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Original article

EVALUATION OF PLASMA TRACE ELEMENTS AND OXIDANT/ANTIOXIDANT STATUS IN BOERBOEL DOGS WITH HIP DYSPLASIA

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Summary

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Changes in plasma concentrations of trace elements and oxidants/antioxidants were evaluated in twenty healthy Boerboels of both sexes and median age of 2 years. Antero-posterior and flexed lateral radiographs of the hip were obtained using digital x-ray machine and hip grading was done according to Fédération Cynologique Internationale (FCI) system. Blood was collected from the cephalic vein for determination of plasma concentrations of manganese (Mn), magnesium (Mg), copper (Cu), cobalt (Co), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPX) and Vitamin E. Correlation between the parameters was done using Pearson's correlation. Eleven (11/20) of the Boerboel dogs had hip dysplasia (HD), comprising five (5/9) males and six (6/11) females. Plasma Mn, Cu and Co were insignificantly higher in Boerboels with normal hips than those with HD. MDA concentration was significantly ($P<0.05$) lower in Boerboels with normal hips (0.75 ± 0.84 µmol/L) than in dogs with HD (1.77 ± 0.78 µmol/L), while SOD was significantly ($P<0.05$) higher in Boerboels with normal hips (0.65 ± 0.22 U/mL) than with HD (0.32 ± 0.16 U/mL). It was concluded that there were differences in plasma oxidants/antioxidants between Boerboel dogs with normal hips and those with hip dysplasia suggesting their role in the pathogenesis of canine hip dysplasia

Key words: antioxidants, Boerboel, prevalence, hip dysplasia, malondialdehyde, trace elements

INTRODUCTION

Canine hip dysplasia is a heritable, polygenic, developmental disease of the coxofemoral joint of rapidly growing larger breeds such as Rottweiler, Labrador Retrievers, German shepherd dogs and Boerboel dogs having both environmental and genetic components (Janutta & Distl, 2006; Coopman *et al.,* 2008). It is characterised by remodelling of the head of the femur and/or acetabulum with resultant

luxation and osteoarthritic changes (Sanchez-Molano *et al.,* 2014). Secondary osteoarthritis results in pain and lameness. Genetic and environmental factors play key roles in canine hip dysplasia. Environmental factors that contribute to canine hip dysplasia include the level of exercise and diet (Smith *et al.,* 2006; Kirkby & Lewis, 2012). High dietary concentrations of calcium and vitamin D have also been implicated in the pathogenesis of canine hip dysplasia (Sanchez-Molano *et al.,* 2014).

Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage. Free radicals are formed in both physiological and pathological conditions in mammalian tissues (Maneesh *et al.,* 2005). The failure of the body's control of lipid peroxidation will result in the subsequent formation of lipid peroxides and free radicals, and the accumulation of their final products as malondialdehyde in different body tissues (Weigel *et al.,* 2013). Canine articular chondrocytes actively produce reactive oxygen species (ROS). Age-related decline in the activity and number of mitochondria plays a critical role in protecting cells from ROS damage. The consequence of increased oxidative stress is deoxyribonucleic acid (DNA) damage and telomere shortening, leading to reduced matrix production, chondrocyte senescence and apoptosis (Davies *et al.,* 2008). Increase ROS also upregulates pro-inflammatory cytokines and matrix metalloproteinase (MMP) factors that mediate cartilage degradation (Nakagawa *et al.,* 2010). Thus, oxidative stress is a relevant part of OA and promotes cartilage destruction and inflammatory transformation (Ziskoven *et al.,* 2010). However, there is no record of the role of oxidative stress in hip dysplasia.

Multiple defense systems against free radical-induced oxidative stress, collectively called antioxidants are present in the serum, plasma and erythrocytes (El-Barbary, 2011). Most of the antioxidant ability of serum is attributed to the presence of ascorbate, transferrin and ceruloplasmin. Erythrocytes are equipped to handle intracellular oxidative stress through the combined activities of catalase, superoxide dismutases, glutathione S-transferase, glutathione peroxidase and glutathione (McCord & Edeas, 2005; Valko *et al.,* 2007). The changes in plasma concentration of antioxidants during osteoarthritis have been well documented in humans and in experimental canine model of osteoarthritis (Goranov, 2007). However, there are no records of changes in plasma concentration of oxidant/antioxidants during hip dysplasia in dogs.

Many trace elements have been recognised to play important role in the pathogenesis and progression of many diseases, including osteoarthritis. Naturally occurring minerals such as magnesium, copper, manganese, selenium and zinc have shown anti-inflammatory effects in both animal and human studies (Wang *et al.,* 2004). Inspite of the involvement of trace elements in the pathogenesis of a number of musculoskeletal diseases, their role in the pathogenesis of hip dysplasia is yet unknown. Recent report showed that there is an association between serum concentrations of trace elements and developmental hip dysplasia in humans (Guner *et al.,* 2018). The present study therefore compares the plasma concentrations of product of lipid peroxidation, antioxidants and trace elements between Boerboels with or without hip dysplasia.

MATERIALS AND METHODS

Animals

Twenty apparently healthy adult Boerboel dogs of both sexes with mean weight 54 ± 7.54 kg and age ranging between 1–5 years (median age: 2 years) were used. All dogs used were micro-chip identified and registered with Boerboel Dog Breeders Association of Nigeria (BDBAN) or Boerboel Alliance. Before the commencement of the study, informed owners consent and ethical approval from the College of Veterinary Medicine Research and Ethics Committee, Federal University of Agriculture, Abeokuta were obtained.

Experimental design

The study was a cross-sectional survey. All dogs used were presented for radiographic elbow and hip screening as part of appraisal criteria for registration under the Kennel Union of South Africa (KUSA). The dogs were divided into two groups based on their radiographic hip classification using the Fédération Cynologique Internationale (FCI) criteria. Dogs with hip classifications A1, A2, B1 and B1 were classified as non-dysplastic or normal, while dogs with hip classifications C1, C2, D1, D2, E1 and E2 were classified as dysplastic.

Radiographic examination

The dogs were premedicated with intramuscular injections of 0.5 mg/kg of 2% xylazine hydrochloride (Xylazine 20 Inj®, Kepro, Holland) and 0.04 mg/kg atropine sulphate (Atocan®, Sishui Xierkang Pharma, China). Thereafter, anaesthesia was induced with 4 mg/kg of 1% propofol injection (Diprivan, ICI - Zeneca Pharmaceuticals) intravenously. Extended antero-posterior and flexed lateral radiographs were obtained in all the dogs. All the radiographs were obtained with a digital X-ray machine using a Potter-Bucky grid. Exposure parameters ranged from 10–16 mAs and 74–80 kVp depending on the size of the dog.

Blood collection and analyses

Prior to anaesthesia, about 5 mL of blood was collected from the cephalic vein of each dog into bottle containing EDTA for the determination of plasma concentrations of malondialdehyde (MDA), superoxide dismutase, glutathione, glutathione peroxidase, Vitamin E, manganese (Mn), copper (Cu), cobalt (CO) and magnesium (Mg). Following blood collection, the samples were centrifuged at 2555×*g* and the plasma was aspirated into Eppendorf tubes and kept frozen until assay.

Malondialdehyde analysis. Plasma concentration of MDA was determined according to the method of Buege & Aust (1978): 0.05 mL of plasma was added to 1.0 mL of thiobarbituric acid (TBA) reagent and then incubated in boiling water bath for 15 min. The tube was placed immediately under a running tap to cool down and was centrifuged at 2555×*g* for 10 min. The absorbance of the clear supernatant was read against blank at 535 nm. The concentration of TBARS (MDA) was calculated as Concentration (M) = Absorbance/ε, where ε is the extinction coefficient of MDA-TBA complex at 535nm (1.56×10^5 $M^{-1}cm^{-1}$).

Superoxide dismutase (SOD). SOD was determined according to the method of Marklund & Marklund (1974). Two test tubes were marked as blank (B) and test (T); 100 µL of potassium phosphate buffer, 830 µL of distilled water (dH_2O) were pipetted into tube B. The tubes were incubated for 10 min at 25 °C and 20 µL of pyrogallol was added to both tubes. They were then mixed by inversion and

the absorbance was recorded on a spectrophotometer for 3 min at 420 nm. The difference between the initial and final absorbance and average absorbance difference were calculated (Δ420/min). SOD activity was calculated as: % Inhibition = [(ΔA 420 nm/min Blank–ΔA 420 nm/min of sample)/ (ΔA 420nm/min Blank] × 100

Glutathione (GSH). GSH concentration was determined according to the method of Ellman (1959). To this end, 0.5 mL of plasma (5 µL of plasma + 495 µL of dH_2O) was added to 0.5 mL of 10% trichloroacetic acid and the mixture was centrifuged for 15 min at 2555×*g*. Then 0.4 mL of the supernatant was added to 0.8 mL of 0.4M Tris buffer pH 8.9 and 20 µL of freshly prepared 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Absorbance was read within 5 min of DTNB addition on a spectrophotometer at 412 nm against blank containing water instead of plasma. The concentration of GSH was calculated as: mM GSH = Absorbance × 2.44 × 14.15, where 14.15 $Mm^{-1}cm^{-1}$ is the molar extinction coefficient of DNTB and 2.44 is the dilution factor of sample in 1.22 mL assay mixture

Glutathione peroxidase (GPX). GPX was determined according to the method of Rotruck *et al.* (1973). Two test tubes were marked blank (B) and test (T), 200 µL of 0.4M potassium phosphate buffer pH 7.0, 100 µL of sodium azide, 200 µL of 10mM glutathione, 100 µL of 0.2 mM hydrogen peroxide and 200 µL of plasma was pipetted into tube T while 400 µL of 0.4M potassium phosphate buffer pH 7.0, 100 µL of sodium azide, 200 µL of 10 mM glutathione and 100 µL of 0.2 mM hydrogen peroxide was pipetted into tube B. They were incubated at 37 °C for 10 min, 10% trichloroacetic acid (TCA) was added to both tubes and the mixtures was centrifuged for 15 min at 2555×*g*. Then 400 µL of the supernatant was added to 800 µL of 0.4M Tris buffer pH 8.9 and 20 µL of freshly prepared DTNB. Absorbance was read within 5 min after adding DTNB at 412 nm against reagent blank. The activity of glutathione peroxidase was calculated in terms of concentration of glutathione utilised as mM GSH = Absorbance / 14.15, where 14.15 is the molar extinction coefficient of DNTB.

Glutathione peroxidase activity was calculated as: {ΔOD/min×GSH Std.×total reaction volume} ÷ {Std. OD×307.32×volume of enzyme source×Hb}, where 307.32 is the molecular weight of GSH.

Copper. Plasma concentration of copper was determined by a colorimetric method using copper colorimetric assay kit (Abnova, Taiwan) as described below: Three tubes were labelled as Sample Test (ST), Sample Blank (SB) and Reagent Blank (RB). Then 100 µL of distilled water was transferred into RB; 20 µL of 1.5 mg/dL standard and 80 µL distilled water (final 300 µg/dL Cu2+) was added into SB; 100 µL of plasma was transferred into ST. To each tube, 35 µL Reagent A (trichloroacetic acid) was added and vortexed. One hundred µL of ST, SB and RB was pipetted into separate wells of a clear flat-bottom 96-well plate, 150 µL of working reagent was added to each well and mixed thoroughly. The mixture was incubated for 5 min at room temperature and optical density read at 359 nm. The corrected absorbance of the sample (ST-{SB + RB}) was used to determine amount of copper by using extinction coefficient derived from the calibration curve.

Manganese. Plasma concentration of manganese was determined by colorimetric method using Manganese Assay Kit (Sigma Aldrich CO, UK): 10 µL of plasma was pipetted into duplicate wells

in the plate. 10 µL of assay buffer was then be added into duplicate wells as Zero calibrator, 50 µL of the substrate preparation was added to each well using a repeater pipet. Twenty-five µL xanthine oxidase preparation was added to each well using a repeater pipette. The sample was incubated at room temperature for 20 min. The optical density was read at 450 nm.

Magnesium. Plasma Mg concentration was measured colorimetrically. Magnesium in plasma reacts with calmagite in alkaline medium forming a coloured complex. Three tubes were labelled as sample test (ST), sample blank (SB) and reagent blank (RB). Twenty µL of the plasma was added to each ST and SB and 20 µL of TRIS solution to RB. Eighty µL of calmagite 80 was added to each tube, vortexed and incubated at room temperature for 10 min. Sixty µL of EGTA was added into each tube except SB, into which 200 µL of dietylamine was added. Absorbance was read at 480 nm after 5 min. The corrected absorbance of the sample (ST-{SB + RB}) was used to determine amount of magnesium by using extinction coefficient derived from the calibration curve

Cobalt. Plasma concentration of copper was determined by colorimetric method using cobalt colorimetric assay kit (Sigma Aldrich CO, UK). Three tubes were labelled as Sample Test (ST), Sample Blank (SB) and Reagent Blank (RB). One hundred µL of plasma was added to each ST and SB and 100 µL of TRIS solution to RB. Ten mL of the cobalt reagent was added to each reaction (well), mixed well using a horizontal shaker and incubated the reaction for 10 min at room temperature. The absorbance is then measured at 475 nm. The corrected absorbance of the sample (ST-{SB + RB}) was used to determine amount of cobalt by using extinction coefficient derived from the calibration curve.

Vitamin E. Plasma concentration of Vitamin E was determined spectrophotometrically using the method described by Rutkowski *et al.* (2005) – 0.5 mL anhydrous ethanol was added to 0.5 mL plasma in a test-tube with a tight stopper and shaken vigorously for 1 min. Then 0.25 mL of xylene was added and shaken vigorously for another minute. The tube was centrifuged to separate the extract (1500×*g*, 10 min), and 0.25 mL batophenanthroline solution added to 1.5 mL of the extract (upper layer). $FeCl_3$ solution (0.25 mL) was then added to the mixture and mixed vigorously. Finally, 0.25 mL H_3PO_4 solution was added to the mixture. The absorbance of the test sample and of the standard sample was then measured at 539 nm against the blank test. Plasma concentration of Vitamin E was calculated as Vitamin E= a/A, where a= difference in absorbance of test samples and standard sample, and A = absorbance of blank.

Statistical analysis

Data were presented as mean and standard deviation. Appropriateness of sample size was determined by N-power analysis. Correlation between the parameters was done using Pearson's correlation coefficient. Differences in plasma concentrations of trace elements, MDA and antioxidants between male and female Boerboels and between Boerboels with normal hips and those with hip dysplasia were determined using un-paired student t-test. Differences were considered significant at $P \leq 0.05$. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The hip scores of the Boerboel dogs in shown in Table 1. Four (4) of the dogs had hip grade of A1 or A2 (on both left and right hip, while five (5) and seven (7) dogs had hip scores of B1 or B2 on left and right hip respectively. In addition, six (6) and three (3) dogs had scores of C1 or C2 on left and right hip respectively, while four (4) and five (5) dogs had hip scores of D1 or D2 on the left and right hip respectively. Only one dog had a hip score of E1or E2 in either left or right hip. The sex distribution of the hip grades in apparently normal Boerboel dogs in this study is shown in Table 2. Five (5/9) of the male Boerboel dogs were dysplastic, vs six (6/11) dysplastic females.

Table 1. Radiographic hip scores in apparently healthy Boerboel dogs

Patient identification	Radiographic hip scores	
	Sex	Left/Right Hip
1	F	D_1/D_2
2	F	A_1/A_1
3	F	C_1/C_2
4	M	D_1/D_2
5	M	D_2/D_2
6	F	D_1/D_2
7	F	E_1/E_2
8	F	A_2/A_2
9	F	C_1/C_2
10	M	A_1/A_1
11	M	C_2/B_1
12	M	B_1/B_2
13	M	A_2/A_2
14	F	B_1/B_2
15	F	B_2/B_1
16	M	C_2/C_1
17	F	B_1/B_1
18	F	C_2/D_1
19	M	B_2/B_1
20	M	C_1/B_2

Table 2. Sex distribution of hip dysplasia in apparently healthy Boerboel dogs

	Hip quality		
	Normal	Dysplastic	Percentage dysplastic
Male	4	5	25%
Female	5	6	30%
Total	9	11	55%

There were no significant differences in the plasma concentration of the trace elements between Boerboel dogs with normal hip and those with hip dysplasia (Table 3). However, the plasma manganese and copper tended to be higher in Boerboel dogs with normal hips than those with hip dysplasia, while that of magnesium tended to be higher in dogs with hip dysplasia.

Plasma concentrations of MDA was significantly lower ($P<0.05$) in Boerboel dogs with normal hip than those with hip dysplasia, while the plasma concentrations of SOD was significantly ($P<0.05$) higher in Boerboel dogs with normal hips than those with hip dysplasia (Table 4). In addition, the plasma concentrations of GSH, GPX and vitamin E tended to be higher in Boerboel dogs with normal hip than those with hip dysplasia. However the differences were not significantly different.

Plasma Mn concentrations were negatively correlated to plasma copper and cobalt, while plasma cobalt was negatively correlated to copper (Table 5). There were no significant ($P>0.05$) correlations between the plasma concentrations of the trace elements. Similarly, there were no significant ($P>0.05$) correlations between MDA and the antioxidants (Table 6). Plasma concentrations of Vitamin E were negatively correlated to GPX.

Table 3. Mean plasma concentrations of trace elements in normal and dysplastic Boerboel dogs

Trace elements	Dysplastic dogs (n=9)	Normal dogs (n=11)	P value
Manganese (μmol/L)	8.01 ± 3.3	9.10 ± 2.9	0.549
Copper (μmol/L)	0.94 ± 1.57	1.57± 2.04	0.555
Cobalt (μmol/L)	0.008 ± 0.004	0.008 ± 0.003	0.581
Magnesium (μmol/L)	0.67 ± 0.08	0.64 ± 0.14	0.976

Table 4. Mean plasma concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPX) in normal and dysplastic Boerboel dogs.

Parameters	Dysplastic dogs (n=9)	Normal dogs (n=11)	P value
MDA (μmol/L)	0.75 ± 0.84	1.77 ± 0.78	0.027
SOD (U/mL)	0.65 ±0.22	0.32 ± 0.16	0.051
GSH (μmol/L)	0.06 ± 0.02	0.05 ± 0.01	0.765
GPX (U/L)	37.03 ± 15.76	32.06 ± 2.93	0.584
Vitamin E (μmol/L)	19.89 ± 3.92	18.13 ± 6.43	0. 048

Table 5. Pearson correlation coefficients (r) and levels of significance (P) among plasma concentrations of trace element in Boerboel dogs with hip dysplasia (n=11)

	Mn	Cu	Co	Mg
Mn		r= –0.044 P= 0.186	r= –0.030 P= 0.231	r= 0.077 P= 0.179
Cu	r= –0.044 P= 0.186		r=–0.036 P=0.440	r=–0.325 P= 0. 670
Co	r=–0.030 P= 0.231	r=– 0.036 P= 0.440		r= 0.287 P= 0.581
Mg	r= 0.077 P= 0.179	r= –0.325 P= 0.670	r= 0.287 P= 0.581	

DISCUSSION

The results of this study showed that the plasma concentration of MDA was significantly lower in Boerboel dogs with normal hip than those with hip dysplasia, while the plasma concentrations of SOD was significantly higher in Boerboel dogs with normal hip than those with hip dysplasia. In addition, the plasma concentrations of other antioxidants and trace elements tended to be higher in Boerboels with normal hips.

In this study, the incidence of hip dysplasia did not differ between the female and male Boerboel dogs. It has been reported that the odd ratio for hip dysplasia is higher in the female than male dogs (Loder & Todhunter, 2017). However, in another prevalence study of hip dysplasia in Rottweilers and Labrador retrievers, there were no significant difference in the prevalence of hip dysplasia between the male and the female (Kirberger, 2017). It appears as though sex did not significantly

influence the risk for the development of hip dysplasia in the Boerboel dogs.

The role of oral antioxidant in the management of canine hip dysplasia has been reported (Impellizeri *et al.,* 1998), although the evidence was considered to be insufficient (Kirkby & Lewis, 2012). Decrease in the expression of SOD has been associated with the early stage of osteoarthritis and linked to an increase in concentration of reactive oxygen species (Scott *et al.,* 2010). In this study, plasma concentration of SOD was significantly lower in Boerboel dogs with hip dysplasia than dogs with normal hips. This finding is similar to that reported in humans with osteoarthritis (Angthong *et al.,* 2013) and further supports the role of ROS in the pathogenesis of canine hip dysplasia and the possible usefulness of antioxidant supplementation in the management of hip dysplasia.

High dietary concentrations of calcium and vitamin D have been implicated in the pathogenesis of canine hip dysplasia (Sanchez-Molano *et al.,* 2014). There are no report of the role of trace elements such as copper, cobalt, manganese and magnesium in the pathogenesis of canine hip dysplasia despite the reported role of these elements in musculoskeletal functions (Wang *et al.,* 2004; King *et al.,* 2005; Strecker *et al.,* 2013). The results of this study showed that there were no significant differences in the plasma concentration of these trace elements between Boerboel dogs with normal hip and those with hip dysplasia, even though the plasma concentrations of copper and manganese tended to be higher in Boerboel dogs with normal hip than those with hip dysplasia. This result despite not being significant may suggest the possible role of these trace elements in the pathogenesis of hip dysplasia.

A recent report showed that there is an association between serum concentrations of trace elements and developmental hip dysplasia in humans (Guner *et al.,* 2018). In the study, it was hypothesised that the association may actually be between trace element concentrations and synthesis of collagen (Guner *et al.,* 2018). In this study, there was no significant difference in blood trace elements between dogs with normal hip and those with hip dysplasia. This result seems to be contrary to earlier findings in humans and may be associated with some of the limitations to this present study.

Table 6. Pearson correlation coefficients (r) and levels of significance (P) among plasma oxidants/antioxidants in Boerboel dogs with hip dysplasia (n=11)

	MDA	SOD	GSH	GPX	Vit. E
MDA		r= –0.276 P= 0.597	r= 0.242 P= 0.992	r= 0.223 P= 0.494	r= 0.110 P= 0.318
SOD	r= -0.276 P= 0.597		r= 0.045 P= 0.478	r= 0.352 P= 0.494	r= 0.232 P= 0.187
GSH	r= 0.242 P= 0.992	r= 0.045 P= 0.478		r= 0.006 P= 0.992	r= 0.116 P= 0.826
GPX	r= 0.223 P= 0.494	r= 0.352 P= 0.494	r= 0.006 P= 0.992		r= - 0.320 P= 0.597
Vit E	r= 0.110 P= 0.318	r= 0.232 P= 0.187	r= 0.116 P= 0.826	r= –0.320 P= 0.597	

Finally, there were few limitations in this study. The number of dogs sampled might be considered to be small, although the N power analysis performed showed that the sample size was sufficient. Another probable limitation is the fact the dogs are from different breeders with possibly different feeding and exercise schedules. These might have influenced the plasma levels of the antioxidants and trace elements in the dogs. In addition, the different age of the dogs may mean that they are at different progression of hip dysplasia, although most of the dogs were up to 2 years of age.

In conclusion, the plasma concentrations of trace elements were higher in Boerboel dogs with normal hips than in those with hip dysplasia, although the differences were not significant. Therefore, we were not able to establish an association between trace elements and hip dysplasia in Boerboel dogs. In addition, the plasma concentrations of MDA and antioxidants changes significantly between Boerboel dogs with normal hip and those with hip dysplasia. This study suggests the role of reactive oxygen species in the pathogenesis of developmental hip dysplasia in dogs.

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