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Amelioration of oxidative stress using *N*-acetylcysteine in canine parvoviral enteritis

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Previously, antioxidants have not been evaluated for treatment of parvoviral diarrhea in dogs. In this study, antioxidant potential of *N*-acetylcysteine (NAC) in dogs infected with canine parvovirus with a nonblinded randomized clinical trial has been carried out. A total 18 parvo-infected dogs were randomly divided into two groups: nine parvo-infected dogs were treated with supportive treatment and nine parvo-infected dogs were treated with NAC along with supportive treatment. Simultaneously, nine healthy dogs were kept as healthy control. In parvo-infected dogs, marked hemoconcentration, leucopenia, neutropenia and oxidative stress were noticed compared to healthy dogs. The NAC treatment progressively improved the leukocyte, neutrophil, monocyte, and eosinophil counts over the time in parvovirus-infected dogs compared to dogs that received only supportive treatment. In addition, NAC treatment significantly improved glutathione S-transferase (GST) activity and decreased nitrite plus nitrate (NOx) and malondialdehyde (MDA) concentrations on day 3 and 5 compared to supportive treatment in parvo-infected dogs. However, supportive treatment alone failed to ameliorate oxidative stress in the infected dogs till day 5. The results of this study suggest that NAC represents a potential additional treatment option that could be considered to improve the health condition and minimize the duration of hospitalization in case of canine parvoviral diarrhea.

1 | INTRODUCTION

Canine parvovirus infection occurs worldwide in domestic dogs and wild canids. Incidence is higher in animal shelters, pet stores, and breeding kennels. Although CPV-2 can affect all breeds of dogs at any age, severe infection is most common in puppies between 6 weeks and 4 months old (Hueffer et al., 2003). The disease condition has been complicated further due to emergence of a number of variants, namely CPV-2a, CPV-2b and CPV-2c over the years and involvement of domestic and wild canines (Buonavoglia et al., 2001; Martella et al., 2004). In absence of suitable antiviral therapy to protect the dogs against CPV infection, supportive treatment is the only option to reduce the mortality due to this disease. Therapeutic efficacy of recombinant feline interferon -ω and recombinant canine granulocyte colony-stimulating factor has been evaluated in CPV, but there are limitations like commercially nonavailability in all the places and exorbitant price make these therapeutics for regular use (De Mari, Maynard, Eun, & Lebreux, 2003; Duffy, Dow, Ogilvie, Rao, & Hackett, 2010; Mylonakis, Kalli, & Rallis, 2016). Oseltamivir, a neuraminidase inhibitor, has also been tried for the treatment of CPV without any substantial benefit in terms of survival or duration of hospitalization (Savigny & Macintire, 2010).

In recent years, oxidative stress has been paid much attention due to its pivotal role in the pathogenesis of viral diseases (Beck, Handy, & Levander, 2000; Schwarz, 1996; Valyi-Nagy & Dermody, 2005). The oxidative stress occurs due to imbalance of production of reactive oxygen/nitrogen species or neutralizing antioxidant enzymes (Kim, Kim, & Hahm, 2012; Lykkesfeldt & Svendsen, 2007). Free radical-induced oxidative stress causes oxidation of the polyunsaturated fatty acids of erythrocyte cell membrane, which results in lipid peroxidation. Malondialdehyde (MDA), the principal and most studied product of polyunsaturated fatty acid peroxidation, is a classical marker of free radical-induced cell damage (Del Rio, Stewart, & Pellegrini, 2005; Kuhn & Borchert, 2002). Determination of MDA in plasma indicates

the degree of lipid peroxidation and the concentration of free oxygen radicals indirectly (Deger, Deger, Bicek, Ozdal, & Gul, 2009). Nitric oxide (NO), another essential messenger molecule under physiological concentrations, becomes a free radical to generate peroxynitrite anion (ONOO) under excess and is considered as a marker of oxidative stress (Pacher, Beckman, & Liaudet, 2007; Pierini & Bryan, 2015). Glutathione S-transferase (GST), a metabolic isozyme, plays a crucial role in defense mechanisms against oxidative injury (Röth et al., 2011). GST protects cells from oxidative stress by detoxifying the secondary reactive oxygen species (ROS) produced when ROS react with cellular constituents (Veal, Toonem, Jones, & Morgan, 2002).

The oxidant/antioxidant imbalance has been reported in pathogenesis of enteric viral diseases like feline coronavirus, bovine herpesvirus-1, porcine reproductive and respiratory syndrome and rotavirus (De et al., 2014; Durgut, Ataseven, & Öztürk, 2013; Kayar et al., 2015; Stukelj, Toplak, & Nemec Svete, 2013). Recently, it has been observed that parvovirus infection is linked with oxidative stress, and marked enhancement of reactive oxygen/nitrogen species, lipid peroxidation, DNA damage and poor antioxidant reserve (Luo & Qiu, 2013; Nykky, Vuento, & Gilbert, 2014; Panda, Patra, Nandi, & Swarup, 2009). In recent years, emphasis has been given on the antioxidants as the potential drugs of interest for management of viral diseases (Beck, 1998; Chandrasena et al., 2014; Crump, Langston, Rajkarnikar, & Grayson, 2013). A strong association of CPV with oxidative stress suggests incorporation of antioxidants in therapeutic regimen in canine parvoviral diarrhea may help in ameliorating the clinical signs.

N-acetylcysteine (NAC), the body's primary cellular antioxidant, is a precursor to glutathione and its role on glutathione maintenance and metabolism is well established (Kelly, 1998). Glutathione counteracts the harmful effect of reactive oxygen and nitrogen species through both direct and indirect scavenging (Dean, Giorlando, & Berk, 2011). Antioxidant efficacy of NAC has been evaluated in hepatitis and cholangiohepatitis of dogs and other noninfectious systemic disease conditions of rat (Dean et al., 2011; Ribeiro et al., 2011; Sadowska, Manuel-Y-Keenoy, & De Backer, 2007; Seguro, Poli de Figueiredo, & Shimizu, 2012; Shahripour, Harrigan, & Alexandrov, 2014; Stanislaus, Gilg, Singh, & Singh, 2005; Webster & Cooper, 2009). Recently, antioxidant effect of NAC has been reported in viral diseases including HIV and influenza (Geiler et al., 2010; Sgarbanti et al., 2014; Staal, 2000; Uchide & Toyoda, 2011). However, the antioxidant effect of NAC in dogs naturally affected with parvoviral diarrhea has not yet been explored. Therefore, this study was aimed to evaluate the antioxidant potential of NAC in diarrheic dogs infected with canine parvovirus.

2 | MATERIALS AND METHODS

2.1 | Animals

The study was conducted at Referral Veterinary Polyclinic and Teaching Veterinary Clinical Complex (RVP-TVCC) of the Institute during February 2016 to May 2016. A total 26 dogs (age group 1.5–6 months) with complains of pyrexia, weakness, reduced appetite, severe dehydration, hemorrhagic diarrhea and vomition were taken for this study. All the dogs were unvaccinated against CPV-2. The fecal samples from clinical cases were collected for disease diagnosis and for conducting this study. Of the 26 fecal samples, 18 were found positive for parvovirus infection by polymerase chain reaction. The parvo-positive dogs were randomly picked up and divided equally into two treatment groups, each group consisting of nine parvo-positive canine gastroenteritis cases. Nine parvo-positive dogs (Group B) received supportive treatment (ST) consisting of intravenous fluid, antibiotics (Ceftriaxone-Tazobactum) and antiemetics, as well as anti-inflammatory drugs (Meloxicam) when indicated, whereas nine parvovirus-positive dogs (Group C) received ST plus NAC (ST-NAC) @ 70 mg/Kg body weight once daily (intravenous route) for 5 consecutive days. Nine healthy dogs of similar age group recruited for the study were presented to the RVP-TVCC either for routine health check up or vaccination, and served as control (group A). All the dogs after examination were treated by expert clinician in the RVP-TVCC. The study was conducted as per the guidelines of Institutional Animal Ethics Committee and care of the animals were undertaken as per the guidelines of committee for the purpose of control and supervision of experiments for animals, India.

2.2 | Diagnosis of canine parvovirus in fecal samples

2.2.1 | Extraction of DNA from fecal samples

The fecal samples were collected from the diarrheic dogs during the episodes of hemorrhagic gastroenteritis in virus transport media (Dulbecco's modified Eagle's medium with 2% Fetal bovine serum and 50 mg/L Gentamycin). The samples were centrifuged at 14,000 *g* for 5 min. After centrifugation, 200 µl of supernatant was separated from each of stool samples in eppendorf tubes and 200 µl of Tris saturated phenol was added, mixed well and centrifuged (14,000 *g* for 3 min). A total volume of 100 µl of phenol and 100 µl of $CHCl_3$ were added in 200 µl supernatant and mixed well. After centrifugation, 200 µl supernatant was collected and 200 µl of chloroform was added and centrifuged. A total volume of 200 µl of supernatant was collected in separate tube and 20 µl of 3M sodium acetate and 1 ml of absolute ethanol were added. After mixing, the tubes were kept at −20°C overnight. Next day the tubes were again centrifuged at 14,000 *g* for 10 min. The supernatant was discarded and 200 µl of 70% ethanol was added and repeated the centrifugation process. The supernatant was discarded and pellet was dried at 90°C and finally dissolved in 20 µl of nuclease-free water.

2.3 | PCR assay

The VP2 gene of CPV was used for diagnosis in this study (Pereira, Monezi, Mehnert, D'Angelo, & Durigon, 2000). The forward (5′ GAA GAG TGG TTG TAA ATA ATA 3′) and reverse (5′ CCT ATA TCA CCA AAG TTA GTA G 3′) primer sets (Imperial Biomedics) were used to amplify part of VP2 gene of CPV to yield a product of 681 bp (Pereira et al., 2000). In PCR, 25 µl of PCR mastermix (2×), 1 µl each of forward and reverse primers (10 pmol), 5 µl of extracted DNA as template and rest nuclease-free water (NFW) to make final volume of 50 µl. Suitable

no template control having NFW instead of template DNA was included as negative control. Whereas the CPV 2 isolated in the laboratory, amplified by PCR, cloned in cloning vector and sequenced was taken as positive control. The PCR was performed in a thermal cycler (Applied Biosystems, USA). The cyclic condition was denaturation at 95°C for 45 s, primer annealing at 55°C for 45 s and extension at 72°C for 45 s. The cyclic condition was repeated for 35 times and a final extension at 72°C was given for 10 min.

After PCR, the amplified products were analyzed on 1.0% agarose gel containing ethidium bromide to a final concentration of 0.5 μg/ml. Ten microliter of amplified product was mixed with 2 μl of bromophenol (6×) dye and loaded into the well and run along with 100 bp to 1 Kbp DNA ladder in 1× TAE electrophoresis buffer at 5 volts/cm^2 and the progress of mobility was monitored by migration of dye. At the end of the electrophoresis, the gel was visualized under the UV transillumiator (Nandi, Pandey, Sharma, & Chauhan, 2008). All the PCR products were gel eluted, cloned using Clone Jet PCR Cloning kit and sequenced to substantiate the authenticity of result.

2.4 | Blood sampling and processing

The blood samples (approximately, 3.0 ml) were collected by venipuncture of either cephalic or recurrent tarsal vein in K_2EDTA-containing vial from each participated dog in association with routine clinical sampling before initiation of any treatment. Of 3.0 ml, 0.5 ml blood was use for hematology and 2.5 ml blood was immediately centrifuged at 200 *g* for 10 min to separate plasma and stored at −20°C until analysis.

2.5 | Hematology

The hematological analysis of blood was performed manually (Jain, 1986). The concentration hemoglobin (Hb) in the whole blood was measured by modified Sahil's acid hematin method. Red blood cell (RBC) and white blood cell (WBC) count was measured by Neubauer's hemocytometer. Methanol fixed blood smear was stained with diluted Giemsa stain (1:10) for 45 min and the differential leukocyte count (DLC) was done. The hematology was performed before treatment (day 0) and thereafter on day 3 and 5 of initiation of treatment.

2.6 | Measurement of oxidative stress

The oxidative stress was evaluated on the basis of measurement of the activity of glutathione S-transferase (GST) and concentration of nitric oxide (NOx) and malondialdehyde (MDA) in plasma before treatment (day 0) and thereafter on day 3 and 5 of initiation of treatment.

2.6.1 | MDA assay

Lipid peroxidation was measured by determining the plasma malondialdehyde (MDA) concentration by double heating method (Draper & Hadley, 1990). In short: 2.5 ml of 10% triochloroacetic acid (TCA) was mixed with 0.5 ml plasma in a test tube. The mixture was kept in a boiling water bath for 15 min. The reaction mixture was cooled and centrifuged at 400 *g* for 10 min. Two milliliters of the supernatant was taken out and mixed with 1.0 ml 0.67% TBA in a separate tube and incubated in a boiling water bath for 15 min. The solution was cooled to room temperature and the absorbance was measured at 532 nm using a spectrophotometer. The concentration of plasma MDA was calculated from the absorbance coefficient (1.56 × 105 $cm^{-1}M^{-1}$) of the thiobarbituric acid–malondialdehyde (TBA-MDA) complex, and value was expressed in nmol/ml.

2.6.2 | NOx assay

The NOx in the plasma was measured by reduction in nitrate with acid-activated copper–cadmium alloy followed by color development with Griess reagent (Sastry, Maudgal, Mohan, Tyagi, & Rao, 2002). Briefly, a reaction mixture consisting of 100-μl plasma sample, 400 μl of carbonate buffer and 150-mg powder of copper–cadmium alloy was incubated for 1 hr at room temperature with frequent shaking. The reaction was stopped by the addition of 0.35-M NaOH and 120-mM ZnSO4. After vortexing, the reaction mixture was centrifuged at 400 *g* for 15 min, and 75-μl supernatant was separated and added with 75-μl Griess reagent (0.1% naphthalene diamine dihydrochloride in 3 N hydrochloric acid and 1% sulfanilamide, 1:1 ratio) in 96-well microplate. The optical density was read at 545 nm in microplate reader after incubation for 10 min at room temperature. The value of NOx production was calculated from a standard curve using different concentrations of potassium nitrate.

2.6.3 | GST activity assay

The plasma GST activity was assayed by using commercially available kit (EZAssayTM GST Activity Estimation Kit, Product Code: CCK028, Himedia, Mumbai, India) and value was expressed in μM $ml^{-1}min^{-1}$.

2.7 | Statistical analysis

The data were analyzed using statistical software package SAS v 9.3 (SAS Institute, Inc. 2011, Cary, NC, USA) to determine whether the average TLC, neutrophils, lymphocyte, monocyte, eosinophil, basophil counts, MDA, NOx concentrations and GST activity were significantly different in NAC-treated dogs when compared to ST-treated and control dogs over the time. The data were taken repeatedly at different time interval from each dog and analyzed using repeated measurement model with dog as subject and period as repeated measurement. The one-way ANOVA was used to compare the treatments to hematology and oxidative stress indices tested and, when an interaction was found, Tukey's post hoc test was used to determine statistical significance between the different treatment groups. An individual dog was considered the experimental unit. A probability level (p) of .05 was selected as the statistical selection limit for all tests. When an interaction was present between the treatment and hematology or oxidative stress indices as determined by one-way ANOVA, the p-value obtained by Tukey's post hoc test for the relevant treatment

groups was presented; when an interaction was not present, the *p*-value obtained from the one-way ANOVA was presented. Results are expressed as means ± *SEM*.

3 | RESULTS

3.1 | Diagnosis of canine parvovirus infections in dogs by PCR

On clinical examination, the canine parvovirus affected dogs exhibited the clinical signs of high rise of temperature, weakness, reduced appetite, severe dehydration, bloody diarrhea and vomition. Of 26 affected dogs, 18 dogs were found positive for CPV infection by PCR using VP2 gene-specific primers in processed fecal sample as evident by presence of expected 681 bp DNA product in agarose gel under UV transilluminator. The rest eight samples were found negative for CPV infection by PCR as there was no specific band visualized (Figure 1).

3.2 | Effect of NAC on Hb and RBC count

The mean Hb concentration and RBC count were significantly higher ($p < .05$) in parvo-infected dogs (group B and group C) compared to control dogs (group A) before treatment. The supportive treatment and supportive treatment plus NAC (ST-NAC) significantly ($p < .05$) improved the Hb concentration and RBC count in parvo-infected dogs on day 3 and 5 as compared to pretreatment value. A statistically significant interaction was found between treatment and Hb ($p = .000$) and RBC count ($p = .020$; Figure 2).

3.3 | Effect of NAC on WBC, neutrophil, lymphocyte, monocyte, eosinophil and basophil count

A marked leucopenia and neutropenia ($p < .05$) was observed in parvovirus-infected dogs compared to healthy dogs. The mean WBC

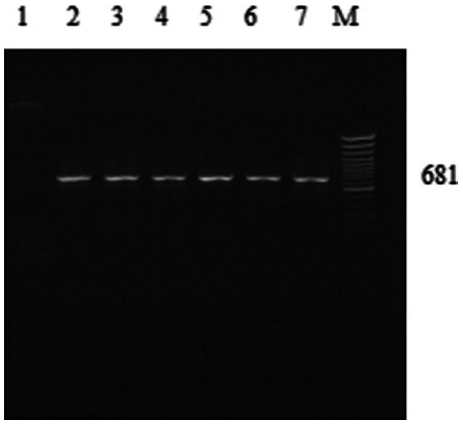

FIGURE 1 Agarose gel electrophoresis of the PCR products for identification of VP2 gene of canine parvovirus in fecal samples collected from dogs suffering from hemorrhagic gastroenteritis. Lane M denotes 100-bp DNA ladder, lane 1 denotes negative control (no template), lane 2 denotes positive control (product size 681 bp), lanes 3–7 denotes positive for canine parvovirus in fecal samples

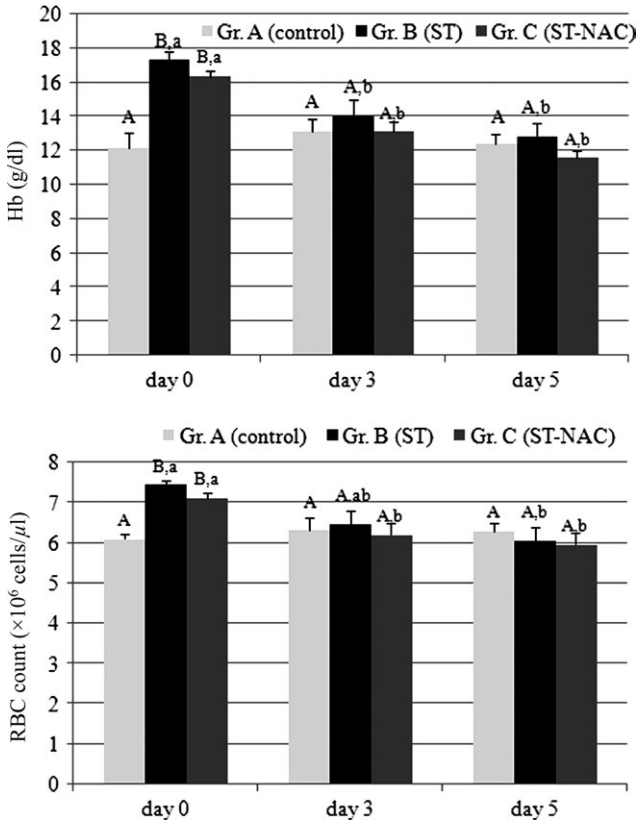

FIGURE 2 Changes of hemogram (Hb and RBC count) over time in nine healthy dogs (Group A), nine parvoinfected dogs treated with supportive treatment (ST, Group B) and nine parvo-infected dogs treated with ST plus *N*-acetylcysteine (NAC, Group C). The data were analyzed by Tukey's Post Hoc test using repeated measure analysis. A statistically significant interaction was found between treatment and Hb ($p = .000$) and RBC count ($p = .020$). Superscripts A, B between the groups within a day and superscripts a, b between the days within the group differ significantly ($p < .05$)

and neutrophil count did not differ significantly over the time in parvo-infected dogs that received only ST. The WBC count on day 5 and neutrophil count on day 3 and 5 improved significantly following the ST-NAC treatment ($p < .0.5$) and reached closer to value of healthy dogs in group A. When the WBC count was compared between parvo-infected dogs (group B and C) and control dogs (group A), it was found that the count was significantly higher on day 5 in ST-NAC-treated dogs than ST-treated dogs and the values did not differ significantly when compared with control dogs. Similarly, the mean neutrophil count in parvovirus-infected dogs was appreciably improved in ST-NAC treatment (Group C) on day 3 and 5 ($p < .0.5$) followed by ST (Group B). However, statistically no significant interaction was noted between treatment and WBC count ($p = .281$) and neutrophil count ($p = .054$; Table 1).

There was marked reduction in the average monocyte and eosinophil counts in parvo- infected dogs (Group B & C) compared to healthy dogs (Group A). There was no conspicuous improvement in average monocyte and eosinophil counts from day 0 to day 5 in dogs that received only ST (Group B). However, ST-NAC-treated dogs (Group C)

showed better result ($p < .05$) in terms of monocyte and eosinophil counts on day 3 and 5. When the mean monocyte and eosinophil counts were compared between parvo-infected dogs and control dogs, no significant difference was noticed on day 5. The mean lymphocyte and basophil counts remain unaffected throughout the study period in both the treated groups. A statistically significant interaction was found between the treatment and monocyte count ($p = .049$), but such interaction was not present for lymphocyte ($p = .886$), eosinophil ($p = .771$) and basophil count ($p = .824$; Table 1).

3.4 | Effect of NAC on GST, NOx and MDA concentrations

In parvo-infected dogs, significantly lower concentration of GST ($p = .004$) but higher concentration of NOx ($p = .000$) and MDA ($p = .000$) were noticed compared to healthy dogs. Although the mean values of GST, NOx and MDA concentrations did not differ significantly throughout the study period in parvo-infected dogs that received only ST (Group B), there was significant elevation of the mean GST ($p = .035$) concentration and appreciable reduction in NOx ($p = .039$) and MDA ($p = .035$) concentrations on day 3 and 5 in ST-NAC-treated dogs (Group C). When the values were compared between ST- and ST-NAC-treated groups, it was found that mean GST ($p = .007, .031$) activity significantly increased and NOx ($p = .001, .033$) and MDA ($p = .007, .037$) concentrations significantly reduced on day 3 and 5 in ST-NAC-treated dogs compared to ST-treated dogs. There was a statistically significant interaction between the treatment and NOx concentration ($p = .036$), but not with GST activity ($p = .063$) and MDA concentration ($p = .100$; Figure 3).

4 | DISCUSSION

In this study, pronounced elevation of Hb% and RBC count in infected dogs indicates hemoconcentration during parvoviral diarrhea. The parvoviral enteritis causes anemia that is attributed to cytotoxic effect

TABLE 1 Changes in leukogram kinetics over time in nine healthy dogs (Group A), nine parvo-infected dogs treated with supportive treatment ST (ST, Group B) and nine parvo-infected dogs treated with supportive treatment plus *N*-acetylcysteine (ST-NAC, Group C)

Groups	Day 0	Day 3	Day 5
WBC ($\times 10^3$ cells/μl)			
Gr. A	7.331 ± 0.581^{A}	8.084 ± 0.580^{A}	7.843 ± 0.711^{A}
Gr. B	3.822 ± 0.385^{B}	4.221 ± 0.325^{B}	4.363 ± 0.413^{B}
Gr. C	$3.978 \pm 0.382^{B,a}$	$4.965 \pm 0.566^{B,a}$	$6.364 \pm 0.466^{A,b}$
Neutrophil ($\times 10^3$ cells/μl)			
Gr. A	4.611 ± 0.421^{A}	4.913 ± 0.418^{A}	4.784 ± 0.452^{A}
Gr. B	1.487 ± 0.149^{B}	1.684 ± 0.150^{B}	2.113 ± 0.317^{B}
Gr. C	$1.637 \pm 0.158^{B,a}$	$2.290 \pm 0.265^{B,b}$	$3.551 \pm 0.189^{C,c}$
Lymphocyte ($\times 10^3$ cells/μl)			
Gr. A	2.006 ± 0.172^{A}	2.368 ± 0.190^{A}	2.307 ± 0.280^{A}
Gr. B	1.939 ± 0.195^{A}	2.105 ± 0.189^{A}	1.822 ± 0.152^{A}
Gr. C	1.977 ± 0.202^{A}	2.083 ± 0.269^{A}	2.144 ± 0.366^{A}
Monocyte ($\times 10^3$ cells/μl)			
Gr. A	0.502 ± 0.042^{A}	0.579 ± 0.035^{A}	0.533 ± 0.045^{A}
Gr. B	0.288 ± 0.036^{B}	0.346 ± 0.047^{B}	0.324 ± 0.026^{B}
Gr. C	$0.251 \pm 0.028^{B,a}$	$0.425 \pm 0.059^{AB,b}$	$0.475 \pm 0.035^{AB,b}$
Eosinophil ($\times 10^3$ cells/μl)			
Gr. A	0.179 ± 0.032^{A}	0.184 ± 0.030^{A}	0.207 ± 0.024^{A}
Gr. B	0.103 ± 0.012^{B}	0.108 ± 0.012^{B}	0.093 ± 0.010^{B}
Gr. C	$0.097 \pm 0.011^{B,a}$	$0.133 \pm 0.019^{AB,ab}$	$0.154 \pm 0.023^{AB,b}$
Basophil ($\times 10^3$ cells/μl)			
Gr. A	0.024 ± 0.012^{A}	0.016 ± 0.011^{A}	0.043 ± 0.014^{A}
Gr. B	0.003 ± 0.003^{A}	0.012 ± 0.006^{A}	0.009 ± 0.006^{A}
Gr. C	0.014 ± 0.007^{A}	0.032 ± 0.017^{A}	0.038 ± 0.009^{A}

The data were analyzed by Tukey's post hoc test using repeated measure analysis. A statistically significant interaction was found between treatment and monocyte count ($p = .049$), but such interaction was not present for TLC ($p = .281$), neutrophil count ($p = .054$), lymphocyte count ($p = .886$), eosinophil count ($p = .771$) and basophil count ($p = .824$).
The values were expressed as mean ± *SEM*.
Superscripts A, B, C between the groups within a day and superscripts a, b, c between the days within a group differ significantly ($p < .05$).

of virus on hematopoietic cells, bone marrow failure and subsequently erythroid hypoplasia during acute stages of the disease (Grimes & Fry, 2015; Nandi & Kumar, 2010; Stann, DiGiacomo, Giddens, & Evermann, 1984). In contrast, hemoconcentration in this study might be due to excessive fluid loss during the episodes of diarrhea. Furthermore, leucopenia and neutropenia are the major hemogram alterations in the parvo-infected dogs (Castro et al., 2013). The leukopenia and neutropenia in parvo-infected dogs could be due to destruction of hematopoietic progenitor cells of the various leukocyte types in the bone marrow, inadequate supply for the massive demand for leukocytes in the inflamed gastrointestinal tract and loss of neutrophils through the damaged gastrointestinal tract (Macartney, McCandlish, Thompson, & Cornwell, 1984; Fulton et al., 199; Goddard, Leisewitz, Christopher, Duncan, & Becker, 2008). However, the WBC count was restored on day 5 in parvo-infected dogs following ST-NAC treatment. In spite of progressive improvement in neutrophil count over time, neutropenia still persisted on day 5 compared with control healthy dogs. It warrants longer duration of treatment with NAC to improve the neutrophil count and health condition of the dogs. Although, there is no report on the effect of NAC to stimulate neutrophil production, however, it has been reported that NAC stimulates pluripotent stem cells to differentiate toward hematopoietic cells in laboratory animal model (Berniakovich, Laricchia-Robbio, & Izpisua Belmonte, 2012). Goddard et al. (2008) opined that blood leukocyte count could be prognostic indicator of canine parvoviral enteritis or outcome of the disease.

Much has been learned about the beneficial effect of antioxidants against viral diseases in recent years (Beck, 2001; Peterhans, 1997; Zhang, Wang, Chen, Chen, & Tian, 2014). Studies derived from influenza virus indicated positive impact of NAC as an antioxidant molecule against viral pathogenesis (Casanova & Garigliany, 2016; Garigliany & Desmecht, 2011). In this study, a pronounced elevation of MDA and NOx and drop of GST activities in infected dogs compared to healthy dogs before initiation of therapy indicate marked oxidative stress in parvovirus infection and corroborated with the finding of other workers (Luo & Qiu, 2013; Nykky et al., 2014; Panda et al., 2009). Oxidative stress has been implicated in several viral infections in human and animals. It has been reported that parvovirus-induced oxidative stress is mediated by damage of mitochondrial membrane, NS1 protein-induced DNA damage besides liberation of proinflammatory cytokines (Barzilai & Yamamoto, 2004; Goddard & Leisewitz, 2010; Nykky et al., 2014). The supportive treatment alone did not succeed to reduce the level of MDA, NOx and improve the GST activities in parvo-infected dogs till day 5 when compared with pretreatment values (day 0). However, inclusion of NAC in supportive treatment significantly ($p < .05$) reduced the MDA, NOx concentrations and increased GST activity in plasma on day 3 onward after initiation of treatment. It indicated the ameliorative potential of NAC in oxidative stress during CPV diarrhea. The NAC inhibits inducible nitric oxide synthase, suppresses proinflammatory cytokine expression/release, reduces lipid peroxidation in serum and restores nonenzymatic antioxidant reserves in human and laboratory animal model (Akca et al., 2005; Caglikulekci et al., 2006; Kasperczyk et al., 2014; Ribeiro et al., 2011; Shahripour et al., 2014). Furthermore, NAC supplementation significantly reduced the plasma malondialdehyde (MDA) in acetic acid (AA)-induced ulcerative colitis of rat model and stabilized erythrocyte glutathione concentrations and decreased the degree of lipid peroxidation in sick dogs that experience systemic oxidative stress during the first 48 hr of hospitalization

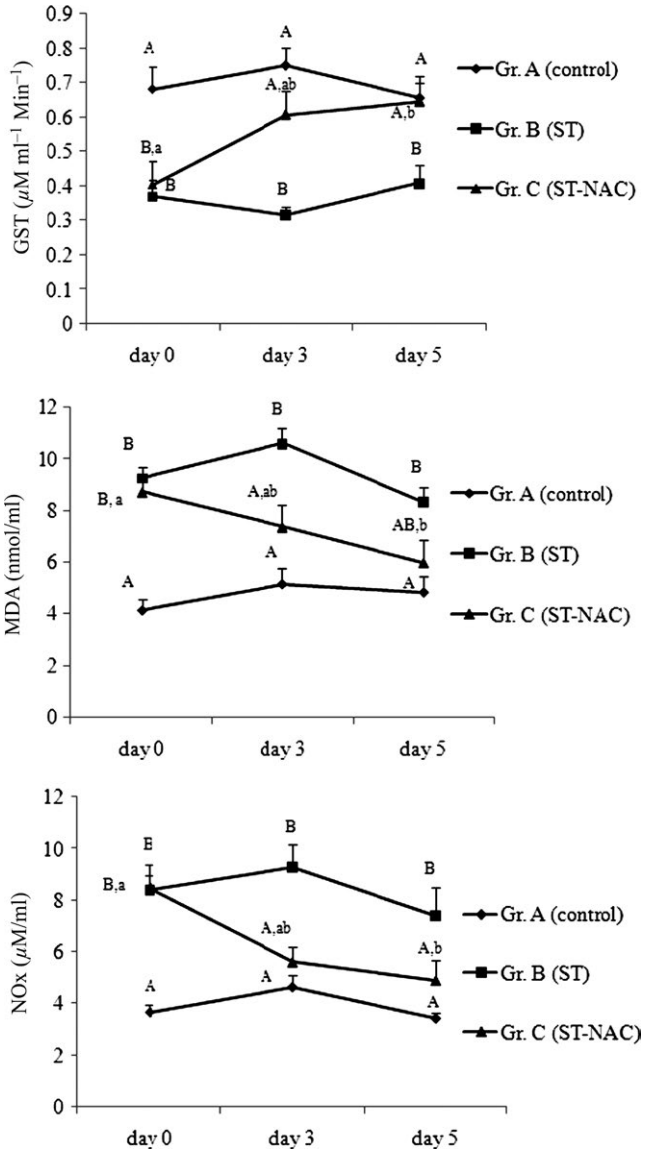

FIGURE 3 Changes of GST activity, MDA and NOx concentrations over time in nine healthy dogs (Group A), nine parvo-infected dogs treated with supportive treatment (ST, Group B) and nine parvo-infected dogst reated with ST plus *N*-acetylcysteine (NAC, Group C). The mean GST activity significantly elevated on day 5 ($p = .035$) from pretreatment value (day 0) in ST-NAC treated dogs, whereas no significant changes was observed in parvo-infected dogs received ST only. The average MDA and NOx concentrations significantly reduced on day 5 ($p = .035, .039$) from pretreatment value (day 0) in ST-NAC treated dogs, whereas no significant changes was observed in parvo-infected dogs received ST only. A statistically significant interaction was found between treatment and NOx concentration ($p = .036$) but, such interaction was not present for GST activity ($p = .063$) and MDA concentrations ($p = .100$). Superscripts A, B between the groups within a day and superscripts a, b between the days within the group differ significantly ($p < .05$)

(Kurutas, Cetinkaya, Bulbuloglu, & Kantarceken,2005; Viviano & Wielen, 2013). Recently, Geiler et al. (2010) observed NAC inhibits the virus replication and proinflammatory cytokines and chemokine gene expression during influenza virus infection. The beneficial effects of NAC have been reported in highly pathogenic H5N1 influenza A virus infection, chronic obstructive pulmonary diseases and rotavirus-associated diarrhea (Dekhuijzen, 2004; Garigliany & Desmecht, 2011; Guerrero, Torres, García, Guerrero, & Acosta, 2014). From the findings of this study, it can be concluded that antioxidants like NAC represent a potential additional treatment option that could be considered in the case of parvoviral diarrhea. However, the therapeutic efficacy of NAC involving large number of dogs with parvovirus infection needs further study to extract the full potential of the compounds with comprehensive results.

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