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Original article

Serum paraoxonase 1 activity and lipid metabolism parameter changes in Dachshunds with chronic mitral valve disease. Assessment of its diagnostic usefulness

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Abstract

Chronic mitral valve disease, which is frequently diagnosed in Dachshunds, leads to structural, hemodynamic and redox state changes in dogs. The aim of this study was to investigate serum paraoxonase 1 (PON1) activity and lipid metabolism in different disease stages. Standardized PON1 activity (PON1/HDL ratio) was lower in asymptomatic dogs, B1 and B2 Stages when compared to healthy ones and symptomatic Dachshunds in Stage C (ACVIM classfication). PON1 paraoxonase activity was elevated in Stage C dogs, with no changes found in PON1 activity towards phenyl acetate. Dachshunds in Stage B2 and C showed increased triglyceride levels, with no changes in cholesterol and lipoprotein concentration in comparison to healthy ones.

Our data suggest that standardized PON1 activity changes could be used in laboratory diagnostics to differentiate the CMVD of affected asymptomatic (Stage B1 and B2) dogs from healthy (Stage A) and clinically affected (Stage C) dogs. Also, a standardized PON1 activity increase might be a prognostic progression signal of the disease to Stage C.

Key words: PON1, chronic mitral valve disease, Dachshunds

Introduction

Chronic mitral valve disease (CMVD), also known as myxomatous mitral valve disease (MMVD) or degenerative valve disease, is the most common canine heart disease, accounting for more than 70% of all cardiovascular diseases in dogs (Parker and Kilroy-Glynn 2012). The disease is frequently seen in the Dachshund, a very popular breed in Poland (Grancarz et al. 2013). Along with Cavalier King Charles Spaniels and Cocker Spaniels, these dogs are at high risk of CMVD development (Lewis et al. 2011). In Dachshunds, as in other predisposed breeds, the disease severity is age related (Guglielmini 2003). The average onset of clinical CMVD occurs at 11-12 years (Garncarz et al. 2013). Disease progression in dogs leads to mitral valve regurgitation in systole, causing heart murmurs over the mitral valve area and, in severe cases, congestive heart failure (CHF) (75% of CHF causes). Clinical diagnostic evaluation of the

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disease, including echocardiography, enables assessment of disease severity according to the American College of Veterinary Internal Medicine (ACVIM) classification scheme.

Morphological and hemodynamic changes in CMVD are associated with oxidative stress and the formation of reactive oxygen species (Freeman et al. 2005, Nishijima et al. 2011). Paraoxonase 1 (PON1) is one of the enzymes involved in redox changes (Kulka 2016) associated with high density lipids (HDL) (Gugliucci et al. 2013). Its antioxidave properties are being intensively examined in human atherosclerosis, diabetes mellitus and familial hypercholesterolemia (Van Himbergen et al. 2005, Soran et al. 2009, El-Lebedy et al. 2014). PON1 addition inhibited induced oxidation of low density lipoproteins (LDLs) in vitro, suggesting a protective role against free oxygen species (Aviram M 1999). PON1 is considered a negative acute phase protein (APP(-)) (Van Lenten et al. 1995), with concentrations decreasing in dogs with pancreatitis and babesiosis (Rossi et al. 2014, Tvarijonaviciute et al. 2015). However, there is minimum information about PON1 changes in canine heart disease, specifically CMVD in Dachshunds. The aim of this study was to examine serum PON1 activity and lipid parameter changes in different ACVIM Stages of CMVD in Dachshunds. Possible PON1 laboratory diagnostic applications were also investigated.

Materials and Methods

Animals

A prospective study was performed on 57 purebred, albeit without pedigrees, Dachshunds (24 males, 33 females) over a 1 year period at the Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences. The dogs were aged 10-176 months, weighing 9.2±2 kg, with a body condition score (BCS) of 4-6 on a scale of 1-9 (Laflamme 1997). A written consent from the owner of each animal was obtained and the study complied with National and Institutional Guidelines on the use of animals in clinical research according to the Polish Legal Act (Dz. U. z 2005 r. Nr 33, poz. 289 z późn. zm.) concerning experiments performed on client owned animals. A high standard of care was applied during the whole clinical examination. All animals were fasted for 12 hours prior to blood collection. All examinations were performed at rest without pharmacological restraint. Dogs with any co-morbidities were excluded from the experiment.

Echocardiography, ECG, radiography

A transthoracic echocardiographic (TTE) examination was performed on all dogs (Aloka 4000, sector transducers 2.5-7 mHz). Examinations were performed according to published standards (Kittleson and Kienle 1998A, Tilley et al. 2008). In some cases the examination was carried out on dogs in a standing position (Kittleson and Kienle 1998A, Haggstrom et al. 2005). The left ventricular diameter in diastole (LVDd) and systole (LVDs) was measured and the dynamic motion of the left ventricular walls was assessed using M-mode in the right parasternal short axis view at the level of the papillary muscles. The aortic diameter (Ao), left atrial size (LA) and left atrial size/aortic root ratio (LA/Ao) were measured from the right parasternal short axis views at the level of the aorta. Mitral regurgitation and tricuspid regurgitation were assessed from the left four-chamber view (Kittleson and Kienle 1998B). Chamber enlargement was assessed by comparing values obtained in each dog to the reference range values for each dog's particular body weight (Kittleson and Kienle 1998B). Information from the echocardiographic examination allowing for a diagnosis of chronic valve disease included lesions of the mitral valve with regurgitation, enlarged left atrium, increased left ventricular end-diastolic diameter, increased left ventricular end-systolic diameter, hyperdynamic left ventricular motion, and hypodynamic left ventricular motion. Electrocardiographic examinations (leads I, II, III, aVL, aVF and aVR) were performed using a BTL-08 MD machine. Radiological examinations of the chest were performed using a G&E Prestige II X-ray machine.

Classification of disease severity

The examination included animal history, clinical examination, echocardiographic examination and, where applicable, electrocardiographic and radiographic examinations. On the basis of this clinical evaluation, dogs were classified according to the ACVIM (American College of Veterinary Internal Medicine) classification. The control group consisted of 10 healthy dogs (3 males and 7 females) at high risk of developing CMVD but with no cardiac structural changes. These healthy dogs (Stage A) had a normal heart rate and rhythm, no heart murmurs were detected during the clinical examination, echocardiographic examinations ruled out organic changes of the mitral valve, and ventricular and atrial size were within normal limits for body weight according to published normal values (Kittleson and Kienle 1998B, Moise and Fox 1999).

Stage B1 dogs (7 males and 7 females) were asymptomatic with the presence of mitral valve disease (auscultatory heart murmur heard during the clinical examination, normal heart rate, mitral valve leaflet changes, and mitral valve regurgitation with normal heart chamber size on echocardiography with values corresponding to normal when compared to published references).

Stage B2 animals consisted of 23 dogs (10 males and 13 females) which were also asymptomatic. However, a heart murmur was present during the clinical examination and echocardiography showed left atrial enlargement (LA), increased LA/Ao ratio, and/or left ventricular enlargement in diastole (LVDd) when compared to published references (Kittleson and Kienle 1998B, Moise and Fox 1999) .

Stage C consisted of 10 patients (4 males and 6 females) with previous or present signs of CHF resulting from CMVD. These dogs had structural heart changes typical for chronic mitral valve disease, enlarged left atria and left ventricles. Congestive heart failure (pulmonary congestion and/or edema) was confirmed with radiographic (GE Prestige II) examinations when clinical signs (tachycardia, tachypnea, dyspnea) were present. Dogs were receiving pimobendan, an ACE inhibitor and furosemide. In some cases they additionally received digoxin and spironolactone.

Blood samples

Blood samples were taken from the cephalic or jugular veins and collected into appropriate collection test tubes: 2 ml into the EDTA-K2 test tube (Collection Test tube; FL Medical s.r.l. Unipersonale, Italy) and 1.5 ml into clotting test tubes (Collection Test tube; FL Medical s.r.l. Unipersonale, Italy). Samples were collected during routine cardiac examinations as part of a full clinical work-up. After routine hematological and biochemical testing and preparation of blood smears, the serum was stored at -80°C until further analysis.

Hematological and biochemical analysis

The peripheral blood count was determined using an Abacus Junior Vet hematology analyzer (Diatron MI PLC, Hungary). Total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride, creatinine, and urea serum concentrations and aspartate aminotransferase (AST) activity were assayed using Pointe Scientific Kits (Pointe Scientific, Inc., USA) and a Miura One fully-automated clinical chemistry analyser (I.S.E. S.r.l., Italy). Blood smears were stained using May-Grünwald Giemsa staining.

Measurement of PON1 activity

PON1 activity toward paraoxon (paraoxonase activity) was evaluated using a slightly modified method (Mackness et al. 1991) with a UV-1800 Shimadzu Spectrophotometer, Japan. 20 µl of serum was added to 800 µl of 100 mM Tris-HCl buffer pH 8.0 containing: 2 mM paraoxon (O,O-diethyl-O-p-nitrophenylphosphate, Sigma Chemical Co.), 2 mM $CaCl_2$ and 1 mM NaCl. Production of p-nitrophenol at 25°C (wavelength 412 nm) was assessed. The molar extinction coefficient used to calculate the rate of hydrolysis was 18.290 $M^{-1}cm^{-1}$. One unit of paraoxonase activity produced 1 nmol of p-nitrophenol per min. A blank sample was run simultaneously to correct for spontaneous substrate breakdown. Activity toward phenyl acetate (arylesterase activity) was measured in a reaction mixture (3 ml) containing: 1 mM substrate and 2 mM $CaCl_2$ in 100 mM Tris-HCl buffer, pH 8.0. After adding 10 µl of serum to the mixture, an increase in absorbance at 270 nm for 1 min at 37°C was measured. 1 U hydrolyzes 1 µmol of phenyl acetate/min. The molar extinction coefficient used to calculate the rate of hydrolysis was 1310 $M^{-1}cm^{-1}$. The results were expressed in U/ml.

Statistics

Statistical analysis was performed using SSPS 21.0 software. A p value of <0.05 was considered statistically significant. For descriptive statistics, values with Gaussian distribution were investigated using multivariate statistics and the differences among groups were calculated using the contrast method. The rest were tested using the Man-Whitney and Kruskal-Wallis tests. The linear relationship between all pairs of results was investigated using Spearman's rank – correlation analysis.

Results

All canine blood parameters were within reference values (Harvey 2012) with no significant differences between ACVIM Stages. The differential leukocyte count was within the reference values (Harvey 2012). No parasites and cells morphological changes were found in stained blood smears. There were no significant differences in biochemical parameters between dogs in different ACVIM Stages.

Table 1. Dachshund sera PON1 activity, HDL, LDL, triglyceride, cholesterol concentrations in dogs of different Stages ACVIM. Letters indicate significant differences ($p<0.05$).

ACVIM Stage		A/control	B1	B2	C
PON1	paraoxon (U/ml)	336.8 ± 22.9	329.0 ± 20.7	333.5 ± 15.2	400.7 ± 22.5[f]
	phenyl acetate (U/ml)	162.0 ± 10.8	156.4 ± 8.5	148.1 ± 6.4	163.6 ± 9.5
PON1/HDL ratio (U/mmol)		81.78 ± 3.94[a,b]	70.52 ± 3.57[a,c]	72.19 ± 2.62[b,d]	84.34 ± 3.88[c,d]
HDL (mmol/l)		4.26 ± 0.28	4.77 ± 0.25	4.66 ± 0.18	4.79 ± 0.27
LDL (mmol/l)		0.28 ± 0.34	0.40 ± 0.55	0.56 ± 0.67	0.37 ± 0.31
Triglyceride (mmol/l)		0.41 ± 0.14[a,b]	0.6 ± 0.35	0.74 ± 0.43[a]	0.81 ± 0.54[b]
Cholesterol (mmol/l)		5.78 ± 0.60	6.59 ± 0.55	6.55 ± 0.40	6.9 ± 0.59

[a,b,c,d] in columns between ACVIM stages, [f] between Stage C and other stages (B1, B2, C).

Table 2. Multivariate analysis for association between factors ACVIM disease stage, gender, age, Body Weight (BW), BCS and measured parameters (PON1 activities, HDL and cholesterol concentrations). p values given. Models have an adjusted R^2 of 0.219 (PON1 paraoxonase activity), 0.331 (PON1 arylesterase activity), 0.303 (PON1/HDL ratio), 0.230 (HDL) and 0.188 (cholesterol).

	PON1 paraoxonase activity	PON1 arylesterase activity	PON1/HDL ratio	HDL	Cholesterol
Stage	0.036[a]	0.376	0.004[b]	0.230	0.520
Gender	0.624	0.296	0.317	0.261	0.199
Age	0.754	0.070	0.583	0.918	0.533
BW (kg)	0.985	0.176	0.789	0.840	0.933
BCS	0.120	0.059	0.057	0.023[a]	0.088

Statistically significant: [a] ($p<0.05$), [b] ($p<0.01$).

ACVIM Stage C serum paraoxonase activity values were higher in comparison to the activity in other Stages (control, B1, B2 respectively). There were no significant changes between ACVIM stages in PON1 activity towards phenyl acetate, or in lipoprotein and cholesterol concentrations. Healthy dogs had lower triglyceride values than animals in B2 and C Stages (Table 1). Our data had shown a decrease in PON1/HDL ratio in ACVIM B1 and B2 Stages in comparison with healthy dogs (control) and symptomatic patients (Stage C) (Table 1). A standardized PON1 activity was expressed as units per millimole of HDL (PON1/HDL ratio) which represents HDL-standardized paraoxonase activity (Bin et al. 2003, Turk et al. 2004, Bionaz et al. 2007). Factors that influence the plasma parameters are summarized in Table 2. Paraoxonase activity and standardized PON1 activity was associated with chronic mitral valve disease. In our model the body condition score (BCS) influenced HDL levels. The majority of examined dogs (about 75%) had a BCS value of 5. There were differences in age between Stages, dogs in B2 and C class were older than animals in the control group. Age was not related with the measured parameters (Table 2).

Overall PON1 activity values in Dachshunds were towards paraoxon 325.2 ± 66.25 U/ml and phenyl acetate 145.4 ± 27.27 U/ml. Paraoxonase/arylesterase P/A ratio was 2.250.

Canine paraoxonase and arylesterase activities ($n=57$) were correlated ($p<0.0001$) $r=0.7511$ (Fig. 1). There was no correlation between biochemical parameters and PON1 activity in dogs. There was no correlation between LVDd, Ao/LA, LA and PON1/HDL ratio or PON1 activity.

Discussion

Our data showed PON1 paraoxonase activity and PON1/HDL ratio changes associated with the CMVD severity, suggesting a possible influence on disease progression on the oxidative status. We found a decrease in a standardized PON1 activity in B1 and B2 Stage dogs. This decrease is treated as a corrected PON1 activity, and it might be due to enchanced oxidative stress (Prasad et al. 1996) in affected asymptomatic dogs. However, there is a minimal insignificant depletion of PON1 paraoxonase activity.

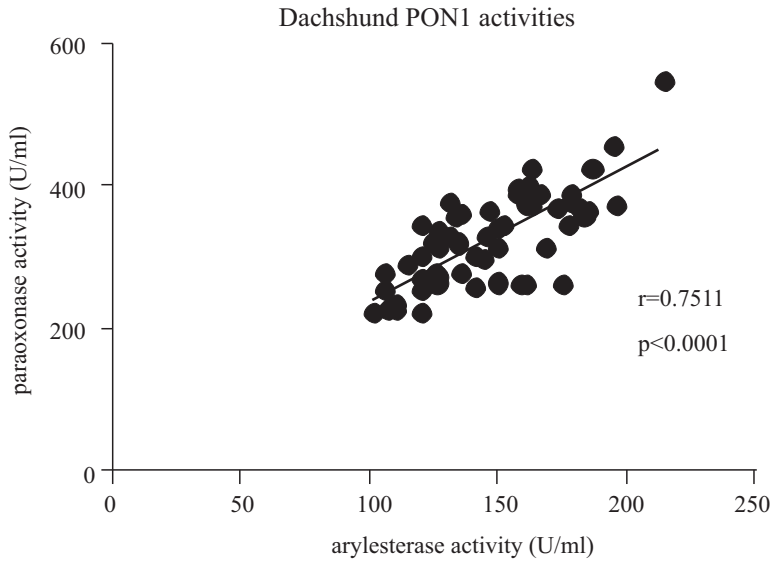

Fig. 1. Correlation between paraoxonase (U/ml) and arylesterase activity (U/ml) in serum of Dachshunds.

With clinical signs of CHF in Stage C and the onset of severe hemodynamic abnormalities, the cardiovascular system and the organism are irreversibly functioning up to their compensatory limits. This significant increase in paraoxonase activity in symptomatic patients may possibly be due to enhanced compensatory responses, which in turn lead to PON1/HDL ratio increase in Stage C dogs compared to asymtpomatic patients. An increased antioxidant reactivity in dogs with mitral endocardiosis and dilated cardiomyopathy is affected by the progression of cardiac insufficiency (Hetyey et al. 2007). A similar response was observed in MMVD development with the NO synthesis cofactor – BH4, which diminishes oxidative stress. Its' levels were elevated in Stage C dogs (Reimann et al. 2014). Similarly increased levels of oxidative absorbance capacity, which primarily involves measurements of hydrophilic, non-lipid-soluble antioxidants, was increased in CHF patients in comparison to healthy subjects (Freeman et al. 2005). These changes may imply an indirect answer to changes in heart morphology (increase of measured echocardiographical LA, LVDd, Ao/LA parameters) because there is no correlation between PON1 activity and the echocardiographic values. It is more probable that variations in PON1 activity reflect redox changes caused by hemodynamic failure, which may be used to classify patients to a higher ACVIM Stage. We found no changes in PON1 arylesterase activity between stages. PON1 activity in Stage C might also be influenced by the treatment given (e.g. spironolactone) (Billecke et al. 2000, Draganov et al. 2005, Kim et al. 2013).

HDLs are the predominant lipoproteins in canine plasma (Watson 1996). They are the main phospholipid, free and ester cholesterol carriers (Maldonado et al. 2001). Their values have been reported as higher in smaller dog breeds such as the Cairn terrier when compared to large breeds, such as Labradors. The opposite is true for LDLs (Downs et al. 1993). The Dachshunds used in our study had no significant differences in HDL levels which would be age related. Studies performed on dogs with a BCS of 5-6, similar to our dogs, showed no significant influence of age on the assessment of HDL concentrations, where LDL levels were increased only in puppies up to 1 year old (Pasquini et al. 2008). From the time of birth until 6 years of age cholesterol and triglyceride values change insignificantly (Pasquini et al. 2008); however in dogs 9 years of age and older these values rapidly increase (Usui et al. 2014). In our study, triglyceride levels where significantly higher in the ACVIM B2 and C Stages in 10-11 year old Dachshunds. Cholesterol concentrations changed insignificantly, probably due to unchanged levels of its transporter, the HDL particle.

Trimodal patterns can be seen in rabbit and human sera based on P/A ratio. Three PON1 phenotypes – A, B and AB can be distinguished with different paraoxonase activity (Eckerson et al. 1983, Watson et al. 2001, Nakanishi et al. 2003). Human PON1 phenotype B with arginine in the 192nd position (192R) has a higher paraoxon hydrolization rate than phenotype A with glutamine in the 192nd position (192Q) (Ginsberg et al. 2009). hPON1 192Q has better antioxidant properties against LDL oxidation in vitro than phenotype 192R (Aviram et al. 1999).

Dachshund sera studies have shown no trimodal pattern, with a high correlation between paraoxonase and arylesterase activities, suggesting no PON1 polymorphism.

We propose that standardized PON1 activity assessment might be a useful tool based on recognizing redox changes associated with increasing CMVD severity in asymptomatic dogs, rather than paraoxonase activity evaluation. In asymptomatic patients, an increase in PON1 paraoxonase activity could be a prognostic marker of progression to Stage C CMVD. However, more validation studies are needed, including assessment of the influence of treatment on paraoxonase activity .

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