

## Total oxidant and antioxidant capacities, nitric oxide and malondialdehyde levels in cats seropositive for the feline coronavirus

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**ABSTRACT:** Feline coronavirus (FCoV) is a highly contagious virus that is ubiquitous in multicat environments and may induce oxidative stress. This virus commonly causes an asymptomatic infection, which can persist in certain individuals. Sporadically and unpredictably, FCoV infection leads to feline infectious peritonitis (FIP), a highly fatal systemic immune-mediated disease. There are no data in the veterinary literature relating to oxidative stress in FCoV. Antioxidant capacity (TAC) can be attributed to single components in the defence systems against free radicals. The measurement of the total oxidant status (TOS) accurately reflects the oxidative status of blood plasma or serum. Nitric oxide (NO) acts as a free radical and contributes to host defences against oxidation. Malondialdehyde (MDA) is a reliable and commonly used marker of overall lipid peroxidation levels and the presence of oxidative stress. This study aimed to determine levels of oxidative stress markers, serum TAC, total oxidant capacity (TOC), NO and serum MDA in 24 cats seropositive for FCoVs and 15 cats seronegative for FCoVs. Significantly higher serum TOC, NO and MDA levels were found in seropositive animals ( $P < 0.001$ ,  $P < 0.05$  and  $P < 0.001$ , respectively) than in seronegative animals. In contrast, serum TAC levels were found to be significantly lower in seropositive cats compared with seronegative cats ( $P < 0.001$ ). The results of the present study suggest that FCoV seropositivity is associated with oxidative stress and decreased antioxidant status.

**Keywords:** oxidative stress; antioxidants; free radicals; total oxidant status

Feline coronaviruses (FCoVs) belong to the family Coronaviridae which is of considerable importance in veterinary medicine. These viruses affect both wild and domestic cats (Addie et al. 2003; Pedersen 2009).

Viruses within the Coronaviridae family infect and often cause a mild or sometimes apparently symptomless enteric and respiratory disease, especially in kittens, and are also associated with a lethal, systemic disease known as feline infectious peritonitis (FIP) (Pedersen 1987; Pedersen 1989; Addie and Jarrett 2001; Rohrbach et al. 2001).

While potentially toxic, reactive oxygen species (ROS) are generated through normal oxidative metabolism, and ROS in low concentrations is necessary for some physiological processes (Gutteridge and Halliwell 1990; Kohen and Nyska 2002). However, oxidative stress has been implicated in the pathogen-

esis of many diseases and inflammatory conditions. It results from an oxidant/antioxidant imbalance, an excess of oxidants or a depletion of antioxidants (Miller et al. 1993; MacNee 2000). Oxidative stress is a secondary aggravating factor in most diseases. Furthermore, an important degree of negative correlation between total antioxidant capacity (TAC) and total oxidant capacity (TOC) in animals suffering from diseases that cause oxidative stress has been reported (Yanik et al. 2004). Oxidative defence mechanisms against ROS, although activated, might not be efficient enough and clinical symptoms of illness may occur (Gutteridge 1993).

Excessive formation of free radicals and concomitant damage at the cellular and tissue level are controlled by antioxidant defence systems (Halliwell 1999), which act in synergy (Gutteridge and Halliwell

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1990). The pathogenesis of several viral diseases such as acquired immunodeficiency syndrome (AIDS), influenza and hepatitis in humans has been linked to oxidative stress (Semba and Tang 1999), and significantly higher levels of lipid peroxides, reduced vitamin-C, and serum selenium levels have been reported in HIV-positive patients (Allard et al. 1998). Oxidative stress has been reported in several infectious or non-infectious diseases of domestic animals (Rodriguez et al. 2011). However, no report has yet been published on the possible involvement of oxidative stress in coronavirus in cats.

The antioxidant capacity of tissues can be attributed to individual components in the defence system against free radicals, which can be measured and used to calculate TAC (Kankofer et al. 2005). The plasma or serum concentrations of antioxidants can be individually determined, but this procedure is time-consuming, labour-intensive and costly, and requires complicated techniques. TAC on the other hand reflects the total antioxidant status of serum; a measurement method has recently been developed (Erel 2004b). In this method, TAC of sera which acts especially against potent free radical reactions which cause strong oxidative damage of biomolecules such as lipids, proteins and DNA, is measured (Kosecik et al. 2005).

Nitric oxide (NO) is generated from the terminal guanidine nitrogen atom of L-arginine by NO synthase (Marletta 1989), and is released from a variety of cells (Degroote and Fang 1999; Gokce and Woldehiwet 2002). NO is an important molecule involved in physiological and pathological processes in mammals. It can be protective or hazardous for organs or tissues in which it is present in biological fluids (Zelnickova et al. 2008). It has been reported that NO has pro-inflammatory and injurious effects on several systems or organs due to the formation of the oxidant peroxynitrite (Bartosz 1996; Van Der Vliet et al. 2000). NO is known to play a major role in the primary defence against several species of bacteria (Degroote and Fang 1999), viruses (Kreill and Eibl 1996) and parasites (Vespa et al. 1994).

There are no data available in the veterinary literature relating to oxidative stress in cats seropositive for feline coronavirus. Therefore, the objective of this study was to determine whether markers of oxidative stress, serum total antioxidant capacity (TAC), total oxidant capacity (TOC), total nitric oxide (NO) and malondialdehyde (MDA) are ab-

normal in cats seropositive for FCoV compared with cats seronegative for FCoVs.

## MATERIAL AND METHODS

**Selection of animals.** Twenty-four cats seropositive for FCoVs and 15 cats seronegative for FCoVs of various breeds, both sexes, aged between six months and two years, were used in this study. Fourteen male cats and 10 female cats were seropositive, while eight male and seven female cats were seronegative. The cats were selected from those that were brought to the Department of Internal Medicine, Faculty of Veterinary Medicine, Istanbul University in the years 2013–2014. Thirty cats with a clinical suspicion of FCoV seropositivity were speed tested and the seropositivity was confirmed. The same cats were speed-tested for FCoV again two weeks later, and 24 cats showed seropositivity. Fifteen seronegative cats were also speed-tested again after two weeks, and their seronegativity was confirmed. The animal groups were based on the speed test results (seropositive or seronegative). Ten of the FCoV-seropositive cats in our study (42%) exhibited one or several very mild clinical symptoms such as anorexia, diarrhoea, dehydration, weight loss, respiratory problems, lethargy, ocular and nasal discharge. Turkey is a country with many stray cats and people usually choose their pets from among them. This increases the risk of viral diseases such as FCoV, and we see this in the cats that are brought to our clinic. Fourteen of the seropositive animals in this study (58%) showed no clinical symptoms whatsoever. Because FCoV is widespread and because oxidative stress parameters in cats with viral diseases had not been determined in our country previously, we decided to carry out this study in cats seropositive for FCoV.

The mild clinical symptoms shown by ten of our FCoV-seropositive cats are found in felines with diseases such as feline panleucopenia (FPL), feline herpes virus (FHV), and feline calicivirus (FCV). However, the cats had been vaccinated against those diseases and did not exhibit the other symptoms usually associated with them (pancytopenia, jaundice, uveitis, multifocal neurological signs, oral ulceration, periodontitis, gingivitis, fever etc.). Also, their haemogram and biochemical blood values were within the reference range values, and most cats are still alive. Based on those findings,

and most importantly, on the seropositivity for FCoV we could make a disease-related distinction. Animals with positive test results were included in this study. Animals brought for vaccination underwent a general examination, and those that tested negative for FCoV constituted our control group. The healthy cats showed no symptoms of any disease and were in good physical condition.

The seropositive cats comprised those that had been treated for internal and external parasites and had been vaccinated with tricat trio vaccination against FHV, FCV and FPL, as well as against the feline leukaemia virus (FeLV), and the feline immunodeficiency virus (FIV). Seronegative cats comprised those that had been treated for internal and external parasites; a vaccination programme had been initiated for these parasites and was ongoing.

The study was not focused on FIP. The FCoV-seropositive cats of our study did not have FIP. However, the cats were brought to our clinic from time to time for vaccinations and for health checks and we talked to their owners. From our observations we arrived at the conclusion that most cats led a normal regular life.

**Collection of blood, serum samples, complete blood count (CBC) and blood serum biochemistry and FCoV test.** Blood samples were collected by jugular or cephalic venipuncture from each cat. Two millilitres of blood without anticoagulant were centrifuged at  $1800 \times g$  for 5 min to separate the serum. The serum samples were then tested with Agrolabo S.p.A.'s Biopronix FCoV IC (Immunochromatographic one step test for the detection of feline coronavirus antibodies). The cats were tested twice for seropositivity and seronegativity at two week intervals. The serum samples taken for the measurement of TOC, TAC, NO and MDA were stored at  $-20^{\circ}\text{C}$  and processed within two months. All oxidative stress parameters were determined twice.

Complete blood count (CBC), blood serum biochemistry (serum glucose, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, globulin) were performed in all patients.

## Estimations

**Total antioxidant capacity (TAC).** TAC values were determined using a novel automated colori-

metric measurement method developed by Erel (2004a). (TAC assay kit, Rel Assay Diagnostic). In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colourless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in colour. Upon the addition of sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the sample, preventing the colour change and thereby providing an effective measure of the total antioxidant capacity of the sample. Briefly, 30  $\mu\text{l}$  serum and 500  $\mu\text{l}$  reagent 1 (acetate buffer 0.4 mol/l, pH 5.8) were added to the test tube and stirred. Seventy five  $\mu\text{l}$  reagent 2 (acetate buffer 0.4 mol/l, and ABTS 30 mmol/l) were then added to the mixture. The absorption of the solution at 660 nm was measured 30 s (value A1) and 5 min (value A2) after mixing. The results are expressed as mmol Trolox equivalents/l (Eq/l) for serum.

**Total oxidant capacity (TOC).** TOC was determined using a novel automated measurement method, developed by Erel (2005) (TOC assay kit, Rel Assay Diagnostic). Oxidants present in the sample oxidise the ferrous ion – O-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a coloured complex with xylenol orange in an acidic medium. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. Briefly, 75  $\mu\text{l}$  serum and 500  $\mu\text{l}$  reagent 1 ( $\text{H}_2\text{SO}_4$  25mM, pH 1.75) were added to the test tube and stirred. Twenty five  $\mu\text{l}$  reagent 2 ( $\text{H}_2\text{SO}_4$  25mM, pH 1.75, ferrous ion 5mM and O-dianisidine 10mM) were added to the mixture. The absorption of the solution at 660 nm was measured 30 s (value A1) and 5 min (value A2) after mixing. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of  $\mu\text{mol H}_2\text{O}_2$  equivalent/l for serum.

**Nitric oxide (NO).** The nitric oxide (total), detection kit (Enzo Life Science) is a complete kit for the quantitative determination of total NO in biological fluids. The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme nitrate reductase, followed by the colorimetric detection of nitrite as a coloured azo dye product of the Griess reaction that absorbs visible light at 540 nm. Briefly,

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200 µl reaction buffer were added to the blank tube (duplicate), and 50 µl sample, 25 µl NADH, and 25 µl nitrate reductase to the sample tube (duplicate). The tubes were incubated for 30 min at 37 °C. Thereafter, 50 µl Griess I and 50 µl Griess II were added to the sample tubes, stirred, and left to stand at room temperature for 10 min before their absorption was measured at 540 nm against the blank. A standard curve was used to calculate the concentration in µmol/l.

**Malondialdehyde (MDA).** The changes in MDA levels in serum samples were measured spectrophotometrically with a method modified by Placer et al. (1966). MDA is the end product of the peroxidation of fatty acids with three and more double bonds. It reacts with thiobarbituric acid (TBA) producing a pink-coloured complex which is measured photometrically at a wavelength of 532 nm. MDA is neither a specific nor a quantitative indicator for fatty acid oxidation; however, it is well-correlated with the degree of lipid peroxidation. Briefly, 250 µl serum and 2.25 ml colouring reagent (a mixture of three parts trichloroacetic acid and one part thiobarbituric acid) were added to the sample tube, while 250 µl physiological serum and 2.25 ml colouring reagent were added to the blank tube. The tubes were heated in a boiling water bath for 20 min. After cooling, the tubes were centrifuged for 5 min at 2500 rpm, and the supernatant was then measured at 532 nm against the blank.

**Statistical analysis.** The Mann-Whitney U-test was used for inter-group comparison. The statis-

tical analysis of the data was carried out with the SPSS software 11.0.

## RESULTS

### Clinical symptoms, complete blood count (CBC) and blood serum biochemistry

Ten of the FCoV-seropositive cats in our study (42%) exhibited one or more very mild clinical symptoms such as anorexia, diarrhoea, dehydration, weight loss, respiratory problems, lethargy, ocular and nasal discharge. Such symptoms are found in cats with diseases such as FPL, FHV, and FCV. However, the cats had been vaccinated against those diseases and did not exhibit the other symptoms usually associated with them (pancytopenia, jaundice, uveitis, multifocal neurological signs, oral ulceration, periodontitis, gingivitis, fever etc.). Also, their haemogram and biochemical blood values were within the reference value range, and most cats are still alive. Based on those findings, and most importantly, on the seropositivity for FCoV we could make a disease-related distinction.

All the animals in the study were found to be within the normal range of CBC parameters. Biochemical blood serum values (Serum glucose, BUN, creatinine, ALT, AST, ALP, total protein, albumin, globulin) were determined for every patient. In our study, these variables were found not to be significant.

Table 1. Mean values ± SE of serum total antioxidant capacity (TAC), total oxidant capacity (TOC) total nitric oxide (NO) and serum malondialdehyde (MDA) levels in 24 cats seropositive for the feline coronavirus and 15 cats seronegative for the feline coronavirus

Parameter	Group	n	Mean ± SE		Median	Minimum	Maximum
TAC (mmol Trolox Equiv/l)	FCoV seropositive	24	1.42***	0.03	1.41	1.17	1.69
	FCoV seronegative	15	1.77	0.06	1.73	1.47	2.21
TOC (µmol H <sub>2</sub> O <sub>2</sub> Equiv/l)	FCoV seropositive	24	2.31***	0.07	2.33	1.73	3.01
	FCoV seronegative	15	1.86	0.06	1.90	1.48	2.21
NO (µmol/l)	FCoV seropositive	24	69.31*	5.17	66.05	41.34	169.97
	FCoV seronegative	15	51.26	2.95	49.91	34.65	75.90
MDA (nmol/ml)	FCoV seropositive	24	37.76***	1.30	37.61	23.75	52.10
	FCoV seronegative	15	30.77	1.08	29.91	22.15	37.24

TAC= total antioxidant capacity, TOC = total oxidant capacity, NO = nitric oxide, MDA = malondialdehyde; \**P* < 0.05,

\*\*\**P* < 0.001



The FCoV-seropositive cats of our study did not have FIP. However, the cats were brought to our clinic from time to time for vaccinations and for health checks and we talked to their owners. From our observations we arrived at the conclusion that most cats led a normal regular life.

### Measures of TOC, TAC, NO and MDA

The serum TAC levels were found to be significantly lower in cats seropositive for FCoV compared with cats seronegative for FCoVs ( $P < 0.001$ ). In contrast, the serum TOC, NO and MDA sera levels were found to be significantly higher in cats seropositive for FCoV compared with cats seronegative for FCoVs ( $P < 0.001$ ,  $P < 0.05$  and  $P < 0.001$ , respectively). The results of analysed parameters are presented in Table 1.

### DISCUSSION

This is the first report documenting the involvement of oxidative stress in FCoV-positive cats. Reactive oxygen and nitrogen species play a complex role in many diseases and in metabolic regulation in disease processes and oxidative stress has been implicated in several viral infections in humans and animals. Virus-induced oxidative stress could be mediated by an early phase of the liberation of pro-inflammatory cytokines. Participation of iron in the Fenton reaction *in vivo* leads to the production of more reactive hydroxyl radicals from superoxide radicals and  $H_2O_2$  and results in increased lipid peroxidation (Halliwell 1994).

Oxidative stress occurs either due to excess production of free radicals or inadequate availability of antioxidants, or a combination of both. The imbalance between increased production of radicals and availability of antioxidant molecules may result in increased oxidative stress (Miller et al. 1993; Halliwell and Gutteridge 1999). Oxidative stress also impairs immune system function (Sies 1991).

Despite the significance of oxidative stress in felines, there is a paucity of articles showing the effect of antioxidant supplementation in cats with naturally occurring diseases (Webb et al. 2008b). Research into the effects of antioxidant supplementation to FIV-infected cats led to the conclusion that antioxidant supplements may have potential

benefits for cats with viral infections, and may be used for therapeutic purposes, but that further studies were needed.

In a study on cats with renal insufficiency, Yu and Paetau-Robinson (2006) reported that the addition of the antioxidant vitamins E and C and of  $\beta$ -carotene to animal diet reduced oxidative damage and may be beneficial for afflicted cats. Serum MDA concentrations in cats with renal insufficiency were found to be higher than in healthy cats; however, antioxidant supplements were not found to have an effect on MDA concentrations.

TAC is a biochemical parameter suitable for evaluating the overall antioxidant status of serum and body fluids resulting from antioxidant intake and/or production, and their consumption by normal or increased levels of ROS production. The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, giving an insight into the delicate balance between oxidants and antioxidants *in vivo* (Ghiselli et al. 2000). The results of our study showed statistically significant differences in TAC values between seropositive and seronegative cats, which suggest that systemic antioxidant defence mechanisms are exhausted in seropositive cats.

TAC encompasses a wide spectrum of activities of the measurable exogenous and endogenous antioxidants (Somogyi et al. 2007). Its value expresses the number of antioxidant molecules present in serum (Halliwell 1994; Erel 2005). An individual antioxidant level or activity indicates the antioxidant characteristics of only one antioxidant, whereas TAC may represent the total antioxidant characteristics of all antioxidants found in the sera (Erel 2004b). When the oxidant/antioxidant balance is tilted towards oxidants and oxidative stress arises, there is a significant negative correlation between the TAC and TOC values (Halliwell 1994; Erel 2005). In this study a negative correlation between TAC and TOC values was found, which corresponds to previously reported findings (Halliwell 1994; Erel 2005). The low TAC level in the FCoV-seropositive group can be explained with excessive depletion of the antioxidant capacity caused by free radical elimination.

Oxidative stress has been reported in other species and diseases such as sarcoptic mange, traumatic reticuloperitonitis, theileriosis and anaplasmosis (Guzel et al. 2008; Camkerten et al. 2009; Atakisi et al. 2010).

Nitric oxide, which has several very different biological effects, is strongly involved in the cellular response to infections caused by a wide range of

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viruses (Bi and Reis 1995; Benenica and Courreges 1999). In a study on contagious ecthyma in sheep, Kandemir et al. (2011) reported considerably increased nitric oxide concentrations in diseased animals. A similar result was found in cats seropositive for FCoV in this study. These findings are supported by the literature.

It has been reported that MDA concentrations often rise under oxidative stress (Jareno et al. 1998). However, Webb et al. (2008a) measured MDA concentrations in FIV-infected cats prior to and after the infection and could not find a significant increase in MDA levels for a period of 16 weeks after inoculation. These results suggest that the type of oxidative stress induced by acute FIV infection may not induce changes in MDA concentrations. Coronaviruses and FIV cause viral diseases in cats. However, in contrast to the results reported by Webb et al. (2008a) for FIV-infected cats, in this study the MDA levels in cats seropositive for FCoV were found to increase with oxidative stress.

An increased level of MDA, an end product of lipid peroxidation, has been reported in *B. gibsoni* infection (Murase et al. 1996; Chaudhuri et al. 2008), in Canine Distemper infection (Karadeniz et al. 2008), in dogs with coccidiosis (Kizil and Yuce 2009) and in canine visceral leishmaniasis (Heidarpour et al. 2012). The present findings are in agreement with these reports.

MDA, an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used marker of the overall lipid peroxidation level and the presence of oxidative stress (Moore and Roberts 1998; Grotto et al. 2009). In the present study, the MDA concentrations of FCoV-seropositive cats were found to be significantly higher than cats seronegative for the feline coronavirus. This finding may be explained by the increased ROS production as a result of higher lipid peroxidation in response to oxidative stress and the generation of MDA as an oxidation end product.

In conclusion, the present study is the first to report oxidative stress markers, TAC, TOC, NO and MDA in cats seropositive for feline coronavirus and provides basic data on FCoV seropositivity which will aid future research. It was found that, while the TAC values were reduced, the serum TOC, NO and MDA concentrations markedly increased in cases of seropositivity for FCoV. The reduced level of TAC may reflect a decrease in the antioxidant capacity. It may be hypothesised that during pathogenesis of

FCoV, excess free radicals are produced resulting in oxidative stress which, among other effects, impairs immune system function. We are of the opinion that the administration of antioxidant compounds in addition to the supportive treatment of seropositive cats, which for various reasons have developed immunosuppression, may have a positive impact on treatment by supporting antioxidative metabolism. Further research is needed to elicit the role of oxidative stress in the pathogenesis of FCoV and the possible use of antioxidants for therapeutic and supportive therapy or preventive purposes. The present results should be corroborated by further studies on a larger number of cats in order to determine the exact role of oxidative stress and treatment efficacy of antioxidants in lifetime asymptomatic carriers and shedders and particularly in FIP.

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