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N-acetylcysteine enhances bone marrow activity in treating pancytopenia induced by canine hemoprotozoan diseases

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Abstract

Canine hemoprotozoan diseases *viz*. ehrlichiosis and babesiosis are mostly associated with critical anemia and thrombocytopenia with pancytopenic changes, leading to multi-organ failure. For faster recovery of patients with complicated hemoprotozoan diseases, whole blood transfusion or bone marrow stimulating agents to produce more red blood cells (RBCs) and platelets might be helpful. Unfortunately, canine specific transfusion procedures are expensive and even not available in many developing countries. Development of alternate therapeutic modality by bone marrow stimulation to augment the production of RBCs and platelets and thus, to treat the critical pancytopenic patients is and an urgent necessity. N-acetylcysteine (NAC), acts as a precursor of reduced glutathione and increases the production of bone marrow B cells. It also improves viability and self-renewal capacity of stem cells and thus, boosts hematopoietic differentiation by protecting induced pluripotent stem cells. This study envisaged to develop alternate therapeutic approach to combat pancytopenia secondary to canine hemoprotozoan diseases. Bone marrow mediated aplastic pancytopenia was induced experimentally by administration of cyclophosphamide in rats. Bone marrow stimulating property of NAC was compared with desmopressin, another bone marrow stimulator, which revealed better in terms of hematobiochemical and histopathological changes. Results of rat model study were extrapolated in clinical canine hemoprotozoan cases having pancytopenia. Dogs treated with hemoprotozoan disease specific therapy along with NAC rendered favorable changes by haltering the progression of critical anemia and thrombocytopenia. Study revealed that supplementation of NAC along with canine hemoprotozoan specific therapy is beneficial to alleviate pancytopenia.

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Introduction

Canine hemoprotozoan diseases namely *Babesia, Ehrlichia, Anaplasma*, and *Hepatozoon* infections are predominant in Indian subcontinent.[1] Multiple organ failure with liver and spleen involvements are common in babesiosis,[2] ehrlichiosis,[3] *Hepatozoon canis* infection,[4] and anaplasmosis.[5] Blood parasites are notorious for bringing about chronic inflammatory reactions owing to their long incubation period, prolonged presence in the body, and an evolutionary preference to infect blood cells or inhabit bone marrow, leading to bone marrow aplasia, myelosuppression, pancytopenia, and high mortality due to severe bleeding or septicemia.[6] Non-regenerative anemia, aplastic pancytopenia, and thrombocytopenia are most obvious findings in canine hemoprotozoan diseases. Thrombocytopenia may be due to the platelet destruction by immune-mediated reactions, excessive consumption due to mild vasculitis, splenic sequestration, or bone marrow failure.[7,8]

Spontaneous clinical recovery of acute phase of canine hemoprotozoan diseases is common. Most of them develop chronic and severe form requiring therapeutic intervention to hasten clinical recovery and prevent clinical exacerbation followed by death. Treating with diminazene aceturate, doxycycline, clindamycin, and imidocarb leads

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to remission of clinicopathological changes, but not invariably eliminates infection. There is a recurrence of signs and symptoms after drug cessation.[7] Hematopoietic growth factor (human granulocyte stimulating factor and recombinant human erythropoietin) is not cost effective, having inconsistent efficacy.[7] Administration of packed red blood cells (RBCs) or whole blood transfusions offer promising results. But, there are limitations, like commercial non-availability in all places and exorbitant price, making these therapeutics not accessible to everyone. Use of glucocorticoid has no evidence-based justification as anti-inflammatory and immunosuppressive agents. It also further predisposes to secondary infection and thus, potentiates gastrointestinal bleeding.[7]

Attention has recently been focused on bone marrow stimulating activity as a major candidate factor in aplastic pancytopenia. Several past studies have proven that anti-oxidants not only help in alleviating oxidative stress but also have effects on the strength and differentiation of stem cells. N-acetylcysteine (NAC), a glutathione precursor mucolytic agent,[9] helps in kidney and liver damage by detoxification,[10] improves immune function, inhibits inflammation,[11] and acts as a nutritional supplement[12] and powerful anti-oxidant in different doses. It also improves viability and proliferation of stem cells of the same cell type,[13] increases production of bone marrow B cells,[14] and boosts hematopoietic differentiation by protecting induced pluripotent stem cells.[15] Desmopressin used in human patients with mild alteration in platelet function.[16] It selectively enhances pro-coagulant platelets formation,[17] increases platelet-dependent thrombin generation *via* enhancing Na^+/Ca^{2+} mobilization,[18] and plays a significant role in hemostasis through releasing von Willebrand factor from the storage sites. Despite substantial recent advances in the knowledge regarding canine tick-borne diseases, we are still unable to attain novel therapeutic regimen for pancytopenia associated with canine hemoprotozoan diseases.

Development of alternate therapeutic modality by bone marrow stimulation to treat the patients with critical pancytopenia, is the need of present hour. It was hypothesized that supplementation of NAC along with disease specific therapy could halter the critical pancytopenia in canine hemoprotozoan diseases.

Materials and Methods

Bone marrow suppression in rats. A pilot study was performed to determine the dose of cyclophosphamide (CP; Baxter, Haryana, India). Male Wistar rats with approximate body weight of 200 to 250 g were selected and pancytopenia was established by bone marrow suppression induction with single dose of CP (37.50 mg kg^{-1}; subcutaneously). This study was performed based on the principles of the Institute Animal Ethical Committee (No. 26-2/2019/JDR dated 06-01-20/19-02-20).

Experimental model in rats. Thirty-six male rats were randomly divided into six groups (n = 6). Duration of the study was 2 weeks. Rats of groups I and II were served as healthy (without CP induction) and disease control (CP induction but with placebo treatment), respectively. On day four after induction of bone marrow suppression, rats of groups III and IV were treated with oral 25.00 and 50.00 mg kg^{-1} NAC (Venus Remedies, Himachal Pradesh, India) for 10 days, respectively. Similarly, rats of groups V and VI received oral 7.50 and 15.00 µg kg^{-1} desmopressin (Taj Pharma, Mumbai, India) on day four after induction for 10 days, respectively. Blood samples were collected on days 0 and 14 of the trial for blood biochemistry. On day 14, rats were sacrificed for histopathological studies.

Hematobiochemical changes in rats. Hematology was performed manually as per the standard protocols.[19] Serum alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total protein (TP), and albumin were estimated using commercial kits (Autospan diagnostic, Arkray, Japan).

Histopathological changes in rats. For histopathological changes, femoral bones were cut into smaller pieces before fixation. Femoral bone pieces were decalcified for 5 - 14 days in formic acid-sodium citrate solution. Liver and spleen samples were rinsed with phosphate-buffered saline, dried, and fixed in 10.00% formalin. Tissues were dehydrated using ascending grades of ethanol, then cleared in xylene, and finally embedded in paraffin wax. Tissue sections in 5.00 µm were prepared and dried at 37.00 ˚C. The sections were deparaffinized in xylene, rehydrated using descending grades of ethanol, and stained with Hematoxylin and Eosin for histopathological examination and scoring using light microscope (BX41; Olympus Tokyo, Japan).

Screening of dogs. Study was conducted to screen clinical pancytopenia associated with canine hemoprotozoan diseases presented at Referral Veterinary Polyclinic, Indian Veterinary Research Institute, Bareilly, India, during the period of March 2021 to May 2022. Dogs found positive for hemoprotozoan diseases with pancytopenia were enrolled in the study.

Experimental details in dogs. Group I dogs received specific anti-hemoprotozoan therapy including doxycycline (Savavet, Pune, India), diminazene aceturate (MSD Animal Health, Rahway, USA), clindamycin (Savavet) along with anti-pyretic, anti-emetic, and anti-histamine, whereas in addition to this therapy, dogs of group II received NAC (15.00 mg kg^{-1}) orally for 10 days, as bone marrow stimulator as per the rat model study. Dose for dog was calculated using formula for dose translation based on body surface area:[20]

$$\textit{Dog equivalent dose} = \textit{Rat dose (mg kg}^{-1}\textit{)} \times \textit{Rat Km/Dog Km}$$

where, Km (correction factor) values for rat and dog are 6.00 and 20.00, respectively).

Hematobiochemical changes in dogs. At days 0 and 14 of the therapy, blood samples were collected for hemato-serum biochemical analysis. Hemoglobin (Hb) level, and platelets, white blood cells (WBCs), RBCs, and reticulocyte counts were determined using an automated hematology analyzer (3000 Vet Plus; URIT, Guangdong, China) The ALT, AST, ALP, TP, and albumin levels were recorded using commercial kits (Arkray, Kyoto, Japan).

Oxidative stress indices in the dogs. The 1,1 diphenyl 2, picryl-hydrazyl (DPPH) assay was performed with slight modifications of Brand-Williams *et al.*, method,[21] and a modified ferric reducing anti-oxidant power (FRAP) assay was performed with minor modifications of Benzie and Strain method.[22]

Prognostic score card to predict therapeutic efficacy. With necessary modifications based on human patient Child - Pugh prognostic score,[23] card was constituted by considering post-therapy improvements in various parameters and assigned score in the scale of (+) 1 to (+) 3, wherein (+) 3 indicated good, (+) 2 moderate, and (+) 1 poor prognosis.

Statistical analysis. The JMP 9.0 Software (SAS, Cary, USA) was used for analysis of the data. Two-way ANOVA was employed to determine the statistical significance of blood parameters. The statistical analysis was considered significant at $p \leq 0.05$.

Results

Bone marrow stimulating property of NAC and desmopressin in rats. Pre- and post-treatment changes in the hematological parameters are given in Table 1. Hemoglobin level, packed cell volume (PCV), total erythrocyte count (TEC), and platelet count were significantly decreased in all groups on day 14 except healthy control group (group I), ($p \leq 0.05$). However, total leukocyte count (TLC) values were non-significant at the end of therapy in all groups except group II. Significantly reduced Hb level was noticed at the end of therapy in group II ($p \leq 0.05$). The Hb levels of rats treated with low (group III) and high (group IV) doses of NAC, and rats treated with low (group V) and high (group VI) doses of desmopressin were significantly increased 14 days after therapy compared to the disease control group (group II), ($p \leq 0.05$). The PCV and TEC values were significantly decreased in all treated groups at the end of therapy compared to the group I ($p \leq 0.05$), whereas PCV and TEC of group IV were significantly higher than group II (disease control), ($p \leq 0.05$). The TLC was significantly increased on day 14 in group II ($p \leq 0.05$), whereas it was non-significant in all other groups. Platelet count was significantly decreased in all groups on day 14 except group I ($p \leq 0.05$). Among the treated groups, hematological parameters including

Table 1. Mean ± SE values of complete blood count (CBC) in rats received different treatments in day 0 (D0) and day 14 (D14).

Groups	Hemoglobin (g dL^{-1})		PCV (%)		RBC (×10^6 µL^{-1})		WBC (×10^3 µL^{-1})		Platelet (×10^5 µL^{-1})	
	D0	D14	D0	D14	D0	D14	D0	D14	D0	D14
I	14.40 ± 0.18^{aA}	14.50 ± 0.21^{aA}	42.50 ± 0.76^{aA}	42.80 ± 1.01^{aA}	8.00 ± 0.16^{aA}	8.00 ± 0.22^{aA}	7.70 ± 0.21^{aA}	7.50 ± 0.19^{aB}	8.00 ± 0.21^{aA}	8.10 ± 0.20^{aA}
II	14.00 ± 0.18^{bA}	8.90 ± 0.20^{aB}	41.60 ± 0.61^{bA}	26.10 ± 0.70^{aB}	7.70 ± 0.17^{bA}	4.70 ± 0.15^{aB}	7.30 ± 0.17^{bA}	12.30 ± 1.46^{aA}	8.00 ± 0.15^{bA}	4.60 ± 0.12^{aB}
III	13.80 ± 0.21^{bB}	9.40 ± 0.21^{aC}	41.0 ± 0.63^{bA}	27.60 ± 0.67^{aB}	7.60 ± 0.37^{bA}	5.00 ± 0.18^{aB}	7.60 ± 0.20^{aA}	9.20 ± 0.28^{aAB}	7.80 ± 0.24^{bA}	4.90 ± 0.09^{aB}
IV	14.00 ± 0.33^{bB}	11.70 ± 0.27^{aD}	41.80 ± 1.01^{bA}	34.80 ± 0.70^{aC}	8.00 ± 0.30^{bA}	6.30 ± 0.12^{aC}	7.70 ± 0.17^{aA}	6.50 ± 0.15^{aB}	8.10 ± 0.21^{bA}	5.40 ± 0.20^{aB}
V	13.90 ± 0.22^{bB}	9.30 ± 0.17^{aE}	41.50 ± 0.67^{bA}	27.30 ± 0.67^{aB}	7.20 ± 0.09^{bA}	4.80 ± 0.06^{aB}	7.60 ± 0.19^{aA}	9.30 ± 1.15^{aAB}	7.40 ± 0.13^{bA}	5.00 ± 0.17^{aB}
VI	13.80 ± 0.22^{bB}	9.60 ± 0.14^{aE}	41.10 ± 0.70^{bA}	28.00 ± 0.63^{aB}	7.70 ± 0.24^{bA}	5.10 ± 0.22^{aB}	7.50 ± 0.14^{aA}	9.00 ± 1.20^{aAB}	7.80 ± 0.26^{bA}	5.20 ± 0.22^{aB}

PCV: Packed cell volume; RBC: Red blood cell; WBC: White blood cell.
Values within same column for a particular parameter (uppercase letter) and in same row (lower case letter) bearing similar superscript do not differ at $p \leq 0.05$.

Hb, TEC, and PCV were significantly increased in group IV compared to the group II ($p \leq 0.05$). However, these values were lower than group I. Critical analysis of the data revealed better efficacy in the rats supplemented with NAC (50.00 mg kg^{-1}) with respect to Hb, PCV, and TEC values.

Pre- and post-treatment changes in the serum biochemical parameters are given in Table 2. The ALT, AST, ALP, TP, and albumin values were non-significant before and after treatment in group I. The ALT, AST, and ALP values of group II were significantly increased on day 14 compared to the group I ($p \leq 0.05$). The ALT values were non-significantly decreased in groups III and IV, and non-significantly increased in groups V and VI on day 14. Similar kind of trend was noticed regarding to AST level. Non-significantly decreased ALP values were noticed in all treated groups on day 14. Significantly decreased TP and albumin were noticed in group II on day 14 compared to the group I ($p \leq 0.05$), whereas it was non-significant in all treated groups except groups V and VI. However, protein profile in treated groups was significantly higher than disease control group ($p \leq 0.05$). Critical analysis of the data revealed better efficacy in animals supplemented with NAC (50.00 mg kg^{-1}) with respect to ALT and TP.

Decalcified bone marrow of healthy control (group I) rats revealed normal cellularity of the myeloid and erythroid precursor cells in the form of sheet, and graded 3 by histopathological score system (HPS). Bone marrow of disease control (group II) showed decrease in the cellularity of the myeloid and erythroid precursor cells compared to the group I, and assigned grade 0. Group III showed compact sheets of hematopoietic cells being denser than group II, and assigned grade 2 by HPS. Bone marrow of group IV was completely packed with hemopoietic cells (megakaryocytes were present), and graded 2.5. Histopathological examination of bone marrow in groups V and VI showed decreased density of hematopoietic cells population in epiphysis of femur with increased cellularity compared to the groups III and IV, and graded 1 and 1.5, respectively.

The liver sections of group I revealed normal architecture of hepatic lobules, including well-organized hepatocytes as cords, sinusoids, inactive Kupffer cells, central vein, and portal triad, and graded 3 by HPS. Liver sections of group II showed swollen hepatocytes with fatty changes in peri-portal and mid-zonal areas, engorged sinusoids around central veins, mild fibro-cellular reaction in peri-portal connective tissue, and necrotic hepatocytes, and graded 0. The liver tissue of group III showed improvement in lesions compared to the disease control group with prominent Kupffer cells, degenerated hepatocytes with fatty changes, and mild fibro-cellular infiltration in the portal connective tissue, and graded 2.

Table 2. Mean ± SE values of serum biochemical profile of rats received different treatments in day 0 (D0) and day 14 (D14).

Groups	ALT (IU L^{-1})		AST (IU L^{-1})		ALP (IU L^{-1})		TP (g dL^{-1})		Albumin (g dL^{-1})	
	D0	D14	D0	D14	D0	D14	D0	D14	D0	D14
I	27.30 ± 2.99^{aA}	27.30 ± 2.99^{aB}	113.30 ± 9.11^{aA}	114.30 ± 8.17^{aB}	132.60 ± 17.32^{aB}	131.50 ± 17.19^{aB}	6.10 ± 0.31^{aA}	6.20 ± 0.15^{aA}	4.10 ± 0.14^{aA}	4.00 ± 0.08^{aA}
II	29.00 ± 1.34^{bA}	51.30 ± 1.63^{aA}	104.50 ± 9.37^{bA}	156.30 ± 4.81^{aA}	180.50 ± 14.79^{bA}	252.50 ± 9.73^{aA}	6.10 ± 0.23^{bA}	4.10 ± 0.17^{aB}	3.90 ± 0.18^{aA}	2.30 ± 0.17^{bB}
III	25.30 ± 3.38^{aA}	21.10 ± 4.85^{aC}	117.60 ± 9.30^{aA}	114.80 ± 9.12^{aB}	132.00 ± 7.07^{aB}	122.80 ± 6.94^{aB}	5.30 ± 0.20^{aB}	5.40 ± 0.20^{aC}	3.80 ± 0.12^{aA}	3.90 ± 0.11^{aA}
IV	35.80 ± 2.57^{aA}	27.40 ± 5.64^{aB}	126.00 ± 6.75^{aA}	119.30 ± 10.06^{aB}	145.10 ± 18.74^{aB}	123.60 ± 17.03^{aB}	5.60 ± 0.36^{aB}	5.90 ± 0.36^{aA}	3.80 ± 0.13^{aA}	4.10 ± 0.15^{aA}
V	27.30 ± 4.86^{aA}	40.60 ± 3.20^{aA}	124.80 ± 14.13^{aA}	140.10 ± 6.75^{aA}	113.10 ± 8.67^{aB}	127.30 ± 12.34^{aB}	5.50 ± 0.22^{aB}	5.30 ± 0.27^{aC}	3.90 ± 0.11^{aA}	3.20 ± 0.12^{bC}
VI	33.60 ± 3.16^{aA}	39.00 ± 3.09^{aA}	123.00 ± 6.98^{aA}	130.50 ± 3.82^{aA}	119.10 ± 5.13^{aB}	124.50 ± 7.37^{aB}	5.60 ± 0.27^{aB}	5.30 ± 0.27^{aC}	3.80 ± 0.14^{aA}	3.30 ± 0.21^{bC}

ALT: Alanine amino transferase, AST: Aspartate amino transferase, ALP: Alkaline phosphatase, TP: Total protein.
Values within same column for a particular parameter (uppercase letter) and in same row (lower case letter) bearing similar superscript do not differ at $p \leq 0.05$.

The liver tissue of group IV showed normal architecture of the hepatic lobules compared to the group III, and graded 2.5. The liver tissue of group V showed disorganized cords, degenerated hepatocytes, sinusoids dilatation, and few mononuclear cells in the sinus spaces and around the portals, and graded 1. Histopathological analysis of group VI showed degenerated hepatocytes and aggregates of hematopoietic cells scattered in the hepatic lobules, and graded 1.5. The spleen sections of group I revealed normal architecture of white and red pulps with normal lymphoid tissue cells or macrophages and normal splenic cords, and graded 3 by HPS. Spleen sections of group II showed reduced lymphoid cells in white pulp and increased number of macrophages with neutrophils in the red pulp sinuses and cords compared to the group I, and graded 0. The spleen of group III showed hyperplasia of lymphoid tissue in peri-arteriolar lymphoid sheath and follicles and activated macrophages and neutrophils in red pulp compared to the group I, and graded 2. The spleen of group IV showed extra-medullary hematopoiesis with aggregation of erythroid and myeloid precursor cells, and plenty of megakaryocytes in red and white pulps zones. The white pulp follicles showed germinal centers with presence of plasma cells being also visible in large numbers in red pulp areas, and graded 2.5.

In group V, the spleen showed hyperplasia of lymphoid tissue in peri-arteriolar lymphoid sheath and follicles with presence of germinal centers in some follicles, and sinusoids were filled with RBCs and moderate number of neutrophils, and graded 1.

The spleen of group VI showed extra-medullary hematopoietic cells in white and red pulps with plenty of megakaryocytes and plasma cells and smaller number of neutrophils in red pulp compared to the group V, and graded 1.5. Histopathological changes in the bone marrow, liver, and splenic tissues of all groups are represented in Figure 1.

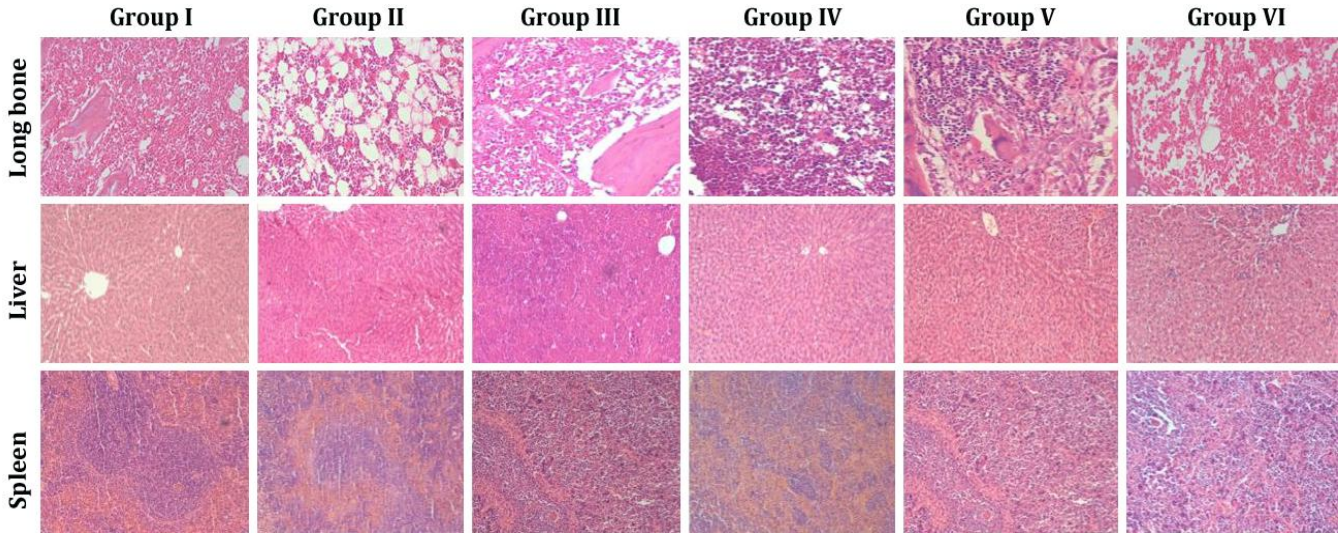

Fig. 1. Histopathological findings of long bone, liver, and spleen depicting the best bone marrow stimulating property along with liver and spleen regeneration properties in aplastic pancytopenia induced rats received N-acetylcysteine (50.00 mg kg^{-1}) orally for 10 days. In long bone; Group I: Normal cellularity of myeloid and erythroid precursor cells in the form of sheet; Group II: Decreased cellularity of myeloid and erythroid precursors compared to Group I; Group III: Compact sheets of hematopoietic cells at places; Group IV: Bone marrow completely packed with hematopoietic cells with presence of megakaryocytes; Group V: Decreased cellularity of myeloid and erythroid precursors compared to Groups III, IV and I; Group VI: Decreased cellularity of myeloid and erythroid precursors compared to Groups IV and I (Hematoxylin and Eosin staining; 20×). In liver; Group I: Normal architecture of hepatic lobules; Group II: Swollen hepatic cells with fatty changes in periportal and midzonal areas; Group III: Degeneration of hepatocytes with fatty changes, mild fibro-cellular infiltration in the portal connective tissue; Group IV: Apparent normal architecture of the hepatic lobules; Group V: Disorganized cords and degenerated hepatic cells, sinusoids dilatation and few mononuclear cells in the sinus spaces and around the portals; Group VI: Degenerated hepatocytes and aggregates of hematopoietic cells scattered in the hepatic lobules (Hematoxylin and Eosin staining; 10×). In spleen; Group I: Normal architecture of white and red pulp with normal lymphoid tissue cells and splenic cords; Group II: Reduced lymphoid cells in white pulp and increased number of macrophages and neutrophils in the red pulp sinuses and cords; Group III: Hyperplasia of lymphoid tissue in PALS and follicles, activated macrophages and neutrophils in red pulp; Group IV: Extramedullary hematopoiesis with collection of erythroid and myeloid precursors and plenty of megakaryocytes in red pulp and white pulp zones. The white pulp follicles showing germinal centers with presence of plasma cells; Group V: Hyperplasia of lymphoid tissue in PALS and follicles with presence of germinal center in some follicles and sinusoids are filled with RBC and moderate number of neutrophils; Group VI: Extramedullary hematopoietic cells in white and red pulp with plenty of megakaryocytes and plasma cells and smaller number of neutrophils in red pulp (Hematoxylin and Eosin staining; 10×).

As per the scoring system, rats of group IV scored the best (2.5) regarding to the bone marrow stimulation and regenerative changes in histopathological examinations of bone marrow, liver, and spleen sections followed by group III (2). Rats of group II showed poor score (0) with severe aplastic pancytopenia and degenerative changes in histopathological analysis of bone marrow, liver, and spleen compared to the group I.

Screening, diagnosis, and treatment of pancytopenia associated with canine hemoprotozoan diseases. Out of 4,536 dogs, presented at Referral Veterinary Polyclinic, Indian Veterinary Research Institute, Bareilly, India, during the study period 96 (2.11%) dogs were suspected for hemoprotozoan diseases with characteristic clinical signs of anemia, fever, tick infestation, lymph node enlargement, etc., and 32 (33.33%) dogs were found positive for hemoprotozoan disease by microscopic examination, being divided randomly into two treatment groups (n = 16). Significantly increased levels of Hb, PCV, TEC, and platelets were observed after 14 days compared to day 0 in groups I and II ($p \leq 0.05$). The Hb level and platelet count of group II were significantly increased after 14 days of therapy compared to the group I ($p \leq 0.05$). The PCV and TEC of group II were non-significantly increased after 14 days of therapy compared to the group I. Non-significantly decreased TLC was observed at the end of therapy compared to day 0 in both treatment groups. Critical analysis of the data revealed better efficacy in dogs supplemented with NAC (15.00 mg kg^{-1}) with respect to Hb and platelet count. Changes in hematological parameters before and after therapy are depicted in Table 3.

Significantly decreased levels of ALT, AST, and ALP were observed in the both groups at the end of trial compared to day 0 ($p \leq 0.05$). In groups I and II significantly increased level of TP was observed after 14 days of treatment. Non-significantly increased value of albumin was also found in group I ($p \leq 0.05$), whereas in group II, albumin value was significantly increased after 14 days of treatment compared to day 0 ($p \leq 0.05$). Critical analysis of the data revealed better efficacy in animals supplemented with NAC (15.00 mg kg^{-1}) with respect to ALT, AST, ALP, and TP. Pre- and post-treatment variations in serum biochemical parameters are described in Table 4.

The FRAP and DPPH assays of both groups depicted significantly increased values in serum samples following 14 days of treatment compared to day 0 ($p \leq 0.05$). Oxidative changes revealed better efficacy in animals supplemented with NAC (15.00 mg kg^{-1}) with respect to FRAP and DPPH (Table 5).

Prognostic score. Response to the treatment was assessed by measuring Hb, TEC, TLC, and platelets and reticulocytes counts, and the result revealed that dogs in group II showed favorable clinical recovery compared to the group I. Prognostic score obtained for each group is detailed in Table 6.

Table 3. Mean ± SE values of complete blood count in hemoprotozoan-infected dogs received different treatments in day 0 (D0) and day 14 (D14).

Groups	Hemoglobin (g dL^{-1})		PCV (%)		RBC ($\times 10^6$ μL^{-1})		WBC ($\times 10^3$ μL^{-1})		Platelet ($\times 10^5$ μL^{-1})	
	D0	D14	D0	D14	D0	D14	D0	D14	D0	D14
Group I	7.10 ± 0.90^{B}	9.70 ± 0.78^{aB}	21.10 ± 2.64^{bA}	29.10 ± 2.21^{aA}	3.40 ± 0.47^{bA}	4.60 ± 0.21^{aA}	16.30 ± 9.85^{aA}	9.40 ± 0.75^{aA}	0.90 ± 0.07^{bA}	2.50 ± 0.18^{aB}
Group II	7.90 ± 0.77^{bA}	11.70 ± 0.76^{aA}	24.10 ± 2.94^{bA}	32.10 ± 2.47^{aA}	3.50 ± 0.34^{bA}	4.70 ± 0.36^{aA}	12.70 ± 1.63^{aA}	11.00 ± 1.48^{aA}	0.91 ± 0.12^{bA}	3.80 ± 0.23^{aA}

Group I: Disease specific therapy treated group; Group II: Disease specific therapy plus N-acetylcysteine treated group; PCV: Packed cell volume; RBC: Red blood cell; WBC: White blood cell.
Values within the same column for a particular parameter (uppercase letter) and same row (lowercase letter) bearing similar superscript do not differ at $p \leq 0.05$.

Table 4. Mean ± SE values of serum biochemical profile in hemoprotozoan-infected dogs received different treatments in day 0 (D0) and day 14 (D14).

Groups	ALT (IU L^{-1})		AST (IU L^{-1})		ALP (IU L^{-1})		TP (g dL^{-1})		Albumin (g dL^{-1})	
	D0	D14	D0	D14	D0	D14	D0	D14	D0	D14
Group I	97.50 ± 16.29^{aB}	82.30 ± 13.89^{bB}	105.40 ± 24.24^{aA}	88.80 ± 22.95^{bA}	110.10 ± 21.15^{aB}	100.80 ± 21.11^{bB}	4.90 ± 0.43^{bA}	5.40 ± 0.38^{aB}	2.80 ± 0.19^{aA}	3.20 ± 0.15^{aA}
Group II	157.60 ± 39.57^{aA}	123.80 ± 35.17^{bA}	100.50 ± 9.84^{aA}	72.60 ± 8.97^{bB}	139.50 ± 21.34^{aA}	116.50 ± 18.97^{bA}	5.20 ± 0.46^{bA}	5.80 ± 0.41^{aA}	2.40 ± 0.27^{bA}	3.10 ± 0.10^{aA}

Group I: Disease specific therapy treated group; Group II: Disease specific therapy and N-acetylcysteine treated group; ALT: Alanine amino transferase; AST: Aspartate amino transferase; ALP: Alkaline phosphatase; TP: Total protein.
Values within the same column for a particular parameter (uppercase letter) and same row (lowercase letter) bearing similar superscript do not differ at $p \leq 0.05$.

Table 5. Mean ± SE values of anti-oxidant changes in the treated dogs in day 0 (D0) and day 14 (D14).

Groups	FRAP (μmol L^{-1})		DPPH (% inhibition)	
	D0	D14	D0	D14
Group I	129.30 ± 26.59^{Ab}	293.30 ± 57.80^{Ba}	76.00 ± 3.40^{Aa}	81.00 ± 3.50^{Ab}
Group II	158.50 ± 22.30^{Ab}	491.80 ± 42.30^{Aa}	68.30 ± 2.80^{Ba}	79.80 ± 1.60^{Bb}

Group I: Disease specific therapy treated group; Group II: Disease specific therapy plus N-acetylcysteine treated group; FRAP: Ferric reducing anti-oxidant power; DPPH: 1,1 diphenyl 2, picryl-hydrazyl.
Values within the same column for a particular parameter (uppercase letter) and same row (lowercase letter) bearing similar superscript do not differ at $p \leq 0.05$.

Table 6. Hematological values and prognostic scores in response to the treatment in day 0 (D0) and day 14 (D14).

Parameters (Normal range)	Group I		Group II	
	D0	D14	D0	D14
Hemoglobin (12.00 - 18.00 mg dL^{-1})	7.10 (1)	9.70 (2)	7.90 (1)	11.70 (3)
TEC (5.00 - 8.00 × 10^6 μL^{-1})	3.40 (2)	4.60 (3)	3.50 (2)	4.70 (3)
TLC (5.00 - 14.00 × 10^3 μL^{-1})	16.30 (1)	9.40 (3)	12.70 (3)	11.00 (3)
Platelet count (1.50 - 4.00 × 10^5 μL^{-1})	0.90 (2)	2.50 (2)	0.90 (2)	3.80 (3)
Reticulocyte count (60.00 - 80,000 μL^{-1})	< 30,000 (1)	46,000 (2)	< 30,000 (1)	66,000 (3)
Total score	7 = Grade C (1^+)	12 = Grade B (2^+)	9 = Grade C (1^+)	15 = Grade A (3^+)
Prognosis	Poor	Moderate	Poor	Good

Group I: Disease specific therapy treated group; Group II: Disease specific therapy plus N-acetylcysteine treated group; TEC: Total erythrocyte count; TLC: Total leukocyte count.

Discussion

Bone marrow is one of the vital parts of the body, being responsible for preservation of immunity and other hemostatic functions by production of RBCs, Hb, WBCs, and platelets.[24] Myelo-suppression or myelo-toxicity often predisposes individuals to life-threatening conditions, such as neutropenia, thrombocytopenia, septicemia, and multi-organ failure.[25] Myelo-suppression is the most common side effect of chemotherapy. Cyclophosphamide is the commonly used myelotoxic anti-cancer drug causing severe oxidative stress and inflammation, and altering hematopoietic activity of bone marrow by producing reactive oxygen species and inflammatory cytokines.[26] Another possibility behind CP-induced myelo-suppression is its effect on the DNA of bone marrow cells. In the present study, single dose of CP (37.50 mg kg^{-1}; SC) was effective in inducing bone marrow suppression. It was in agreement with Iqubal *et al.*, recorded significantly reduced level of Hb, RBCs, WBCs, granulocytes, lymphocytes, monocytes, and platelets after single dose of CP (200 mg kg^{-1}; intravenously) in rats.[27] In the present study, induction of aplastic pancytopenia with single dose of CP (37.50 mg kg^{-1}; SC) revealed better and effective experimental model to induce aplastic pancytopenia in rats with reductions of Hb, PCV, TEC, and platelet count of 5.00, 15.00, 3.00, and 3.50%, respectively.

The N-acetylcysteine has been reported for the management of anemia and oxidative stress in hemodialysis patients and considered as a promising anti-oxidant.[28] Desmopressin is an analogue of anti-diuretic hormone, interacting with type 2 vasopressin receptors of endothelial cells and thereby, inducing the release of von Willebrand factor and associated factor VIII, being hemostatically effective in mild hemophilia and von Willebrand type 1 diseases.[29] Furthermore, desmopressin depicted better results in treating hemostasis in thrombocytopenia associated with bone marrow failure and platelet disorder.[30] In the current study, it was found that NAC (50.00 mg kg^{-1}; orally *per* day) depicted better efficacy over desmopressin in relation to bone marrow cellularity and total blood count by 10 days of therapy. Neboh and Ufelle have demonstrated myelo-protective activity of crude methanolic leaf extract of *Cassia occidentalis* in CP-induced bone marrow suppression in terms of increased Hb production and leukocytosis in *Wistar* rats.[31] Similar changes were observed with nerolidol for myelo-protection and amelioration of hematological toxicity in CP-induced bone marrow suppression in Swiss albino mice.[27] Similar findings were observed in the current study with oral administration of high dose (50.00 mg kg^{-1}) of NAC in terms of improved hematological parameters in CP-induced bone marrow suppression. The current study showed significantly increased hematological parameters *viz*. Hb, PCV, TEC, and platelet count in group IV (NAC at a dose of 50.00 mg kg^{-1}) compared to the group II (disease control). Significant increase in the hematological parameters of group IV could be attributed to the NAC protective and anti-oxidant effects on bone marrow cells. Significant reduction was observed in the hematological parameters of group II, and this could be due to the oxidative stress and direct effect of toxin and its metabolites on the DNA of bone marrow cells.[27] The CP causes liver damage, being indicated by increase in ALT, AST, and ALP levels in serum.[32] In the present study, significant increased levels of ALT, AST, and ALP in serum of group II rats were noticed, being in agreement with findings of Shokrzadeh *et al*.[32] It was also

found that administration of aqueous carob extract exhibited protection against hepatic damage induced by CP through oxidative stress reduction.[33] Similarly, in the current study, group IV rats being treated with high dose of NAC showed improvements in the liver biomarkers. This effect may be due to the free radical scavenging activity of NAC.

Histopathological findings of CP-treated rats in the current study revealed reduction in hematopoietic count with increase in adipose tissue. These findings were in accordance with previous studies, wherein bone marrow was markedly hypo-cellular with distortion of the myeloid and erythroid tissues and more empty spaces in the CP-administered group.[27,34] Treatment with high dose of NAC significantly reversed these histological aberrations of bone marrow towards normalcy. Hepatic damage produced by CP in group II was characterized by swollen hepatocytes with fatty degeneration in peri-portal and mid-zonal areas, engorged sinusoids around central veins, mild fibrocellular reaction in peri-portal connective tissue, and hepatocytes necrosis. This was in accordance with Al-Salih *et al*., report,[35] defining fatty changes in the hepatocytes with diffuse Kupffer cells proliferation and dilated portal vein. Rats of group IV being treated with NAC (50.00 mg kg^{-1}) showed regenerative changes, like completely packed bone marrow with hematopoietic cells and presence of megakaryocytes. This hepato-protective effect may be due to the anti-oxidant activity of NAC. Similar hepato-protective effect was reported by Cai *et al.*,[36] concluded that NAC significantly reduced the necrotic area in the liver tissue. Spleen can perform compensatory hematopoiesis in the milieu of impaired bone marrow function to restore the hematological process.[37] The CP administration showed reductions of the diameter and lymphoid cells in white pulp and increased number of macrophages and neutrophils in the red pulp sinuses and cords in group II rats. This was in accordance with Khazaei *et al.*,[38] noticed disorganization in splenic structures, such as hemosiderin deposition and reduction of the diameter of white pulp. These alterations were significantly improved in NAC-treated group IV. Significantly increased diameter of white pulps, extra-medullary hematopoiesis with aggregation of erythroid and myeloid precursors cells, and plenty of megakaryocytes in red and white pulps zones were noticed in group IV rats. Additionally, the white pulp follicles also showed germinal centers with presence of plasma cells, being also visible in large numbers in red pulp areas, indicated that NAC (50.00 mg kg^{-1}) promoted the recovery of this damage after CP administration.

In the study of pancytopenia associated with canine hemoprotozoan diseases, significantly increased levels of Hb, PCV, TEC, and platelet were observed in the dogs of groups I and II after 14 days of therapy compared to the day 0. Post-treatment hematological values in group II dogs (NAC at a dose of 15.00 mg kg^{-1}) were closer to the reference values. Potent anti-oxidant efficacy of NAC has been revealed by several researchers.[35,39,40] The present study depicted potent anti-oxidant activity with NAC (15.00 mg kg^{-1}) for regeneration of the hepatocytes in the dogs with hepato-biliary diseases, which in turn might improve post-treatment hematopoietic values. Anemia observed in dogs affected with hemoprotozoan infection is considered as one of the factors causing hypoxia and hypoxic liver injury, which can result in elevated levels of ALT, AST, and ALP.[41] Similar trend was also noticed in both treated groups on day 0.

The N-acetylcysteine was found to be useful in fulminant hepatic failure associated with paracetamol over-dose.[42] The NAC improved hepatic hemodynamic in patients with fulminant hepatic failure and this effect was mediated by cyclic 3, 5-guanosine monophosphate.[43] The NAC increased the concentrations of hepatic adenosine triphosphate and glutathione by ameliorating the oxidative stress in obstructive jaundice.[44] In the present study, significantly decreased levels of ALT, AST, and ALP were more pronounced in NAC-supplemented dogs (group II) at the end of trial, denoted NAC potent anti-oxidant property for regeneration of the hepatocytes in the dogs with hepato-biliary diseases, which in turn might improve post-treatment serum biochemical and liver profile values. Dogs infected with hemoprotozoa showed increased oxidative markers, like nitric oxide and advanced oxidation protein products.[45] In the present study, the anti-oxidants levels were significantly higher in group II received NAC compared to the group I, which may be due to the protective and anti-oxidant effects of NAC.

Critical evaluation of hematological parameters in both treated groups showed that the Group II dogs being treated with NAC revealed remarkable clinical recovery compared to the group I. Prompt therapeutic interventions are very essential in treating aplastic pancytopenia in hemo-parasitic diseases. Otherwise, the animal may develop critical anemia warranting blood transfusions. In contrast to human beings, well-established blood banks are not widely prevalent for companion animals. In the present study, dogs supplemented with NAC (15.00 mg kg hepato-biliary diseases) orally for 14 days along with hemoprotozoan disease specific therapy and supportive therapy rendered favorable changes in a better way, characterized by haltering the progression of critical anemia and thrombocytopenia in dogs with compromised liver due to the hemoprotozoan diseases. These findings also recommend alternate approach to rejuvenate therapeutic modalities of canine transfusion medicine.

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Conflict of interest

There is no conflict of interest to report.

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