the antioxidant defence, resulting in an excessive production of reactive oxygen species (ROS). Their deleterious action induces lipid and protein peroxidation and nuclear DNA damage at a cellular level, ultimately leading to impaired cardiac contractile function, fibrosis resulting from fibroblast stimulation, and activation of the pathways responsible for pathological myocardial remodelling (26). The major systemic antioxidant defence includes superoxide dismutase (SOD), which converts superoxide radical anions (O_2^-) into H_2O_2, later detoxified into water by catalase (CAT) and glutathione peroxidase (GPx) (12). This group of enzymes acts both intra- and extracellularly, playing a pivotal role in protecting the system from ROS-induced injury. It was shown that SOD and CAT overexpression in desmin-

deficient mice hearts decreased intracellular ROS, minimised myocardial degeneration, and markedly improved cardiac function (22). Various *in vivo* models have repeatedly proven that dietary supplementation with compounds possessing antioxidative properties, such as vitamins or polyphenols, may reduce the oxidative burden in heart failure (18). Alpha-tocopherol supplementation ameliorated the progression of heart failure in many animal models; however, human clinical trials failed to show positive effects (24). The administration of vitexin, an apigenin flavone glycoside, significantly reduced the myocardial infarct size and enhanced SOD activity in a rat ischaemia/reperfusion injury model, while resveratrol was found to alleviate oxidative damage by increasing the activity of SOD, CAT, and GPx in cisplatin-treated rats (6, 28).

Atrial fibrillation may potentially contribute to enzymatic antioxidant defence imbalances and oxidative stress to a greater extent than chronic heart failure (CHF) with a sinus rhythm (SR) (29). This may be due to a markedly increased heart rate, a lower cardiac output and a greater degree of atrial tissue remodelling. Therefore, this study aims to evaluate the serum total antioxidant capacity (TAC) and antioxidant defence of dogs with advanced CHF + AF in comparison to subjects with CHF + SR and healthy controls.

Material and Methods

Animals and blood sample preparation. A total of 33 dogs, patients of the Faculty's Cardiology Service, were qualified for the study. Twelve dogs were used as controls because they were apparently healthy following a detailed anamnesis, physical examination, a 6-lead ambulatory electrocardiographic examination, a transthoracic echocardiography, and a routine blood evaluation (complete blood count, albumin, alanine transaminase, aspartate transaminase, total protein, total bilirubin, cholesterol, phosphorus, glutamate dehydrogenase, glucose, creatinine, urea, chloride, sodium, potassium, and calcium contents). Two-dimensional, M-Mode and Doppler imaging modes were used to obtain images from standard echocardiographic views, including right parasternal short- and long-axis and apical four- and five-chamber views. Left atrium to aorta ratio (LA/Ao) was measured in the right parasternal short axis transaortic view, while left ventricular M-Mode measurements were taken from the right parasternal long axis 4-chamber view. The same examination protocol together with chest radiographs and, where necessary, further diagnostic procedures to exclude any other systemic diseases, was carried out on animals assigned to the study groups. Based on the results, the animals were divided into two study groups: dogs with CHF + AF (n = 12) and dogs with CHF + SR (n = 9). Chronic heart failure was diagnosed based on the presence of modified New York Heart Association (NYHA) class III or IV disease signs together with data collected from clinical and additional examinations confirming or discounting atrial fibrillation with a fast ventricular rate (8). Dogs with any systemic diseases, as well as those with more than a twofold elevation in urea or hepatocellular enzyme activity were excluded from the study.

Tachycardiomyopathy was defined as persisting AF with fast ventricular response and preserved left ventricular systolic function and pathological remodelling of the heart characteristic of sustained tachycardia, without clinical, echocardiographic or radiographic evidence of cardiopulmonary disease, or other identifiable cause of the arrhythmia. Patent ductus arteriosus (PDA) was diagnosed by significant ductal flow with left-to-right shunt on colour and continuous-wave Doppler echocardiographs, together with clinical signs of the condition. Mitral valve disease (MVD) and dilated cardiomyopathy (DCM) were diagnosed based on consensus guidelines (1, 7). The group of dogs suffering from CHF was additionally subdivided into animals undergoing pharmacological treatment for the underlying medical condition (n = 13) and animals without cardiac treatment (n = 8).

All animals were fasted at least 12 h before blood sampling. Blood samples were drawn into EDTA and plain sample tubes from a peripheral vein using a 21-gauge needle as part of a routine clinical evaluation. EDTA tubes were centrifuged immediately at 2,000 × g at 4°C for 15 min and the plasma was removed. To separate the serum, blood was allowed to clot for a minimum of 15 min at room temperature. It was then centrifuged and the serum was decanted. The obtained material was aliquoted and immediately frozen at −80°C until analysis. All samples were measured within four weeks of blood collection.

Determination of TAC and antioxidant enzyme activity. Commercially available kits were used to determine serum TAC, CuZn-SOD, CAT, and plasma GPx (all from Cayman Chemical, Ann Arbor, MI, USA) activity, according to the manufacturer's protocol with some modifications. Cayman Chemical does not define the species specificity of those assays because the enzymes of which the activity is measured are conservative. These assays had been validated for use in dogs by measuring parallelism and dilution linearity. The serum was diluted at 1:1.5 for TAC and 1:5 for SOD, while CAT activity was determined in undiluted serum. The EDTA–plasma solution was diluted at 1:20 for a GPx assay. Coefficients of variation were as follows: 19.74% for CAT, 22.57% for SOD, 15.63% for GPx, and 8.95% for TAC. The absorbance of the coloured reaction products was measured spectrophotometrically using a Spark 10M microplate reader (Tecan, Grödig, Austria). All the samples were assayed in the same assay run in duplicate and the average was used in calculations.

Statistical analysis. All the data were subjected to statistical analysis using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) and Statistica 13 (Statistica, now Tibco, Palo Alto, CA, USA). A Shapiro–Wilk normality test was used to assess the distribution of the data. The variables with a skewed distribution, excluding LA/Ao and left ventricular internal diameter in diastole, normalised (LVIDDN), were ln transformed to normality. A one-way analysis of variance with the Tukey *post hoc* test was used to determine statistically significant differences between the groups. As the groups' age and body weight differences were not the subject of our study, these variables were ln transformed to normality and multivariable linear regression analyses followed by analyses of covariance were conducted to remove the possible bias of these variables. The unpaired *t*-test was used to compare two sets of data. The intergroup differences of LA/Ao and LVIDDN were calculated with non-parametric Kruskal–Wallis analysis followed by Dunn's multiple comparisons *post hoc* test. The mean ± standard deviation (SD) was calculated for normally distributed data and the median (range) was calculated for skewed data. $P < 0.05$ was considered significant.

Results

A total of 33 dogs, 20 of which were males, were included in the analysis. The mean (± SD) age across all the groups was 8.45 ± 3.31 years, and the median (range) weight was 24.6 (6.5–74) kg. In the control group, there were two beagles, two border collies, two Nova Scotia duck tolling retrievers, two German pointers, and a mixed-breed dog, an Alaskan Malamute, a Polish greyhound, and a Bernese mountain dog. The CHF + SR group included four mixed-breed dogs, two bull terriers, two Cavalier King Charles spaniels, and a Pekingese. In the CHF + AF group there were five German shepherds, two mixed-breed dogs, and a dachshund, a golden retriever, an Irish wolfhound, a St. Bernard, and a German pointer. The general characteristics of the study groups are summarised in Table 1.

The subjects in the CHF + SR group were diagnosed with advanced MVD (n = 6) or DCM (n = 3). The arrhythmia in the patients with atrial fibrillation occurred as a result of left atrial enlargement secondary to end-stage MVD (n = 3), DCM (n = 3) or PDA (n = 2). Four patients were diagnosed with tachycardiomyopathy.

The serum activity of all analysed enzymes differed among the groups. SOD activity was significantly lower in the study groups (CHF + AF 1.13 ± 0.32; CHF + SR 1.14 ± 0.17 U/mL) than in the control animals (1.54 ± 0.32 U/mL; $P < 0.001$) (Fig. 1). In the multivariable linear regression model, body weight was significantly associated with CAT, whereas there were no associations between body weight and SOD, GPx, or TAC. There were also no associations between age and studied oxidative stress parameters. CAT activity after adjustment for body weight was significantly higher in dogs with CHF + AF (13.98, 95% confidence interval (CI):12.44–15.52, $P < 0.001$) and only by a small margin not significantly higher in dogs with CHF + SR (13.84, 95% CI:12.1–15.58, $P = 0.06$) than in the control group (11.12, 95% CI: 9.7–12.54). Plasma GPx activity was significantly lower in the CHF + AF group (1,535.24 ± 260.44 nmol/min/mL) than in the CHF + SR group (1,933.1 ± 380.93 nmol/min/mL) and in the healthy controls (2,173.05 ± 622.64 nmol/min/mL; $P < 0.0001$) (Fig. 2). Serum TAC did not differ significantly among the three groups. However, it was significantly lower in the merged group of diseased animals (0.29 ± 0.038 mM Trolox) compared to healthy controls (0.312 ± 0.015 mM Trolox; $P < 0.05$) (Fig. 3).

Table 1. Demographic, echocardiographic, and treatment characteristics of the study groups. Statistical significance of the intergroup differences is shown in the superscript letters

Variable	CHF + AF	CHF + SR	Control	P value
total number	12	9	12	-
age (yrs)	8.15 ± 3.58	10.84 ± 1.88^{A}	6.96 ± 2.83^{B}	P < 0.05
body weight (kg)	37.41 ± 20.51^{B}	16.3 ± 9.18^{C}	21.54 ± 9.61	P < 0.05
sex (male/female)	8/4	7/2	5/7	ns
LA/Ao	2.42 (1.68–3.92)^{A}	2.29 (2.01–3.29)^{A}	1.4 (0.97–1.48)^{B,C}	P < 0.0001
LVIDDN	2.2 (1.92–3.27)^{A}	2.2 (1.99–2.43)^{A}	1.52 (1.39–1.61)^{B,C}	P < 0.0001
HR	228.08 ± 34.34^{A,B}	142 ± 29.81^{C}	105.33 ± 13.68^{C}	P < 0.0001
NYHA class III	4/12	4/9	0/12	
NYHA class IV	8/12	5/9	0/12	
cardiac treatment	8/12	5/9	0/12	
Drugs				
ACE-I	6/12	5/9	0/12	
spironolactone	6/12	5/9	0/12	
digoxin	3/12	0/9	0/12	
loop diuretics	8/12	5/9	0/12	
β-blockers	3/12	0/9	0/12	

LA/Ao – left atrium to aorta ratio; LVIDDN –left ventricular internal diameter in diastole (normalised) according to Cornell *et al.* (4); HR – heart rate; NYHA – New York Heart Association; A – $P < 0.05$ *vs*. controls; B – $P < 0.05$ *vs* CHF + SR; C – $P < 0.05$ *vs* CHF + AF

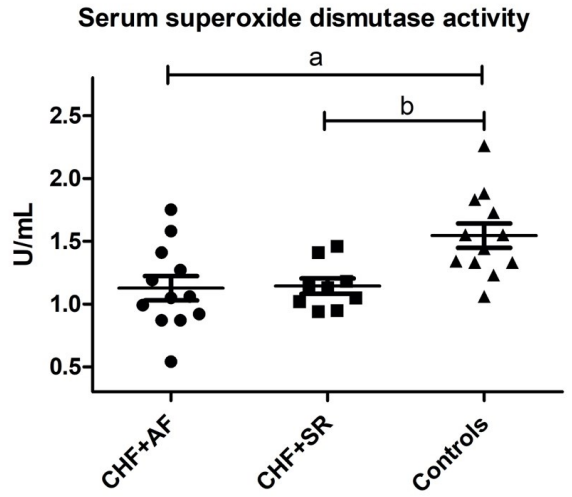

Fig. 1. Comparison of the serum superoxide dismutase activity among the three groups. a – $P < 0.01$; b – $P < 0.05$

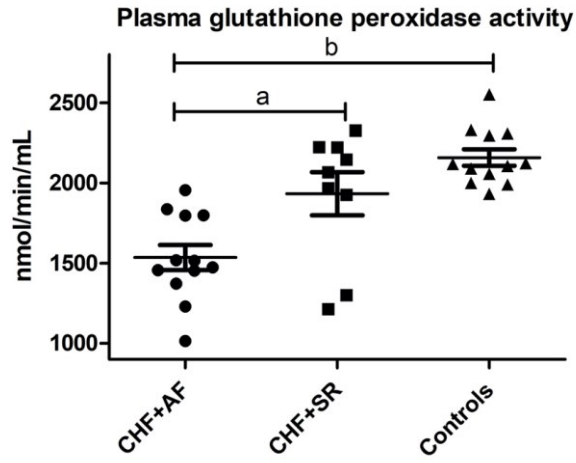

Fig. 2. Comparison of the plasma glutathione peroxidase activity among the three groups. a – $P < 0.05$; b – $P < 0.001$

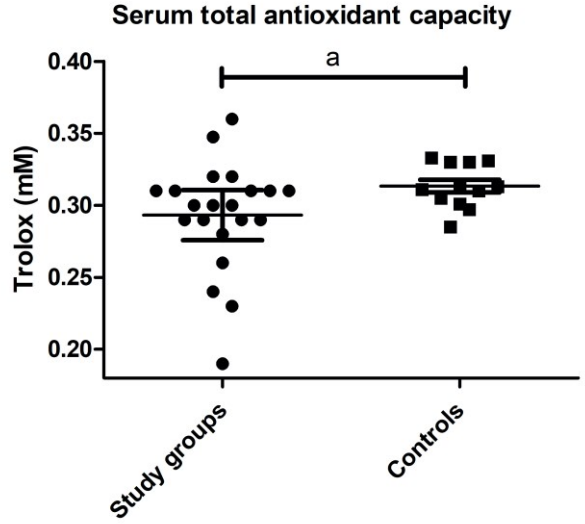

Fig. 3. Comparison of the serum total antioxidant capacity between cardiac patients (merged CHF + AF and CHF + SR groups) and healthy controls. a – $P < 0.05$

In addition, the dogs suffering from cardiac disease were divided into subgroups of untreated and treated dogs. However, no significant differences were observed in either TAC or enzymatic activity between these subgroups.

Discussion

We compared the antioxidant status of dogs affected by CHF + AF with subjects with CHF + SR and healthy controls. To the best of the authors' knowledge, this is the first report comparing the antioxidant enzymatic profile in dogs with chronic heart failure with and without atrial fibrillation.

In mammals, superoxide dismutase is present in three isoforms: cytosolic, mitochondrial, and extracellular copper-zinc tetrameric, the last also known as SOD3 (17). Superoxide dismutase is a key antioxidative biomarker and its level is routinely assessed in human patients with heart failure in various samples, including blood serum. The majority of human studies show an inverse relationship between SOD activity and chronic heart disease outcome (9). Its activity was also shown to be decreased in the cardiac tissue of dogs with experimentally induced chronic volume overload (21). The results of the current study are in agreement with these findings and show that oxidative stress was significantly enhanced in both study groups. Another study, however, showed no differences in the SOD activity measured in washed erythrocytes between dogs with idiopathic DCM and healthy controls (10). It is important to emphasise, that enzymatic activity will differ depending on the studied biological material and activity intensities in dissimilar sample types should not be directly compared. In addition, the SOD activity did not differ between dogs with CHF + AF and those with CHF + SR.

Catalase is primarily an intracellular enzyme mainly found in erythrocytes and hepatocytes. Nonetheless, its serum activity has been widely linked to oxidative stress in both humans and dogs (15). To date, no published reports exist on the catalase activity in cardiovascular disease in dogs. In humans, both the catalase protein levels and the catalase activity in heart muscle tissue were found to be significantly increased in end-stage heart failure due to DCM and ischaemic cardiomyopathy compared with healthy control hearts (5). This is in line with our results, which showed an increasing trend of catalase activity, where the lowest values were obtained in healthy controls, and the highest values were noted in subjects with atrial fibrillation. A possible reason for this phenomenon may be a compensatory mechanism that adapts to the overproduction of reactive oxygen species and the need for increased scavenging activity, and it seems particularly credible in light of the significantly decreased GPx activity in the same study group. Nonetheless, as catalase is mainly found intracellularly,

we also hypothesised that the increased serum activity might merely reflect ongoing cardiomyocyte damage in the course of atrial fibrillation. What is more, it has been shown that the catalase activity in the red blood cells of patients having undergone resynchronisation therapy was significantly lower than the activity before the procedure, which could be attributed to the reduced level of oxidative stress and improved cardiac function after the therapy (16). Decreased catalase activity was also seen in patients with refractory heart failure with clinical improvement after five days of intensive therapy, compared to their pre-therapy catalase activity levels (3). We failed to show such a relationship between dogs before cardiac treatment and those which had been treated pharmacologically. However, it should be borne in mind that this study was not specifically designed to address this issue, as our patients had pharmacological protocols individually tailored to their needs and thus they were not unified.

Glutathione peroxidases are a family of selenoproteins that exert their antioxidant effect by reducing hydroperoxide to water, using glutathione as a reducing substrate. Its main isoforms are found in mitochondria and cytosol, the intestinal epithelium, cellular membranes, and plasma (2). The last of these isoforms, also known as GPx3, was measured in this study. GPx activity was intensively studied in human patients with cardiovascular disease and yielded discrepant results (9). In dogs, its activity decreased in experimentally induced mitral regurgitation, while no differences were reported between its activity in the whole blood of dogs with cardiovascular disease and that in the whole blood of healthy controls (21, 27). As we found no differences between the healthy dogs and the CHF + SR group, our findings are consistent with the latter study. Interestingly, one study found a significant increase in the GPx concentration in the red blood cells of dogs with idiopathic DCM compared to healthy controls, where 6 out of 12 dogs in the DCM group suffered from atrial fibrillation (10). However, this finding cannot be fully compared to the results of our study, as we measured the catalytic activity of a different isoform of the enzyme. Nonetheless, we found that the plasma GPx3 activity was significantly decreased in dogs with atrial fibrillation. This may be explained by an overproduction of reactive oxygen species and a depletion of antioxidant reserves, possibly including a substrate for the reaction, *i.e.* glutathione.

The TAC assay is designed to measure different elements of the antioxidant defences of a system and their ability to withstand an oxidative assault. It has been widely used to estimate the antioxidant status of animals with cardiovascular disease and has delivered conflicting results. A mixed group of dogs with CHF secondary to MVD and DCM were found to have significantly higher plasma TAC compared to healthy controls (11). Another study yielded similar results, proving that dogs suffering from MVD and DCM have significantly higher TAC than healthy subjects, measured according to the ferric reducing ability of the plasma. However, the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method failed to show this dependence in the same study group (14). It may, therefore, be concluded that the method used to estimate TAC may markedly influence the results, as each of them measures different components of the complex antioxidant defence system (23). Moreover, Svete *et al.* (25) found significantly lower plasma TAC measured using a Trolox assay in a group of dogs with asymptomatic cardiovascular disease than in healthy controls. Interestingly, no differences were found between dogs with heart failure and healthy dogs. In our study, utilising a Trolox equivalent antioxidant capacity assay, animals suffering from cardiac disease were found to have significantly lower TAC than controls. It indicates that cardiac patients are exposed to a significantly greater degree of oxidative damage and subsequent depletion of antioxidant reserves.

This study has some limitations. First and foremost, the study groups are rather small and heterogeneous, hence further investigations are recommended to confirm the results observed in this preliminary study. Furthermore, the differences in the age and weight of the patients may also have impacted the results. This could not be prevented due to the specificity of the examined medical condition.

In conclusion, the results of the study suggest that chronic heart failure in dogs significantly impacts the serum total antioxidant capacity and the antioxidant enzymatic defence, while plasma glutathione peroxidase activity is markedly lower in dogs with CHF + AF in our study conditions. However, the role of that imbalance in the pathogenesis of the disease is not clear and needs further investigation. Further research should focus on testing the levels of oxidation damage products. It would also be worth testing whether boosting the antioxidant defence would ameliorate progression of the disease and its clinical outcome.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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