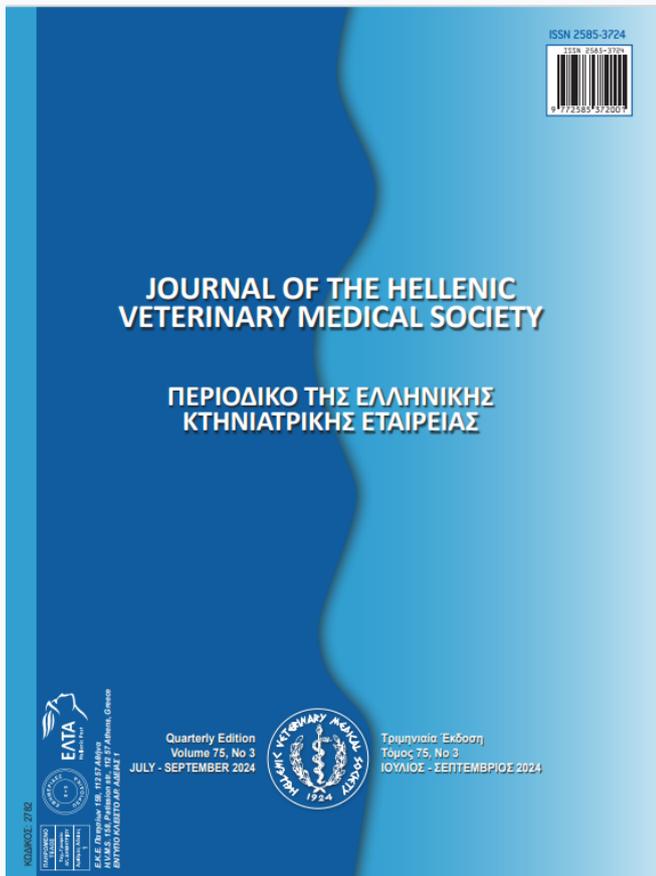


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Oxidative stress markers in canine *Parvovirus* infection

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ABSTRACT: *Parvovirus* infections are among the deadly viral diseases in dogs and sick puppies. Young dogs are more prone to the virus. Antioxidants are important components of the defense system which prevent cell damage by neutralizing reactive oxygen species. Malondialdehyde, enzymatic and non-enzymatic antioxidants are reliable and commonly used marker of oxidative stress. The present study was performed to determine the role of oxidative stress markers in canine *Parvovirus* disease. Thirty Cane Corso male dogs with an average age of 3-6 months were enrolled in this study out of which 15 animals were spontaneously infected with the *Parvovirus* and the other 15 were healthy. The activity of superoxide dismutase (SOD), glutathione peroxidase (GPX), Catalase, total antioxidant capacity (TAC), malondialdehyde, vitamin D and vitamin E levels were measured by standard methods. The increase in malondialdehyde in infected dogs demonstrates that oxidative stress plays a role in the pathogenesis of the *Parvovirus*. In spite of reduction in vitamin D, vitamin E, superoxide dismutase and glutathione peroxidase activity, the markedly elevation in TAC and catalase activity are indicative of a good response by the enzymatic antioxidant system against oxidative stress induced by the *Parvovirus*. In conclusion, oxidative stress is involved in the pathogenesis of *Parvovirus*. Thus, strengthening the antioxidant system can be effective in the prevention and treatment of canine *Parvovirus* infection.

Keywords: Antioxidant system; Dog; Oxidative stress; *Parvovirus*; Vitamin E

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INTRODUCTION

Parvovirus infection is one of the most common causes of morbidity and mortality in dogs [1]. The infection is more prevalent among puppies and young dogs, with symptoms being significantly more severe in puppies compared to adult dogs [2].

By infecting the intestines and bone marrow, Parvovirus causes diarrhea, vomiting, and a reduction in the immune system's ability to fight infections. It also impedes the proliferation of healthy intestinal epithelium. The damage inflicted on absorbing cells leads to a higher incidence of intestinal infections, resulting in diarrhea and dysentery [3]. Additionally, the virus can infiltrate the bone marrow and disrupt its function [2]. Since the bone marrow serves as the primary source of red and white blood cells, its destruction results in white blood cell depletion, weakening the dog's immune system, and heightening the risk of exposure to other infections [4]. The prognosis for survival often hinges on the severity of the clinical signs at the time therapy is initiated [1].

Oxidative stress is the imbalance between free radical production and antioxidant status, leading to oxidative damage to macromolecules such as fats and proteins [5]. In simpler terms, any situation resulting in an increase in the level of reactive oxygen species (ROS), or decreased antioxidant action, or incomplete ROS elimination is termed oxidative stress. ROS are toxic to cells, causing enzyme inactivation, protein denaturation, DNA destruction, and lipid peroxidation [6].

Oxidative stress and inflammatory reactions have been associated with many diseases and inflammatory conditions [7]. Reactive oxygen species (ROS), which are perceived as a defense mechanism of the body, represent one of the earliest cellular responses following successful pathogen recognition. Evidence of systemic oxidative stress manifests as increased lipid peroxidation and depletion of antioxidant status. This is evidenced by the production of increased free radicals in infected animals [8]. Cells undergoing oxidative stress are damaged and release lipid biomarkers and enzymes, forming the basic mechanism of several diseases such as tissue inflammation, infection, obesity, cancer, and chronic disease [9].

Antioxidants protect the body from reactive oxygen species by directly modifying them or inhibiting the activity of oxidizing enzymes, encompassing both enzymatic and non-enzymatic components. The im-

balance between oxidants and antioxidants has been observed in the pathogenesis of enteric viral diseases such as Feline Coronavirus, Bovine Herpesvirus-1, respiratory syndrome, and Rotavirus [8].

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acid peroxidation within cells. The concentration of MDA is commonly recognized as a marker of oxidative stress and antioxidant status in animals [10]. Determining MDA levels in plasma reflects the degree of lipid peroxidation and the concentration of free oxygen radicals, indirectly indicating the presence of oxidative stress [11,12]. Previous research has demonstrated that acute gastroenteritis in dogs is associated with altered erythrocytic lipid peroxidation and the activities of catalase and superoxide dismutase [13].

Rubio et al. (2017) reported that various serum antioxidant biomarkers, such as Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), and paraoxonase 1 (PON1), are decreased in the sera of dogs with idiopathic inflammatory bowel disease (IBD) [14]. Total antioxidant capacity appears to be a suitable biochemical parameter for measuring various antioxidants simultaneously. This parameter reflects the production and increased consumption of antioxidants following elevated levels of oxidative stress. Similarly, Miller et al. (1993) developed a new test to measure total antioxidant capacity (TAC), which encompasses a range of antioxidant enzymes and compounds. The significant advantage of the TAC test is its ability to measure the concentration of various antioxidants present in a biological sample, rather than focusing on a single compound [15].

Vitamin E is an important fat-soluble antioxidant involved in many antioxidant enzyme systems, including superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase, and non-enzymatic agents [16].

Considering the significance of oxidative stress in diseases resulting from cell damage, it is essential to acknowledge the ability of Parvovirus to damage cells. Moreover, the activity of antioxidants in combating reactive oxygen species (ROS) is of particular interest. Thus, the purpose of this study was to evaluate and compare a panel of various serum biomarkers assessing both the antioxidant response and oxidative damage response in the sera of 15 dogs with Parvovirus infection compared to 15 sera from healthy dogs.

MATERIALS AND METHODS

This experimental study was approved by Institutional Animal Care. It was kept and handled according to the European Union Animal Rights. To eliminate the effect of breed differences on the results of this study and due to the availability of Cane Corso dogs, thirty unvaccinated Cane Corso male dogs with an average age of 3-6 months were enrolled. The 30 dogs, including 15 healthy and 15 dogs which had spontaneously been infected with clinical *Parvovirus*, were selected in coordination with several small animal clinics. By CPV-DNA amplification technique involving specific primers for CPV-2 and polymerase chain reaction (PCR), feces from the animals were used to confirm the diagnosis [17]. 15 healthy dogs were selected as a control group. The clinical examination was performed by a specialist and under sterile conditions. Thereafter, blood samples were collected from the cephalic vein of all the dogs. The serum was isolated by centrifugation at 2500 RPM for 15 min at 4°C, and stored at -20°C to later be analyzed.

Total antioxidant capacity was measured by a diagnostic kit made by RANDOX (Randox Laboratories Ltd., Crumlin, County Antrim, UK) according to Miller et al., (1993) and expressed nmol/ml of serum [15]. The measurement is based on the decrease of free radicals (ABTS^{•+} - at 3 min by antioxidants. The ABTS^{•+} radical cation is formed by the interaction of ABTS with ferryl myoglobin radical species, generated by the activation of met myoglobin with hydrogen peroxide. The suppression of the absorbance of the ABTS^{•+} radical cation by serum antioxidants was compared to that from a Trolox (6-hydroxy-2, 5, 7, -tetramethylchroman-2-carboxylic acid), which is included as part of the TAC kit. The results are expressed as nmol/ml of Trolox equivalents. In addition, control serum (Randox, Crumlin, UK) with TAC value of 1.0 to 1.36 nmol/ml was measured in each batch of samples for the estimation of analytical imprecision (between-batch coefficient of variation). [15].

SOD activity was evaluated using diagnostic RANSOD kit manufactured by RANDOX (Randox Laboratories Ltd., Crumlin, County Antrim, UK) according to Arthur and Boyne, (1985) and expressed in U of SOD /ml of serum [18]. In this method xanthine and xanthine oxidase was added to serum to produce superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to generate a red formazan dye. SOD activity was then assayed by the degree of inhibition

of this reaction spectrophotometrically at 505 nm.

GPX activity was assayed using a diagnostic RANSEL kit manufactured by RANDOX (Randox Laboratories Ltd., Crumlin, County Antrim, UK) according to Paglia and Valentine, (1967) and explanation in U of GPX/ml of serum [19]. In this method glutathione (GSH) was oxidized by cumene hydroperoxide catalysed by GPX. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The reduction in absorbance was then assayed by spectrophotometry at 340 nm.

MDA was assayed using a diagnostic ELAB-SCIENCE kit (Elabsience Laboratories, USA), by colorimetric method and the results were stated in nmol/ml. In brief, malondialdehyde (obtained from the catabolite of lipid peroxide) reacts with thiobarbituric acid (TBA) and produces a red compound, which has a maximum absorption peak at 532 nm [20].

The activity of catalase was assayed by a rapid, spectrophotometric assay using a diagnostic ELAB-SCIENCE kit (Elabsience Laboratories, USA). This method is a combination of optimized enzymatic conditions and the spectrophotometric evaluation of hydrogen peroxide based on formation of its stable complex with ammonium molybdate [21].

The concentration of Vitamin D and E were evaluated by chromatography (ng/ml). The chromatography was carried out using a Shimadzu system (Columbia, MD) composed of two LC-10ADvp pumps, SIL 10ADvp autosampler, CTO-10ASvp column oven, RF-10Ax1 fluorescence detector, and SCL-10ADvp controller. The data was achieved by CLASSvp software, v. 5.03. The separation was acquired on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 µm particle size) connected with a guard column RX-C8 (12.5 mm × 4.6 mm i.d., 5 µm particle size) (Agilent Technologies, Palo Alto, CA). A Shimadzu UV-160A spectrophotometer was used to determine the absorbance of standard solutions.

All the data were analyzed using IBM SPSS Statistics for Windows (Version 20.0. Armonk, New York: IBM Corporation) [22]. Distributions of the residuals of continuous data were evaluated for normality by the Kolmogorov-Smirnov test. Residual variances were normally distributed for all the parameters. For the detection of possible statistically significant differ-

ences between healthy and infected groups, Duncan and student's tests were used. Pearson's correlation coefficient was evaluated to determine statistically significant relationships among different parameters. Statistical significance was accepted at $P < 0.05$.

RESULTS

In this research, thirty Cane Corso male dogs with an average age of 3-6 months and an average weight of 18 - 28kg were studied and the results are presented in Tables 1 to 3 and figure 1. The T-test indicates that there is a statistically significant reduction in the mean concentrations of superoxide dismutase in level $P < 0.001$ in sick dogs (Table 1). The mean glutathione peroxidase activity ($P < 0.0001$), the mean

concentrations of vitamin D ($P < 0.0001$), and the mean concentration of vitamin E ($P < 0.0001$) were significantly lower in infected dogs compared to the healthy ones (Table 1). Also, the mean malondialdehyde concentration ($P < 0.0001$), total antioxidant capacity concentration ($P < 0.0001$) and catalase ($P < 0.01$) were markedly higher in infected dogs (Table 1). There was a significant positive correlation between the mean activity of catalase and the total antioxidant capacity ($P < 0.0001$) and a significant negative correlation was seen between the mean concentration of glutathione peroxidase and the TAC ($P < 0.0001$) in healthy dogs. In infected dogs, a significant positive correlation was observed between the mean activity of catalase and the mean concentration of malondialdehyde ($P < 0.0001$).

Table1 The mean and standard error of oxidative stress markers in healthy and patient groups

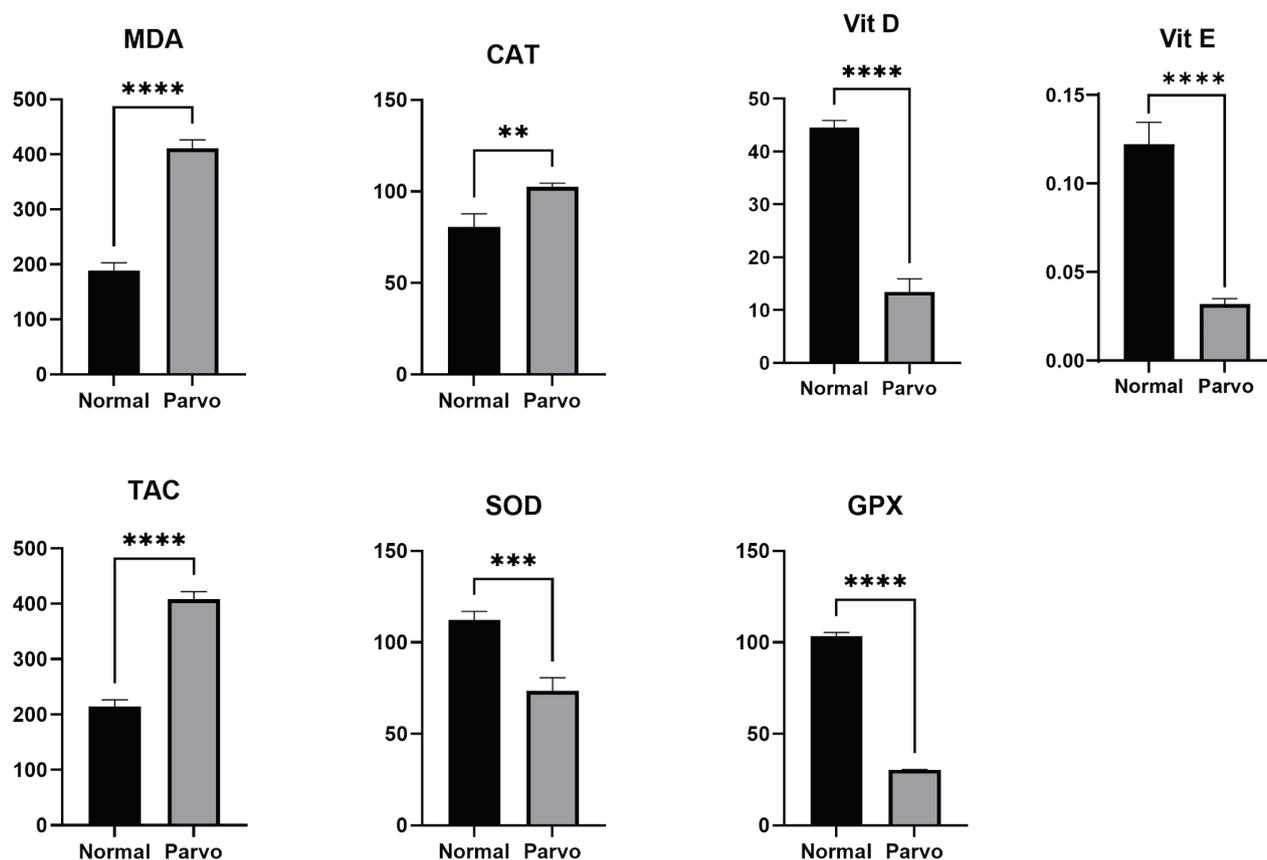
Group		CAT	MDA	TAC	VitD	VitE	SOD	GPX
Normal	Mean	80.6571	189.3559	215.1996	44.6023	.1222	112.3539	103.6265
	Std. Error of Mean	7.17203	13.59463	11.07739	1.28851	.01232	4.53246	.82743
	Minimum	45.34	139.21	153.41	38.40	.07	93.94	99.64
	Maximum	107.06	259.00	253.06	52.02	.19	133.33	106.99
Disease	Mean	102.6000	411.5563	408.5882	13.4861	.0320	73.5574	30.4421
	Std. Error of Mean	1.99203	15.10701	13.43640	2.46982	.00288	7.07859	.20932
	Minimum	94.20	341.06	341.06	6.46	.02	33.33	29.63
	Maximum	111.34	497.21	467.53	27.82	.05	98.18	31.50

Table2 The correlation coefficient between the mean of different parameters in the healthy group

		CAT	MDA	VitD	TAC	VitE	SOD	GPX
CAT	Pearson Correlation	1	.440	-.806**	.384	-.090	-.123	-.040
	Sig. (2-tailed)		.204	.005	.273	.805	.735	.914
	N	10	10	10	10	10	10	10
MDA	Pearson Correlation	.440	1	.071	.171	.214	-.698*	-.054
	Sig. (2-tailed)	.204		.847	.637	.553	.025	.882
	N	10	10	10	10	10	10	10
VitD	Pearson Correlation	-.806**	.071	1	-.359	.112	-.362	.009
	Sig. (2-tailed)	.005	.847		.309	.758	.304	.979
	N	10	10	10	10	10	10	10
TAC	Pearson Correlation	.384	.171	-.359	1	-.109	-.019	.014
	Sig. (2-tailed)	.273	.637	.309		.763	.958	.970
	N	10	10	10	10	10	10	10
VitE	Pearson Correlation	-.090	.214	.112	-.109	1	-.521	.300
	Sig. (2-tailed)	.805	.553	.758	.763		.123	.400
	N	10	10	10	10	10	10	10
SOD	Pearson Correlation	-.123	-.698*	-.362	-.019	-.521	1	.129
	Sig. (2-tailed)	.735	.025	.304	.958	.123		.723
	N	10	10	10	10	10	10	10
GPX	Pearson Correlation	-.040	-.054	.009	.014	.300	.129	1
	Sig. (2-tailed)	.914	.882	.979	.970	.400	.723	
	N	10	10	10	10	10	10	10

Table 3 The correlation coefficient between the mean of different parameters in Parvovirus infected group

		CAT	MDA	VitD	TAC	VitE	SOD	GPX
CAT	Pearson Correlation	1	-.365	.203	-.220	.024	.465	-.653*
	Sig. (2-tailed)		.300	.573	.541	.948	.176	.041
	N	10	10	10	10	10	10	10
MDA	Pearson Correlation	-.365	1	-.024	-.231	-.187	-.009	.182
	Sig. (2-tailed)	.300		.948	.520	.604	.980	.614
	N	10	10	10	10	10	10	10
VitD	Pearson Correlation	.203	-.024	1	.217	.034	.167	.248
	Sig. (2-tailed)	.573	.948		.547	.925	.645	.489
	N	10	10	10	10	10	10	10
TAC	Pearson Correlation	-.220	-.231	.217	1	.674*	-.545	.100
	Sig. (2-tailed)	.541	.520	.547		.033	.103	.783
	N	10	10	10	10	10	10	10
VitE	Pearson Correlation	.024	-.187	.034	.674*	1	-.258	-.221
	Sig. (2-tailed)	.948	.604	.925	.033		.472	.539
	N	10	10	10	10	10	10	10
SOD	Pearson Correlation	.465	-.009	.167	-.545	-.258	1	-.033
	Sig. (2-tailed)	.176	.980	.645	.103	.472		.927
	N	10	10	10	10	10	10	10
GPX	Pearson Correlation	-.653*	.182	.248	.100	-.221	-.033	1
	Sig. (2-tailed)	.041	.614	.489	.783	.539	.927	
	N	10	10	10	10	10	10	10



** : $P < 0.01$, *** : $P < 0.001$, **** : $P < 0.0001$

Fig.1: The levels of studied parameters in normal and disease groups.

DISCUSSION

Enzymatic and non-enzymatic antioxidant systems play a crucial role in the animal defense mechanism, safeguarding cells and their components against oxidizing agents [23]. In various diseases, pathogens can directly or indirectly elevate the concentration of reactive oxygen species (ROS) and modulate the activity of antioxidants [4, 24].

Rubio et al. (2017) found significantly higher concentrations of ferrous oxidation-xylenol orange (FOX), thiobarbituric acid reactive substances (TBARS), and reactive oxygen species production (ROS) in the sera of dogs with inflammatory bowel disease (IBD) compared to healthy dogs ($P < 0.0001$). The researchers suggested that oxidative stress might play a significant role in the pathogenesis of canine IBD [14].

Another study revealed that acute cases of gastroenteritis in dogs are linked to altered erythrocytic lipid peroxidation, as evidenced by changes in malondialdehyde (MDA) concentration. Furthermore, the activities of catalase and superoxide dismutase are affected in this disease. These alterations in oxidative stress indices are more pronounced in cases involving canine Parvovirus compared to parvo-negative cases [13].

In this study, statistical analysis revealed a significant increase in the mean concentration of malondialdehyde in dogs infected with Parvovirus ($P < 0.0001$). Since malondialdehyde is a product of lipid peroxidation, its elevation in dogs with ongoing Parvovirus infection indicates the involvement of oxidative stress in the pathogenesis of this virus. Elsayed et al. (2020) reported that in dogs with CPV-2 infection, elevated levels of MDA and H₂O₂ associated with oxidative stress state, along with alterations in antioxidant biomarkers such as SOD, GPX, catalase, and trace minerals such as Zn, Cu, and iron were recorded to counteract the expected cellular damage [25].

The statistically significant increase in malondialdehyde in our study suggests that Parvovirus increases the production of reactive oxygen species (ROS), resulting in the following outcomes: (a) damage to intestinal cells and interference with the intestine's absorbing ability, leading to diarrhea, and (b) damage to the bone marrow, resulting in a decrease in white blood cell count [25,26]. The latter finding further weakens the infected animal's immune system, increasing susceptibility to infection [26].

Consistent with our findings, Ukwueze et al. (2020) reported a significantly higher mean concentration of serum MDA in puppies infected with canine Parvovirus type 2 compared to healthy puppies [8]. In another study, acute cases of gastroenteritis in dogs were associated with altered erythrocytic lipid peroxidation, as evidenced by the estimation of malondialdehyde (MDA) concentration [14]. Harizan et al. (2021) reported that oxidative stress induces a significant increase in MDA and nitrite plus nitrate (NO_x) concentrations in comparison to the decreased activity of glutathione S-transferases (GST) and catalase in dogs affected by parvoviral enteritis [27].

In this research, the response of the antioxidant defense system against *Parvovirus* was investigated by analyzing enzymatic and non-enzymatic antioxidant concentrations. The mean concentrations of TAC ($P < 0.0001$) and catalase activity ($P < 0.01$) were significantly higher in the experimental group relative to the control group. However, superoxide dismutase ($P < 0.001$) and glutathione peroxidase activities ($P < 0.0001$) were significantly lower in infected dogs in compared to healthy dogs (Table 1, Fig.1).

Consistent with our findings, Ukwueze et al. (2020) observed a significant reduction in SOD activity in puppies infected with canine Parvovirus type 2, but no significant changes in catalase and GPX activity, which differs from our research findings on catalase and GPX activity. These differences between the studies may be attributed to variations in the age, breed of the studied dogs, and the course of the disease [8].

In contrast, Panda et al. (2009) noted higher activities of SOD and CAT in dogs affected by canine parvoviral gastroenteritis [28]. These discrepancies in findings could be associated with differences in breeding, age, and sex among the studied dogs. Elsayed et al. (2020) reported that antioxidant supplementation may bolster the body's defense mechanism and reduce stress conditions in dogs with canine *Parvovirus* enteritis [25].

Kataria et al. (2020) observed that CPV-positive dogs endure higher oxidative stress than CPV-negative dogs [29]. In another study, all gastroenteritis dogs (both CPV-positive and negative) exhibited reduced activities of antioxidant enzymes and trace elements [28].

Based on the findings of our study, there was a sta-

tistically significant decrease in the mean serum concentrations of vitamin D ($P < 0.0001$), vitamin E ($P < 0.0001$), and glutathione peroxidase ($P < 0.0001$) in dogs infected with *Parvovirus*, indicating a relatively robust antiviral response. The significant reduction in the concentrations of vitamins D and E in infected dogs suggests that these vitamins are utilized in neutralizing free radicals. Additionally, it has been demonstrated that these vitamins protect the cell membrane against oxidative agents and free radicals [30]. Bohn (2019) reviewed previous research on the effect of carotenoids (precursors of vitamin A) on chronic diseases caused by oxidative stress and concluded that there is an inverse correlation between carotenoid concentrations and the incidence of these diseases [31]. Jewells et al. (2002) reported that high concentrations of vitamin E in the serum prevent the development and spread of skin diseases and enhance the healing process [32]. These findings underscore the crucial role of antioxidants in the treatment protocol [7].

According to the findings of the present study, *Parvovirus* infection is associated with oxidative stress and changes in the antioxidant system. In order to neutralize ROS non-enzymatic antioxidants like vitamins D and vitamin E are consumed. The outcome of this action presents a significant reduction in the serum concentration of these vitamins. Therefore, strengthening the non-enzymatic antioxidant system can be effective in the prevention and treatment of this disease. At the same time, the enzymatic antioxidant system is activated and the synthesis of enzymes is stimulated to fight the ROS. However, in the process of cellular protection i.e. antioxidants against oxidative stress, the total antioxidant capacity and catalase show statistically significant increase in dogs with *Parvovirus* infection.

Hence, there are changes observed in the concentration of various enzymatic antioxidants in dogs infected by *Parvovirus*. These findings may be indicators of the possible differences in the defense system considering the production of antioxidants which functions to counteract the elevated level of ROS production. Also, significant increase in the serum total antioxidant capacity and catalase in dogs infected with the *Parvovirus* show an adequate response of the antioxidant system in neutralizing ROS. Antioxidants were used to neutralize free radicals [30]. Despite this, the concentration of antioxidants was significantly increased in dogs infected with the *Parvovirus* (Table

1). Moreover, the significant reductions in the serum activities of glutathione peroxidase and superoxide dismutase in animals infected with the *Parvovirus* indicate that these antioxidant enzymes are the most consumed compared to the other enzymes. Furthermore, the consumption of these antioxidants is more than their production (Table 1). There is a significant increase in the serum activity of catalase in infected animals relative to the one seen in healthy dogs. This suggests that the increase in its production is more than the rise in its consumption due to the neutralization of ROS produced by the presence of *Parvovirus*. The increase in catalase in infected dogs may indicate an increase in production relative to its consumption, but in the case of glutathione peroxidase and superoxide dismutase this is the opposite.

The presence of a significant positive correlation between the mean concentrations of catalase and TAC in healthy dogs ($P < 0.0001$) suggests a direct relationship between catalase production and total antioxidant capacity in healthy animals. Conversely, a significant negative correlation observed between the mean concentrations of glutathione peroxidase and TAC in healthy dogs ($P < 0.0001$) indicates that the production of this enzyme in the healthy state is opposite to that of TAC.

In infected dogs, a significant positive correlation was observed between the mean activities of catalase and malondialdehyde ($P < 0.0001$). This suggests that the rate of catalase synthesis is proportional to the production of free radicals during *Parvovirus* infection. Statistical tests showed no significant correlation between the mean concentrations of other parameters in healthy and infected dogs.

In summary, the pathogenesis of *Parvovirus* infection is associated with oxidative stress, impacting enzymatic antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, as well as non-enzymatic antioxidants like vitamin D and vitamin E. While the antioxidant system's response to reactive oxygen species (ROS) is acceptable, bolstering this system with antioxidants may effectively prevent and treat cellular damage caused by *Parvovirus*. In line with this, Gaykwad et al. (2018) demonstrated that treatment with N-acetylcysteine (NAC) in *Parvovirus*-infected dogs significantly improved glutathione S-transferase (GST) activity, decreased both nitrite plus nitrate (NOx) and MDA concentrations on days 3 and 5, leading to progressive recovery of the leukocyte count [12].

CONCLUSION

The MDA is elevated in canine *Parvovirus* infection due to an increase in lipid peroxidation. The production of antioxidants in subjects infected with the *Parvovirus* is great enough that despite its involvement in the neutralization of ROS, the total serum antioxidant capacity shows a marked significant increase. Vitamin D and vitamin E support cells against oxidative stress produced by the presence of *Parvovirus*, resulting in a significant reduction in their serum concentrations. In conclusion and based on the find-

ings of this study, the pathogenicity of the canine *Parvovirus* infection is in dogs associated with oxidative stress although the animal's antioxidant system reacts well.

CONFLICT OF INTEREST

None declared

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