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Comparative evaluation of darbepoetin therapy in non-regenerative anaemia associated with *Babesia gibsoni* infection in dogs

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Abstract

The present study describes a comparative evaluation of haemato-therapeutic response to darbepoetin therapy in non-regenerative anaemia associated with Babesia gibsoni infection in dogs. A total of 68 dogs, presented at Teaching Veterinary Clinical Complex, Mannuthy with signs of anaemia, weakness, anorexia and pallor of mucous membranes were screened for non-regenerative anaemia. Twenty dogs with a reticulocyte count of < 60,000/µL, haematocrit of < 30 per cent and positive for B. gibsoni were selected for detailed study. The twenty dogs were divided into two groups of ten animals and were subjected to estimation of complete blood count and analysis of serum protein and iron status. Ten dogs brought to the hospital for vaccination or health check-up served as healthy control. Haematological examination revealed significant anaemia and thrombocytopenia in both the groups. Serum total protein, albumin and total iron binding capacity revealed no difference between diseased groups with the healthy control. Serum iron and percentage transferrin saturation were similar in Group I and II, but significantly higher than healthy control. To compare darbepoetin efficacy, Group I dogs were administered with clindamycin and doxycycline for three weeks, whereas Group II was given with darbepoetin at the dose rate of 0.5 µg/kg body weight at weekly intervals for three weeks in addition to clindamycin-doxycycline as in Group I. Dogs of both groups showed complete remission of clinical signs within 10 days of therapy. Statistical analysis revealed no significant difference in haematological parameters between the treatment groups. It can be concluded from this study that there is no added advantage of darbepoetin therapy over conventional therapies against non-regenerative anaemia associated with B. gibsoni infection in dogs.

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Non-regenerative anaemia refers to minimal reticulocytosis in severe cases of anaemia, which continued even after sufficient time needed for an adequate bone marrow response (Fleischman, 2012). The causes of non-regenerative anaemia in dogs are chronic diseases, bone marrow disorders, renal disease, acute blood loss and endocrine diseases (Couto, 2020). According to Paltrinieri (2014), a lack of regeneration in infectious diseases was either due to infection of stromal cells that sustain haematopoietic cells in the bone marrow or due to the existence of anaemia of inflammation in chronic diseases. Non-regenerative anaemia in chronic kidney disease resulted primarily from decreased production of erythropoietin from renal tissues (Chalhoub and Langston, 2015). The diminished levels of cortisol and subsequent bone marrow arrest resulted in normocytic, normochromic non-regenerative anaemia in hypoadrenocorticism whereas decline in erythropoiesis in response to declined levels of thyroid hormones contributed to non-regenerative anaemia in hypothyroidism (Lanen and Sande, 2014). Bone marrow disorders are the major causes of non-regenerative anaemia in dogs which can be attributed to an ineffective haematopoiesis and subsequent hastened apoptosis of progenitor cells within the marrow (Weiss, 2003). Anaemia due to blood parasites is generally considered as regenerative. Allison and Meinkoth (2010) reported regenerative anaemia in *Babesia gibsoni* infection in dogs. In contrast, in a pilot study on regenerative status of anaemia in *B. gibsoni* infected dogs in Kerala, nearly 50 per cent had non-regenerative anaemia (Arun, 2021). The absence of regeneration in haemoprotozoal infections could be attributed to secondary suppression of bone marrow activity by underlying inflammation (Grimmes and Fry, 2015). Arun *et al*. (2022) reported non-regenerative anaemia in a dog co-infected with *Babesia gibsoni* and *Anaplasma platys*.

The incidence of *B. gibsoni* infection in dogs in Kerala is high as per Jain *et al*. (2017) who reported that nearly 47.33 per cent of anaemic dogs were infected with piroplasms of *Babesia gibsoni*. Relapse of infection and persistent anaemia remains a challenge even after standard therapeutic regimens with doxycycline, imidocarb, clindamycin, metronidazole or their different combinations. Persistent anaemia is a real challenge in the treatment of *B. gibsoni* infected dogs. A well-designed study to evaluate the effect of haemopoietic stimulating agents in non-regenerative anaemia in *B. gibsoni* infected dogs is lacking. This study intended to estimate serum iron and protein status and to evaluate haemato-therapeutic response to darbepoetin therapy in non-regenerative anaemia associated with *B. gibsoni* infection in dogs.

Materials and methods

Dogs presented at the Teaching Veterinary Clinical Complex, Mannuthy, with signs of anaemia, such as weakness and pallor of mucous membranes, were screened for non-regenerative anaemia associated with *B. gibsoni* infection. The criteria for inclusion of dogs for the study were a haematocrit of <30 per cent, reticulocyte count of <60,000/µL (Briggs and Bain, 2017) and blood smear positive for *B. gibsoni* organisms. The presence of *B. gibsoni* was later confirmed by PCR using species-specific primer sets as described by Jain *et al*. (2017). Twenty dogs which met the inclusion criteria were randomly divided into two groups (I and II) of ten animals each for therapeutic evaluation. Wet film, blood smear, buffy coat smear, complete blood count and faecal examination were done to rule out co-morbidities.

Haematology and serum biochemistry

Complete blood count was obtained from whole blood in EDTA in an automated haematology analyser (Make: Orphee Mythic TM 18 Vet) within half an hour of collection of blood samples. Quantitative analysis of protein and iron status was performed for both the groups. Serum levels of albumin and total protein were estimated in serum biochemistry analyser (Hospitex, Master-T) using commercially available kits (Spin React make) as per standard instructions of the manufacturer. Serum levels of iron and total iron binding capacity (TIBC) were determined using assay kits (Euro make) in an automated serum biochemical analyser

Table 1. Species-specific primer sequences of *Babesia gibsoni* organisms

Organism	Primer	Sequences	Product length
Babesia gibsoni	Forward	BAGI F-5'-TTG GCG GCG TTT ATT AGT TC-3'	481 bp
	Reverse	BAGI R-5'-AAA GGG GAA AAC CCC AAA AG-3'	

(SelectraPRO S Lite) and the percentage transferrin saturation (PTS) was calculated.

Reticulocyte count

Reticulocytes were stained using azure B stain. Dissolved 1.0 g of azure B in 100 ml of 3 per cent trisodium citrate-saline solution prepared by mixing 30 g of trisodium citrate dihydrate in 1.0 litre of saline (Briggs and Bain, 2017). Mixed two drops of whole blood in EDTA with two drops of azure B stain and incubated for 20 minutes at room temperature to stain the reticulocytes. Prepared the smear, air dried and examined under oil immersion of microscope (Olympus Model CH-20i). The number of reticulocytes in different microscopic fields were counted manually and calculated the absolute reticulocyte count as given below.

Number of fields counted = n

Total number of reticulocytes in 'n' fields = x

Average number of red cells per field = y

Total number of red cells in 'n' fields = n * y

Reticulocyte percentage = [x / (n * y)] * 100

Absolute reticulocyte count/ µL = (Reticulocyte percent/100) * (no. of RBCs/ µL)

Polymerase chain reaction

The genomic/ parasitic DNA extracted from whole blood using DNeasy™ blood and tissue kit (Qiagen, Germany) was quantified using Nanodrop UV-VIS spectrophotometer (Thermofisher, USA) at 260nm and 280 nm. The presence of *Babesia gibsoni* was confirmed by amplifying a 481 bp sequence of 18S rRNA of the organism using species specific primer sets (Table 1) as per Jain *et al*. (2017).

Bone marrow evaluation

Bone marrow evaluation for myeloid to erythroid (M:E) ratio was performed in two cases on the day of presentation as per Moritz *et al*. (2010). Briefly, the animal was restrained in lateral recumbency (Fig.1) under local anaesthesia (2.5 ml of 2 % lignocaine) and intramuscular butorphanol @ 0.5 mg/ kg. An 18G Jamshidi needle with stylet was inserted by clockwise-anti-clockwise rotational movement into the inter-trochanteric fossa of femur, after which the the stylet was removed to expose the hub of the needle. A 5.0 ml sterile syringe pre-filled with 0.25 ml acid-citrate-dextrose anticoagulant solution was fixed to the hub of the Jamshidi needle and approximately 0.5 ml of bone marrow was aspirated aspirated by repeated forceful withdrawals of the syringe plunger. Examined the stained bone marrow smears (Field stain, Make: Nice) under the oil immersion objective of a microscope (Olympus Model CH-20i) for a 500-cell differential count and calculated the M:E ratio .

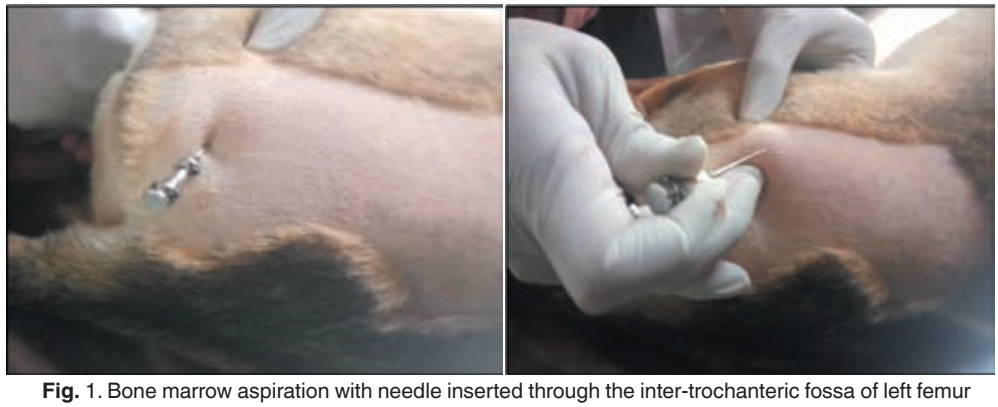

Fig. 1. Bone marrow aspiration with needle inserted through the inter-trochanteric fossa of left femur

Therapeutic study

Babesia gibsoni infected dogs with non-regenerative anaemia were divided into two groups. Dogs in Group I (n=10) received doxycycline @ 10 mg/Kg PO, OD and clindamycin @ 11 mg/Kg PO, OD for three weeks. Inappetent dogs were given intravenous preparations of these drugs until oral alimentation was possible. Animals of Group II (n=10) were given doxycycline and clindamycin as in Group I along with darbepoetin @ 0.5 µg/kg subcutaneously once in a week for three weeks. Response to therapy was evaluated on the basis of clinical signs and haematology. Complete blood count and reticulocyte count were re-assessed on days 7, 14 and 21.

Healthy control

Ten animals brought to the hospital for vaccination or health check-up served as healthy control for obtaining normal values for the parameters under study.

Statistical analysis

Pre-treatment and post-treatment haematological values were compared by paired t-test and between-group comparison by repeated measure ANOVA using the statistical software SPSS version 24.0.

Results and discussion

All the peripheral blood smear samples revealed signet-ring shaped intra-erythrocytic organisms suggestive of small babesia (Fig. 2) as per Birkenheuer (2012) and the presence of *Babesia gibsoni* was confirmed by PCR (Fig. 3). Wet film, blood smear and faecal samples of all the dogs were negative for other pathogenic parasites or their infective stages.

Haematology revealed severe anaemia and thrombocytopenia in animals of both the groups. A mean haematocrit of 18.67 per cent and 12.38 per cent was observed on the day of presentation in Group I and II, respectively. The reason for anaemia is attributed either to the direct action of *B. gibsoni* or by the action of metabolic products that impaired erythropoiesis as suggested by Grimmes and Fry (2015). Thrombocytopenia in *B. gibsoni* infection is due to sequestration of platelets in spleen as documented by Revathi *et al*. (2021). In this study, the mean thrombocyte count was 76 × 10^3/µL and 78.40 × 10^3/µL in Group I and II, respectively on the day of presentation. Leucogram did not vary significantly between groups and from the healthy control.

Protein status of study population was assessed on the basis of serum total protein and albumin (Table 2). The mean total protein and albumin values were similar in both the treatment groups and the healthy dogs. Normal levels of total protein and albumin ruled out the possibility of long-term protein deficiency as the reason for non-regenerative anaemia.

With respect to iron status (Table 2), serum iron concentration was statistically higher in both Group I (263.39 µg/dL) and Group II

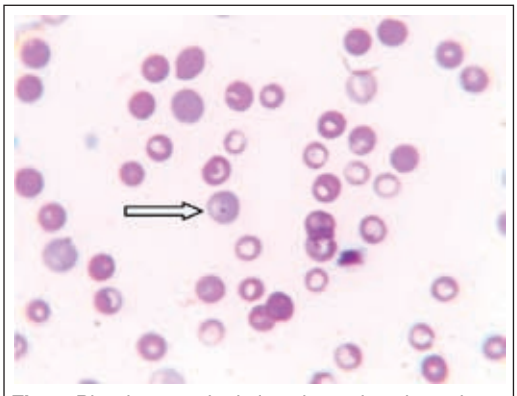

Fig. 2. Blood smear depicting signet ring shaped *Babesia gibsoni* organisms in an erythrocyte

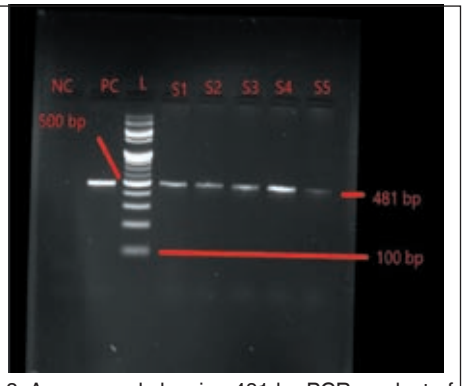

Fig. 3. Agarose gel showing 481 bp PCR product of Babesia *gibsoni* (NC-negative control, PC-positive control, L- ladder, positive samples-S1, S2, S3, S4, S5

Table 2. Serum biochemical parameters of dogs with non-regenerative anaemia associated with *Babesia gibsoni* infection

Parameters	Group I (n=10) Mean ± SE	Group II (n=10) Mean ± SE	Healthy control (n=10) Mean ± SE	F value	p-value
Total protein (g/dL)	6.39 ± 0.26	6.60 ± 0.24	6.98 ± 0.21	1.47	0.25
Albumin (g/dL)	2.65 ± 0.21	2.74 ± 0.14	3.20 ± 0.15	2.99	0.07
Serum iron (µg/dL)	263.39 [a] ± 50.58	254.47 [a] ± 20.74	158.51 [b] ± 32.88	3.55*	0.04
Total iron binding capacity (µg/dL)	447.49 ± 61.06	366.82 ± 30.42	385.38 ± 96.52	0.95	0.39
Percentage transferrin saturation	72.16 [a] ± 13.48	71.50 [a] ± 6.55	40.07 [b] ± 7.45	3.59*	0.04

**Significant at p ≤ 0.05, Values with different superscripts differ significantly*

(254.47 µg/dL) than the healthy controls (158.51 µg/dL) whereas no significant difference was observed between Group I and II. The mean TIBC was within normal range in all the groups studied. Percentage transferrin saturation was significantly higher in Group I (72.16 per cent) and Group II (71.5 per cent) than the control population (40.07 per cent). A high serum iron, a normal TIBC and an elevated PTS indicated a normal to high level of iron in the body which in turn ruled out the possibility of iron deficiency to be the reason for anaemia. Surprisingly, the presence of microcytic red cells in peripheral blood smear, i.e., erythrocytes less than 6 µm in diameter, which is typically seen associated with iron deficiency anaemia was noticed in many of the cases. The major difference was that the microcytes observed in *B. gibsoni* infected dogs were microcytic-normochromic (Fig. 5) in appearance unlike the characteristic microcytic-hypochromic red cells seen in iron-deficiency anaemia. The presence of more than 11 per cent microcytes in peripheral blood smear is considered as moderate microcytosis (Palmer *et al*., 2015). Moderate microcytosis was noticed in 65 per cent (13/20) of the cases studied. Hence, it might be concluded from this study that the reason for microcytic-normochromic erythrocytes in *B. gibsoni* infected dogs with non-regenerative anaemia is not due to true iron deficiency, but possibly a functional deficiency of iron.

The myeloid to erythroid ratio (M:E) observed in two cases were 0.58 and 0.75 (Fig. 6). The normal M:E ratio in dogs ranged from 0.75 to 2.53 (Stacy and Harvey, 2017). According to the authors, a normal M:E ratio was observed not only in regenerative anaemia, but also in non-regenerative anaemia which indicated an ineffective erythropoiesis in the event of anaemia. The decreased M:E

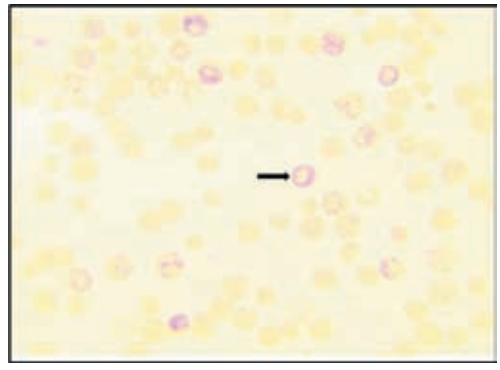

Fig. 4. Reticulocytes, 1000 X magnification, Azure B stain

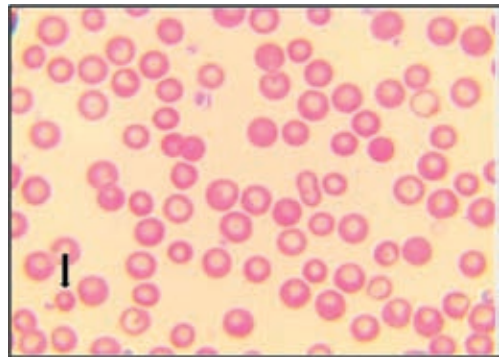

Fig. 5. Numerous normochromic-microcytes in a peripheral blood smear, 1000 X magnification, Field stain

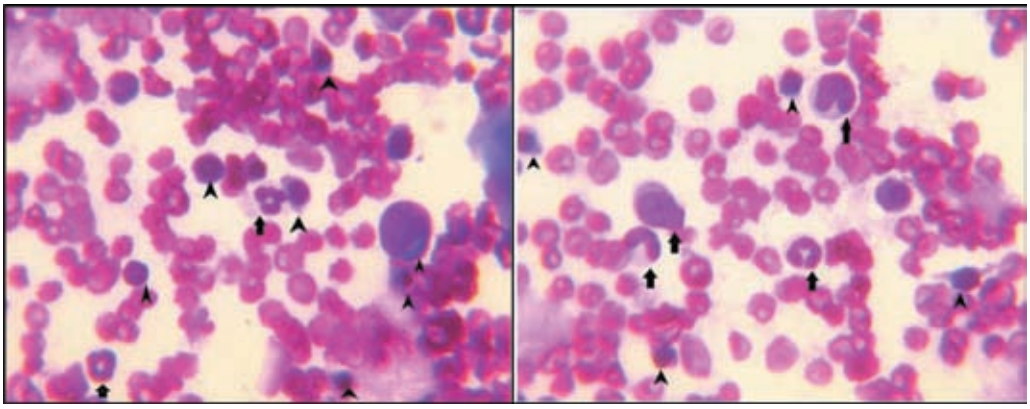

Fig. 6. Bone marrow smear, 1000 X magnification, Field stain. Arrow head indicate cells of erythroid series and arrows indicate cells of myeloid series.

ratio in one case indicated a higher number of nucleated erythroid cells compared to myeloid series, but the number of reticulocytes in peripheral circulation was lower than 60,000/ µl suggestive of ineffective erythropoiesis.

The outcome of therapeutic study was assessed on the basis of clinical response to drug therapy, and changes in haemogram and reticulocyte counts on days 7, 14 and 21. Following drug therapy, dogs in both groups recovered from anorexia, dullness and lethargy within three to five days and complete remission of clinical signs was noticed in 10 days. Vishnurahav *et al*. (2016) reported similar therapeutic response in *B. gibsoni* infected dogs. Haemoglobin, RBC count, haematocrit and thrombocyte count improved significantly with therapy in both the groups I and II as given in table 3 and table 4 respectively. However, this study found no significant difference in haemogram and thrombogram between Group I and II, *i.e*., with and without darbepoetin on days 7, 14 and 21 (Table 5).

Reticulocytes are immature erythrocytes in peripheral circulation. Interestingly, the reticulocyte count (Figure 4) was lower than 60,000/ µL, *i.e*., the criteria set to differentiate regenerative and non-regenerative anaemia (Briggs and Bain, 2017), in nine out of ten dogs during the study period in both Group I and Group II on days 7, 14 and 21. A reticulocyte count of more than 60,000 cells/µL, suggestive of strong erythroid regeneration, was noticed only in one dog each in Group I and Group II. The dog in Group I had a reticulocyte count of 20,600/µL, 87,309/ µL, 84,630/µL and 39,600/µL on days 1, 7, 14 and 21 respectively. The corresponding figures in the dog with strong regeneration in Group II, *i.e*., with darbepoetin therapy were 32,524/ µL, 130,464/ µL, 117,488/ µL and 147,250/ µL respectively. Though a direct comparison of these figures indicated a higher erythroid regeneration in the dog in Group II, erythroid regeneration was low in 90 per cent (9/10) of the cases in Group II. Erythroid regenerative response to higher doses of darbepoetin may be explored in a future therapeutic study.

A lack of strong regeneration in 90 per cent (18/20) of the total study population in both the groups points towards the active presence of certain factors that inhibit erythropoiesis. The inhibitory factor could be a persistent low-grade infection. Iron and protein are not the limiting factors as serum iron and albumin were normal in both the study groups. In other words, in this study the reason for non-regenerative anaemia in *B. gibsoni* infected dogs is not a low protein status or not a low iron status. Though erythropoietin level was not assessed in this study, the possibly of deficiency of erythropoietin is low as there is no response to darbepoetin therapy. The level of erythropoietin may be included as an additional criterion in future trials. The poor regenerative response in *B. gibsoni* infected dogs can be attributed to a functional deficiency of iron due to chronic low-grade infection as reported by Chikazawa and Dunning (2016) in anaemia of inflammatory disease.

Table 3. Comparison of haematology and reticulocyte count before (day-0) and after treatment (day-21) of Group I

Parameters	Pre-treatment (Mean ± SE)	Post-treatment (Mean ± SE)	t-value	p-value
Total leucocyte count (× 10^3/µL)	9.38 ± 1.24	9.42 ± 1.15	0.02	0.98
Lymphocyte count(× 10^3/µL)	3.64 ± 0.73	3.06 ± 0.43	0.60	0.56
Monocyte count (× 10^3/µL)	0.67 ± 0.11	0.46 ± 0.04	1.63	0.14
Granulocyte count (× 10^3/µL)	5.06 ± 0.57	5.92 ± 0.82	0.84	0.42
Red blood cell count (× 10^6/µL)	2.61 ± 0.18	5.78 ± 0.10	17.08**	<0.00
Haemoglobin (g/dL)	5.77 ± 0.37	13.83 ± 0.42	14.35**	<0.00
Volume of packed red cells (per cent)	18.67 ± 1.31	39.51 ± 0.81	13.25**	<0.00
Platelet count (× 10^3/µL)	76.00 ± 17.51	288.30 ± 41.75	6.39**	<0.00
Reticulocyte count (/µL)	30692.80 ± 4504.75	9499.70 ± 3820.11	3.96*	0.01
**significant at p ≤ 0.05 and **significant at p≤ 0.01*				

Table 4. Comparison of haematology and reticulocyte count before (day-0) and after treatment (day-21) of Group II

Parameters	Pre-treatment (Mean ± SE)	Post-treatment (Mean ± SE)	t-value	p-value
Total leucocyte count (× 10^3/µL)	10.28 ± 1.63	8.76 ± 1.05	0.73	0.48
Lymphocyte count(× 10^3/µL)	3.96 ± 0.51	2.98 ± 0.62	1.17	0.27
Monocyte count (× 10^3/µL)	0.79 ± 0.13	0.59 ± 0.09	1.48	0.17
Granulocyte count (× 10^3/µL)	5.53 ± 1.12	5.49 ± 0.97	0.98	0.98
Red blood cell count (× 10^6/µL)	2.34 ± 0.22	6.00 ± 0.12	14.14**	<0.00
Haemoglobin (g/dL)	5.63 ± 0.59	15.26 ± 0.65	16.53**	<0.00
Volume of packed red cells (per cent)	18.62 ± 1.81	40.39 ± 1.10	10.06**	<0.00
Platelet count (× 10^3/µL)	78.40 ± 20.97	353.30 ± 34.84	5.78**	<0.00
Reticulocyte count (/µL)	38464.50 ± 3799.39	24795.10 ±13933.51	0.90	0.39
**significant at p ≤ 0.05 and **significant at p≤ 0.01*				

To conclude, there is no added advantage in the use of darbepoetin to induce erythroid regeneration in *B. gibsoni* infected dogs with non-regenerative anaemia. The reason for non-regenerative anaemia in *B. gibsoni* affected dogs is not a true deficiency of iron, or protein. Poor erythroid regeneration can be attributed to a functional deficiency of iron subsequent to persistent low-grade *B. gibsoni* infection. Moreover, *B. gibsoni* infected dogs with non-regenerative anaemia had high serum iron and hence supplementation of iron should be done with caution.

Conclusion

Haemato-therapeutic response to darbepoetin therapy was studied in non-regenerative anaemia associated with *Babesia gibsoni* infection in dogs. Twenty dogs with a reticulocyte count of less than 60,000/µL, haematocrit less than 30 per cent, and associated with *B. gibsoni* infection were studied in detail. These dogs were randomly divided into Group I and II of ten animals each. Haematology revealed severe anaemia and thrombocytopenia in both the groups. The mean total protein and albumin values were similar in both the treatment groups and the healthy dogs. Normal levels of total protein and albumin ruled out the possibility of long-term protein deficiency as the reason for non-regenerative anaemia. A high serum iron, a normal TIBC and an elevated PTS indicated a normal to high level of iron in the body which in turn ruled out the possibility of iron deficiency to be the reason for anaemia. Dogs of both groups *i.e*., with and without darbepoetin,

Table 5. Comparison of response to treatment between Group I and Group II

Parameter	Group	Days				Between groups
		0th	7th	14th	21st	p-value
Total leucocyte count (× 10^3/µL)	G1	9.38 ± 1.45	11.55 ± 1.88	9.71 ± 1.97	9.42 ± 1.11	0.87
	G2	10.28 ± 1.45	11.55 ± 1.88	8.49 ± 1.09	8.76 ± 1.11	
Lymphocyte count(× 10^3/µL)	G1	3.64 ± 0.63	4.89 ± 1.04	3.62 ± 0.61	3.06 ± 0.54	0.93
	G2	3.96 ± 0.63	5.13 ±1.04	3.16 ± 0.61	2.98 ± 0.54	
Monocyte count (× 10^3/µL)	G1	0.67 ± 0.12	0.78 ± 0.17	0.63 ± 0.69	0.46 ± 0.74	0.70
	G2	0.79 ± 0.12	0.92 ± 0.17	0.56 ± 0.07	0.59 ± 0.07	
Granulocyte count (× 10^3/µL)	G1	5.06 ± 0.89	6.81 ± 1.06	5.72 ± 0.85	5.92 ± 0.89	0.84
	G2	5.43 ± 0.89	5.72 ± 1.06	4.56 ± 0.24	5.49 ± 0.89	
Red blood cell count (× 10^6/µL)	G1	2.61 ± 0.20	3.12 ±0.31	4.57 ± 0.24	5.78 ± 0.11	0.43
	G2	2.34 ± 0.20	3.40 ± 0.31	4.90 ± 0.24	6.00 ± 0.11	
Haemoglobin (g/dL)	G1	5.77 ± 0.49	7.08 ± 0.89	11.51 ± 0.69	13.83 ± 0.55	0.53
	G2	5.63 ± 0.49	7.67 ± 0.89	11.87 ± 0.69	15.26 ± 0.55	
Volume of packed red cells (%)	G1	18.67 ± 1.58	22.75 ± 2.56	33.36 ± 1.62	39.51 ± 0.97	0.80
	G2	12.38 ± 1.58	25.00 ± 2.56	35.90 ± 1.62	40.39 ± 0.97	
Platelet count (× 10^3/µL)	G1	76.00 ± 19.32	115.40 ± 42.83	206.40 ± 34.79	288.30 ± 38.45	0.22
	G2	78.40 ± 42.83	215.70 ± 42.83	323.00 ± 34.79	353.30 ± 38.45	
Reticulocyte count (/µL)	G1	30692.80 ± 4167.27	38446.00 ± 9259.29	24348.70 ± 9253.84	9499.70 ± 10216.06	0.85
	G2	38464.50 ± 4167.27	43984.90 ± 9259.29	34298.00 ± 9253.84	24795.10 ± 10216.06	

showed complete remission of clinical signs within 10 days of therapy. However, this study found no significant difference in haemogram and thrombogram between the two treatment groups on days 7, 14 and 21.To conclude, there is no added advantage in the use of darbepoetin to induce erythroid regeneration in *B. gibsoni* infected dogs with non-regenerative anaemia. Non-regeneration in *B. gibsoni* infection in dogs is not associated with low iron or low protein status, but could be attributed to the suppression of bone marrow by chronic blood parasitism.

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Conflict of interest

There is no conflict of interest reported by the authors.

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