Original Article

Serum biomarkers of oxidative stress in dogs with idiopathic inflammatory bowel disease

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Highlights

- Decreased total antioxidant capacity, measured by CUPRAC and TEAC assays, was demonstrated in sera from dogs with IBD.
- Serum thiol and PON1 activity was significantly decreased in sera from dogs with IBD compared with healthy dogs.
- Oxidative damage in dogs with IBD was demonstrated by increased serum FOX, ROS and TBARS.

Abstract

The objective of this study was to evaluate and compare a panel of various serum biomarkers evaluating both the antioxidant response and oxidative damage in dogs with idiopathic inflammatory bowel disease (IBD). Eighteen dogs with IBD and 20 healthy dogs were enrolled in the study. Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of the plasma (FRAP), total thiol concentrations, and paraoxonase 1 (PON1) activity were evaluated in serum to determine antioxidant response. To evaluate oxidative status, ferrous oxidation-xylenol orange (FOX), thiobarbituric acid reactive substances (TBARS) and reactive oxygen species production (ROS) concentrations in serum were determined.

Mean concentrations of all antioxidant biomarkers analysed, with exception of FRAP, were significantly lower (P <0.0001) in the sera of dogs with IBD than in healthy dogs. The oxidant markers studied were significantly higher (P <0.0001) in sera of dogs with IBD than in healthy dogs. These findings support the hypothesis that oxidative stress could play an important role in the pathogenesis of canine IBD.

Keywords: Antioxidant; Cupric; PON1; Reactive oxygen species; Thiol

Introduction

Idiopathic inflammatory bowel disease (IBD) is characterised by persistent or recurrent activation of the mucosal immune system accompanied by infiltrations of inflammatory cells in the intestinal mucosa (Allenspach et al., 2007; Simpson and Jergens, 2011). It is the most common cause of chronic intestinal disease in dogs, and results in diverse and often debilitating clinical signs (Jergens et al., 2003; Allenspach et al., 2016).

The pathogenesis of IBD in dogs is not completely understood; however it is believed that intestinal inflammation results from a dysregulated immune response to intestinal antigens (Allenspach et al., 2010). There is evidence that oxidative stress plays an important role in the pathogenesis of IBD in human patients particularly in the initiation and perpetuation of inflammation and in subsequent tissue damage. Oxidative stress occurs when there is a marked imbalance between the production of reactive oxygen species (ROS) and their removal by antioxidants (Rezaie et al., 2007). Recent studies suggest that oxidative stress could also represent a significant factor in the pathogenesis of IBD in dogs. One study that evaluated the metabolomics profile in dogs with IBD using an untargeted metabolomics approach suggested the presence of oxidative stress and a functional alteration of the GI microbiota in dogs with IBD, which persisted even in the face of a clinical response to medical therapy (Minamoto et al., 2014). Other recent studies have reported that various serum antioxidant biomarkers, such as Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC) and paraoxonase 1 (PON1), were decreased in the sera of dogs with IBD (Rubio et al., 2016a,b; Segarra et al., 2016), again suggesting that oxidative stress could play an important role in the pathogenesis of canine IBD.

The purpose of this study was to evaluate and compare a panel of various serum biomarkers evaluating both the antioxidant response and oxidative damage response in sera from dogs with IBD. These included previously the described antioxidant serum biomarkers TEAC, CUPRAC and PON1, and additional antioxidants that have not previously been studied in canine IBD, such as ferric reducing ability of plasma (FRAP) and total serum thiol concentrations. To investigate oxidative damage, we measured ferrous oxidation-xylenol orange (FOX) and thiobarbituric acid reactive substances (TBARS), which measures products of lipid peroxidation in serum. Finally, we measured serum concentrations of reactive oxygen species.

Material and methods

Animals

In this retrospective study, a group of 18 dogs diagnosed with IBD at the Royal Veterinary College (RVC), London, were included. Dogs with a history typical for chronic enteropathy (≥3 weeks of vomiting, diarrhea or both, with or without weight loss) were included. The diagnosis of chronic enteropathy was confirmed based on established criteria (no clinically relevant abnormalities on routine hematology, serum biochemistry; trypsin-like immunoreactivity [TLI], canine pancreatic lipase [cPL] and adrenocorticotropic hormone [ACTH]-stimulation test results within the reference ranges; no abnormalities on abdominal imaging [radiographs, abdominal ultrasound examination or both]; Allenspach et al., 2007). The histopathological criteria for IBD were based on the guidelines for evaluation of gastrointestinal inflammation in companion animals, as established by Washabau et al. (2010). In all cases biopsies were obtained and reviewed by a board-certified pathologist.

In addition, 20 clinically healthy dogs were included in this study as a control group.

Archived sera from healthy dogs and those with IBD and biopsy samples were originally obtained in 2007 for diagnostic purposes only and were residual samples, stored and available in the RVC archive. They were originally obtained with informed owner consent under the Veterinary Surgeons Act (residual clause) and approved by the RVC Ethics and Welfare Committee and were frozen immediately after blood sampling and stored at -80 ºC until further analysis.

Antioxidant capacity

The TEAC assay, based on the inhibition of the radical ABTS by the sample, was performed according to the assay described by Arnao et al. (1996) validated by Rubio et al. (2016a). The CUPRAC assay, based on the capacity of the sample in reducing Cu(II) to Cu(I), was performed as previously described for use in canine serum (Rubio et al., 2016b). The FRAP assay was performed following the method by Benzie and Strain (1996) which measures the ferric to ferrous ion reduction by the sample.

Serum thiol was determined according to the method by Jocelyn (1987), and serum PON1 activity was analysed following a previously described method for use in canine serum (Tvarijonaviciute et al., 2012).

All analyses were performed at Murcia University using an automated biochemistry analyser (Olympus AU600 Automatic Chemistry Analyser, Olympus). All

assays showed inter- and intra-assay imprecision of <15%. Lower detection limits of TEAC, CUPRAC and FRAP were 0.090, 0.003, and 0.031 mmol/L, respectively. The lower detection limit of serum thiol and PON1 were 4.0 µmol/L and 0.6 U/mL, respectively.

Oxidant biomarkers

The FOX assay was based on the automated method described by Arab and Steghens (2004) and performed using the Olympus AU600 Automatic Chemistry Analyser (Olympus). The TBARS assay was determined following the method by Buege and Aust (1978) using a microplate reader (Powerwave XS, Biotek instruments).

Reactive oxygen species (ROS) were estimated by luminol-mediated chemiluminescence assay (Vong et al., 2014) using a microplate reader (Victor 2 1420 Multilabel Counter; PerkinElmer, Finland) and expressed in counts per second (cps). All oxidant assays showed inter- and intra-assay imprecision less than 15% when evaluated in our laboratory. In addition, the lower detection limits of FOX and TBARS were 55.91 and 0.81 µmol/L, respectively. The ROS assay showed a lower detection limit of of 1,300 cps.

Statistical analysis

Data were analysed using Graphpad Prism software (version 5 for Windows). Concentrations of antioxidants and oxidant biomarkers were compared between dogs with IBD and healthy control dogs. The results for each parameter were evaluated for normality using the Shapiro-Wilk test. Thiol, PON1, TBARS and ROS results were not normally distributed, therefore they were presented as median and interquartile range

(IQR). Normally distributed data were presented as means ± standard deviation. We determined statistical differences between healthy dogs and dogs with IBD using unpaired t test (normally distributed data) and Mann Whitney U test (not normally distributed data). Correlations between variables were determined using Spearman correlation analysis. A *P*-value (two-tailed) of <0.05 was taken as statistically significant in all cases.

Results

Animals

The 18 dogs with IBD included Border collie (*n*=1), Boxer (*n*=3), Cocker spaniel (*n*=2), East-European shepherd (*n*=1), Greyhound (*n*=1), Labrador (*n*=2), Mixed breed (*n*=1), Old English sheepdog (*n*=1), Polish Lowland sheepdog (*n*=1), Rottweiler (*n*=1), Schnauzer (*n*=1), Staffordshire terrier (*n*=2), West Highland white terrier (*n*=1). Their ages ranged from 7 months to 11 years; eight were females (*n*=6 spayed; *n*=2 intact) and 10 were males (*n*=4 neutered; *n*=6 intact). The clinical status of each dog was evaluated at the time of diagnosis using the canine chronic enteropathy clinical activity index (CCECAI) scoring system established by Allenspach et al. (2007), which is based on nine variables, including attitude and activity, appetite, vomiting, stool consistency, stool frequency, weight loss, ascites, pruritus and serum albumin concentration. Based on CCECAI, the disease was mild (score 4-5) in two dogs, moderate (score 6-8) in 11 dogs, severe (score 9-11) in two dogs, and very severe (score ≥12) in three dogs.

Histopathologic findings of intestinal mucosal biopsies showed in all cases a lympho-plasmacytic inflammation, in some cases with eosinophils. There was no neutrophilic or macrophagic inflammation in any case.

The 20 control dogs consisted of various breeds (Mixed breed, Staffordshire terrier, Italian Spinone, Labrador, Cavalier King Charles spaniel, English Springer spaniel, Akita Inu, Golden retriever, Border collie, Dogue de Bordeaux, Rottweiler, Great Dane, Japanese Spitz, Leonberger). Their ages ranged from 2 to 13 years; seven were female (*n*=6 spayed; *n*=1 intact) and 13 were male (*n*=11 neutered; *n*=2 intact).

Antioxidant response

Dogs with IBD had lower TEAC concentrations (0.35±0.06 vs. 0.51±0.04 mmol/L; *P* <0.0001), lower CUPRAC concentrations (0.3±0.05 vs. 0.44±0.05 mmol/L; *P* <0.0001), lower serum thiol concentrations (median, 63; IQR, 34-94 vs. median, 245; IQR, 216-277 µmol/L; *P* <0.0001), and lower PON1 activity than control dogs (median, 2.2; IQR, 1.7-2.8 vs. median, 3.5; IQR, 3.1-3.8 IU/mL; *P* <0.0001; Fig. 1).

However, serum FRAP did not differ between dogs with IBD and control dogs (0.41±0.09 vs. 0.42±0.07 mmol/L; *P* =0.6459).

Oxidant biomarkers

Dogs with IBD had higher serum ROS counts (median, 8,861; IQR, 5,900-14,763 cps vs. median, 1,502; IQR, 1,384-1,648 cps; *P* <0.0001), higher FOX concentrations (148±65 vs. 72±13 µmol/L; *P* <0.0001) and higher TBARS

concentrations than control dogs (median, 8.0; IQR, 5.9-10.5 vs. median, 2.4; IQR: 1.9-2.8 µmol/L; P <0.0001; Fig. 2).

Correlation study

We observed correlations between all antioxidant biomarkers except for FRAP and TEAC, and FRAP and CUPRAC (Table 1). The highest correlations ($\rho > 0.90$) were observed between TEAC, CUPRAC and serum thiol.

Similarly, all oxidant biomarkers were positively correlated, with the highest coefficient of correlation being between TBARS and ROS ($\rho = 0.84$; P <0.001). In addition, TBARS and ROS correlated negatively with TEAC, CUPRAC, and PON1 (all $\rho \leq 0.45$; P <0.01). A negative correlation was also observed between all oxidant biomarkers and serum thiol (all $\rho \leq 0.54$; P <0.001).

The CCECAI was not correlated with any of the biomarkers studied (all $P \geq 0.05$).

Discussion

Our study, evaluating the oxidative stress in dogs with IBD compared to healthy control dogs using a comprehensive panel of serum biomarkers, suggests increased oxidative stress status in canine IBD.

Serum TEAC and CUPRAC were significantly reduced in dogs with IBD compared with healthy dogs. These results corroborate recent studies in dogs (Rubio et al., 2016a,b; Segarra et al., 2016). Furthermore, Rubio et al. (2016a) observed similar

228 decreases (about 30%) in the TEAC concentration in dogs with IBD, as we did in the
229 present study. In this cohort of dogs, dogs with IBD had mean CUPRAC serum
230 concentrations that were 33% lower than in healthy dogs, compared to a 17% decrease
231 observed by Rubio et al. (2016b). Decreased TEAC and individual antioxidant (biotin,
232 folate, β-carotene, and vitamins A, C, and B) concentrations have previously been
233 reported in human patients with ulcerative colitis (UC; Fernandez-Banares et al., 1989;
234 Geerling, 1999; Aslan et al., 2011). The decreased antioxidant response in dogs with
235 IBD could be due to severe, persistent oxidative stress that depletes antioxidant
236 resources and overtakes the ability of the body to produce more antioxidants (Rezaie et
237 al., 2007). We failed to detect a difference in serum FRAP between healthy dogs and
238 dogs with IBD. This might be explained by the different individual antioxidants that
239 contribute to serum FRAP compared with other total antioxidant capacity (TAC) assays.
240 Ascorbic acid, α-tocopherol and principally uric acid are the contributors to FRAP in
241 humans, whereas thiols and albumin are the main contributors to CUPRAC and TEAC.
242 Therefore, we recommend that several different methods be used to measure TAC in
243 biological samples, because individual assays have different biochemical bases,
244 resulting in different results and interpretations (Cao and Prior, 1998; Hetyey et al.,
245 2007; Jansen and Ruskovska, 2013).
246
247 Studies have shown that mucosal thiol proteins are targets of oxidative injury
248 (Grisham et al., 1990). In the present study, serum thiol and PON1 were also
249 determined as individual antioxidants and both were significantly diminished in dogs
250 with IBD. Paraoxonase 1 (PON1) is widely distributed among tissues, including the
251 intestine. This protein is considered to be an antioxidant enzyme as it hydrolyses lipid
252 peroxides, in addition to its anti-inflammatory role in disease (Ceron et al., 2014). One

253 of the explanations for decreased PON1 activity could be its inactivation due to an
254 exacerbated oxidative environment (Nguyen and Sok, 2003). Our results are in
255 agreement with other studies that reported diminished PON1 activity in dogs with IBD
256 and human patients with IBD (Baskol et al., 2006; Boehm et al., 2009; Segarra et al.,
257 2016). In addition, there is evidence that diminished PON1 activity could also be a
258 consequence of a reduced number of the free SH groups on PON1, because of
259 exposition to oxygen free radicals (Jaouad et al., 2006), which could explain the high
260 correlation between serum PON1 activity and total serum thiol found in this study.
261
262 We demonstrated increased ROS and products of lipid peroxidation in the sera
263 of dogs with IBD by TBARS and FOX assays. These results are in agreement with
264 reports of IBD in humans (Levy et al., 2000; Sampietro et al., 2002; Baskol et al.,
265 2006). It has been reported that phagocytic cells (neutrophils and macrophages) isolated
266 from inflamed intestinal tissue in human patients with IBD release large amounts of
267 ROS during stimulation that could act locally or be secreted into the circulation to
268 produce different systemic effects (Kitahora et al., 1988; Alzoghaibi, 2013). However,
269 in our study, inflamed intestinal mucosa in the dogs with IBD contained lymphocytes
270 and plasma cells, which could suggest that these cells might also be a source of
271 systemic ROS, as has been suggested in humans (Lantow et al., 2006). Nevertheless,
272 studies using larger group sizes are needed to evaluate the association between the type
273 of cells detected in the inflamed mucosa of the dogs with IBD and the systemic
274 concentrations of oxidants (ROS and lipid peroxides) during the disease.
275
276 The relatively low number of dogs with IBD in our study might have limited our
277 ability to detect correlations between the various serum biomarkers and clinical disease

activity. However, our data show that antioxidants, which act to control oxidative stress, are decreased, and oxidants generated in association with oxidative stress are increased in dogs with IBD. These findings could help explain the pathophysiology of the disease. However, further studies with larger group sizes are necessary to evaluate the potential of these markers as possible predictors of disease and biomarkers for treatment monitoring.

Although we did not determine the stability of the oxidant and antioxidants we measured in canine sera, previous reports demonstrated that antioxidants were stable in human serum stored at -80 ºC for at least 1 year (Jansen et al., 2013). In addition, the values of the control group were inside the reference values of our laboratory, determined with fresh samples. However, possible changes during the long storage of the samples in any of the analytes measured cannot be disregarded and this should be considered as a limitation of our study.

Conclusions

Our study demonstrated the presence of oxidative stress in dogs with IBD. Based on our results, a profile including biomarkers of total antioxidant status such as TEAC and CUPRAC, individual antioxidant biomarkers such as PON1 and thiol, and biomarkers of oxidant status measuring lipid peroxidation, such as FOX, or TBARS, or directly measuring ROS production, might be useful in the comprehensive evaluation of the oxidative stress response in dogs with IBD.

Conflict of interest statement

The authors have no financial and personal relationships with people or organisations that could have inappropriately influenced his work.

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Fig. 1. Comparisons of antioxidant biomarkers in healthy dogs and dogs with IBD. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). TEAC, Trolox equivalent antioxidant capacity; CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric reducing ability of plasma; PON1, paraoxonase 1.

Fig. 2. Comparisons of oxidant biomarkers in healthy dogs and dogs with IBD. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). ROS, reactive oxygen species; FOX, ferrous oxidation-xylenol orange; TBARS, thiobarbituric acid reactive substances.

Table 1. Spearman correlation coefficients, levels of statistical significance (95% confidence intervals) between all biomarkers studied.

	TEAC	CUPRAC	FRAP	Thiol	PON1	FOX	TBARS
CUPRAC	0.92*** (0.84 to 0.95)						
FRAP	0.26 (- 0.07 to 0.55)	0.27 (- 0.07 to 0.55)					
Thiol	0.90*** (0.81 to 0.95)	0.90*** (0.81 to 0.95)	0.04 (- 0.30 to 0.37)				
PON1	0.68*** (0.45 to 0.82)	0.72*** (0.51 to 0.85)	0.34* (0.01 to 0.61)	0.65*** (0.41 to 0.81)			
FOX	-0.40* (-0.65 to -0.08)	-0.39* (-0.64 to -0.06)	0.46** (0.16 to 0.69)	-0.54*** (-0.74 to -0.26)	-0.20 (-0.50 to 0.13)		
TBARS	-0.54*** (-0.74 to -0.26)	-0.59*** (-0.77 to -0.32)	0.12 (-0.22 to -0.44)	-0.65*** (-0.81 to -0.41)	-0.45** (-0.68 to -0.14)	0.74*** (0.54 to -0.86)	
ROS	-0.70*** (-0.84 to -0.49)	-0.77*** (-0.88 to -0.58)	-0.07 (-0.40 to -0.27)	-0.77*** (-0.88 to -0.60)	-0.65*** (-0.81 to -0.40)	0.61*** (0.35 to 0.78)	0.84*** (0.70 to 0.91)

* P <0.05; ** P <0.01; *** P <0.001.

CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric reducing ability of plasma; FOX, ferrous oxidation-xylenol orange; PON1, paraoxonase 1; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant capacity.

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