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Assessment of Serum Proinflammatory Cytokines and Some Associated Haematobiochemical Indices in An experimental Canine Trypanosomiasis

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THIS research assessed the concentrations of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and interleukin-6 (IL-6) in Nigerian indigenous dogs (NID) experimentally infected with *Trypanosoma congolense* (*T. congolense*) and correlated with some haematological and biochemical indices. Fifteen NID of weight (9.3±1.04 kg) were randomly assigned into three groups of five each. Group A was uninfected and untreated. Group B received *T. congolense* (2.5 x 10^5), but untreated. Group C received *T. congolense* (2.5 x 10^5) and treated with diminazene aceturate on day 17 post-infection (pi). Blood was obtained pre-infection, pi and post-treatment (pt) for cytokines assessment using ELISA. Serum TNF-α increased significantly ($p < 0.05$) in groups B and C on days 10 and 17 pi but decreased on day 5 pt in group C compared to groups A and B. Serum IL-6 increased significantly in groups B and C on days 1 to 22 pi. Both TNF-α and IL-6 correlated positively with monocytes count, creatinine, and urea. Serum TNF-α correlated negatively with PCV, erythrocytes count, haemoglobin concentration (Hb) and albumin, while IL-6 correlated negatively with on PCV, Hb and albumin. In conclusion, IL-6 was elevated post infection and remained elevated post treatment. Tumour necrosis factor - alpha concentration was elevated in NID infected with *T. congolense*, but decreased significantly post treatment. Both IL-6 and TNF-α positively or negatively associated with assessed haematological and biochemical indices. In conclusion, proinflammatory cytokines are elevated in NID experimentally infected with *T. congolense* and caused some pathology.

Keywords: Proinflammatorycytokines, dogs, ***Trypanosoma congolense*** , diminazene aceturate.

Introduction

African trypanosomiasis (AT) is a complex, debilitating and zoonotic protozoan disease of man and animals which constitutes a major impediment to livestock production and economic development in several parts of sub-Saharan Africa, including Nigeria [1,2] despite desperate and calculated decades of attempts to control the disease and its vectors[3,4]. In tsetse flies infested sub-Saharan African countries, pathogenic protozoan trypanosome species are transmitted to a wide range of susceptible mammalian hosts, including dogs, through infective tsetse fly (*Glossina spp.*) bites when taking blood meals [5,6]. This zoonosis had led to losses estimated at billions of dollars in the livestock industry [7]. *Trypanosoma congolense* (*T. congolense*) is considered to be the most pathogenic trypanosoma species in cattle, with anaemia being the most prominent pathological feature of AT [8]. Infection with AT induces multiple disorders in the immune system. African trypanosomiasis is difficult

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to understand due to the complex interactions between the host immune response and the parasite survival strategies [9,10]. Cytokines can be used to understand the pathogenesis ofthis one of the most economically important and neglected zoonotic diseases in sub-Saharan Africa. They are signaling proteins secreted by components of the innate and adaptive immune systems, and act as effectors or biomarkers of inflammatory responses [11]. Wide range of physiological responses, including immunity, inflammation, and haematopoiesis are mediated by these important cell signaling proteins. Various studies [12,13] have suggested that cytokine responses influence the outcome of AT. Immunological hyperactivation of cells, particularly macrophages and T-cells, leading to massive production of pro-inflammatory cytokines and systemic inflammatory response syndrome (SIRS) contributes to the organ damage or death of infected hosts [14]. Proinflammatory cytokines likeIL-6, TNF-α and IFN-γ are elevated in most, if not all, inflammatory states and are recognized as targets of therapeutic intervention [15-18]. They are produced predominantly by activated macrophages and are involved in up-regulation of inflammatory reactions [19]. Interleukin – 6 is a potent pyrogenic cytokine and has an essential role in organizing lymphocyte trafficking to lymphoid organs during febrile events [20]. It has been implicated in the progression of various infectious diseases and considered to be one of the most important cytokines during infection, along with TNF-α [19, 21]. Clinical studies in humans and animals have also linked the increased systemic levels of IL-6 with the exacerbation of clinical outcomes [22, 23]. Tumour necrosis factor alpha mainly produced by activated macrophages, T - lymphocytes and natural killer cells [24], is a proinflammatory cytokine with pleiotropic effects, which has been identified as a major regulator of inflammatory responses and is involved in the pathogenesis of some inflammatory and autoimmune diseases [25]. It has two different receptors initiating signal transduction pathways which lead to various cellular responses, including cellular death, differentiation, and proliferation. Interferon gamma is a pro-inflammatory cytokine secreted primarily by T- lymphocytes and natural killer (NK) cells with a role in innate immunity and as an inducer of the adaptive immune response. Serum IFN-γ in human patients with African trypanosomiasis, was found to be significantly elevated in the early stage compared to late stage and higher for both stages than the control [26].

Efforts to enhance knowledge of the pathophysiology of systemic inflammation and identification of more accurate predictors of prognosis in human and animals have been studied [12,27,28], however, there is a paucity in the expression of cytokines in dogs with trypanosomiasis. This research was aimed at assessing TNF-α, IL-6 and IFN-γ and their association with some haematobiochemical parameters in Nigerian indigenous dogs (NID) infected with *T. congolense,* early and peak stage of infection, and post treatment.

Methods

Ethical approval

Ethical approval was obtained from the College of Veterinary Medicine Ethical Committee (COLVETEC) of the Federal University of Agriculture, Abeokuta. The proposal was subjected to screening to ensure adequate care to the experimental animals and appropriate standard animal welfare monitoring (PG/08/0313).

Experimental animals

Fifteen apparently healthy Nigerian indigenous dogs (7 males and 8 females) between 1 and 3 years old were randomly assigned and used for the study. The dogs were purchased from pet owners in Abeokuta, Ogun State, Nigeria and housed in wire mesh experimental animal house unit where they were allowed to acclimatize for two weeks before the commencement of experiment. During the period of acclimatization, the dogs were screened for vector borne diseases and certified negative for trypanosomes. They were treated for endoparasites using Albendazole 2.5% suspension (Shanu-zole®, Jawa International Limited, Nigeria) at dose of 20 mg/kg, orally. They were also treated prophylactically against some haemoparasites (babesiosis, ehrlichiosis) and bacterial organisms with oxytetracycline 5% (KC Oxytet, Pantex, Holland), intravenously for 5 days at the dose of 10 mg/kg. The dogs were fed on cooked rice and fish, once daily, and water was provided *ad libitum.*

Experimental Procedures

Prior to commencement of the experiment, the rectal temperature and weight were measured for baseline value. Also the blood was collected for baseline biochemical and hematological values.

Test organism (Trypanosomes)

Field strain of *T. congolense* from naturally infected dog was typed and sequenced to confirm the species.

*Experimental design*A simple randomized experimental design was used for the study. The dogs were randomly assigned into three groups (A, B and C) comprising of 5 dogs each.

Group A was neither infected with *T. congolense* nor treated with diminazene aceturate (DA), but was given 5 ml of normal saline intravenously.

Group B was infected with *T. congolense* (2.5 x 10^5), but was not treated with DA.

Group C was infected with *T. congolense* (2.5 x 10^5) and treated with a single dose of DA at the dose of 3.5mg/kg intramuscularly on day 17 post infection (pi).

Infection of experimental animals

Blood sample was taken from each dog before infection to determine baseline Pack Cell Volume (PCV), red blood cells (RBC), haemoglobin concentration (Hb), neutrophil, lymphocytes and monocytes counts, albumin, creatinine or blood urea nitrogen (BUN), serum concentration of tumor necrosis factor alpha (TNF-α), interleukin – 6 (IL-6) and interferon gamma (IFN-γ). Half millitres (1/2 ml) containing 2.5 x 10^5 trypanosomes from the naturally infected dog was diluted with normal saline and injected intravenously through the cephalic vein into the experimental dogs.

Determination of parasitemia

Levels of parasitemia were determined by taking 0.5ml of blood from each dog in each group on days 3, 5, 7, 8 and 10 using wet mount method. Briefly, a drop of blood was put on a glass slide and covered with a cover slip. It was viewed immediately under the light microscope and density of parasites per field matched as described by Herbert and Lumsden [29].

Serological assessment of cytokines

Blood samples (5 millitres) were taken from each dog in the groups (A, B and C) on days 1 (24hrs), 10, 17 and 22 pi through the cephalic vein into bottles containing EDTA for full blood count (FBC)and plain bottles for determination of serum TNF-α, IFN-γ and IL-6 using ELISA kit (Ray Biotech Laboratory, USA). Serum concentrations of TNF-α, IFN-γ and IL-6 were determined based on the manufacturer's instructions. Concentrations of the samples were determined using ELISA analysis software (elisaanalysis.com). The standard minimum detectable concentrations of the used TNF-α, IFN-γ and IL-6 were 2pg/ml, 0.1ng/ml and 0.1ng/ml respectively.

Determination full blood count and some biochemical parameters

The haematological parameters were determined manually by haemocytometery method [30] using the improved Hawksley Haemocytometer (Hawksley and Sons, London). Biochemical indices were assessed using spectrometric and enzymatic colorimetric methods.

Treatment of infected (group C) dogs on day 17 post infection

Infected (group C) dogs were treated with DA at dose of 3.5 mg/kg intramuscularly on day 17 pi when the parasitemia at its peak and the PCV(15%) was below the average normal value which is one of the classical clinical manifestation of trypanosomiasis in the experimental dogs. Blood (0.5 ml) was taken from the treated dogs of group C on D22 pi (D5 pt) to examine for rate of clearance of parasitemia. All infected dogs were treated at the termination of the research using DA at the dose rate of 3.5 mg/kg.

Results

Serum TNF-α (pg/ml) concentration in dogs experimentally infected with T. congolense

TNF-α was detectable at a very low level in all the groups on day 0 (D0) and increased slightly on day 1 pi without significant differences ($p > 0.05$) between all the groups. The concentrations in the infected groups (Groups B and C) increased significantly ($p < 0.05$) on days 10 and 17 (D 10 and D 17) compared to the non – infected group (Group A). There was a sharp drop of the concentration in group C on D 22 pi (D5 pt) while that of group B was significantly (p < 0-05). The uninfected group A maintained low concentrations throughout the study period (Fig. 1).

Serum IFN-γ(ng/ml) concentration in dogs experimentally infected with T. congolense

Interferon gamma (IFN-γ) was detectable in serum of all the groups A, B and C dogs on D0 with no significant difference ($p > 0.05$).The serum concentration of IFN-γ of groups A decreased slightly on days 1, 10 and 17 and then increased to the same level of D0. The concentration in Group B increased from D1 to D22 pi with significant differences ($P < 0.05$) between D10 and D22 pi. The concentration of IFN-γ group C dogs decreased on D1 pi but, became elevated on days 10 and 17 pi with a significant difference ($P < 0.05$) between groups A and B. The concentration decreased sharply close to baseline after treatment (D5 pt) without a significant difference ($P < 0.05$) between groups A and B (Fig. 2).

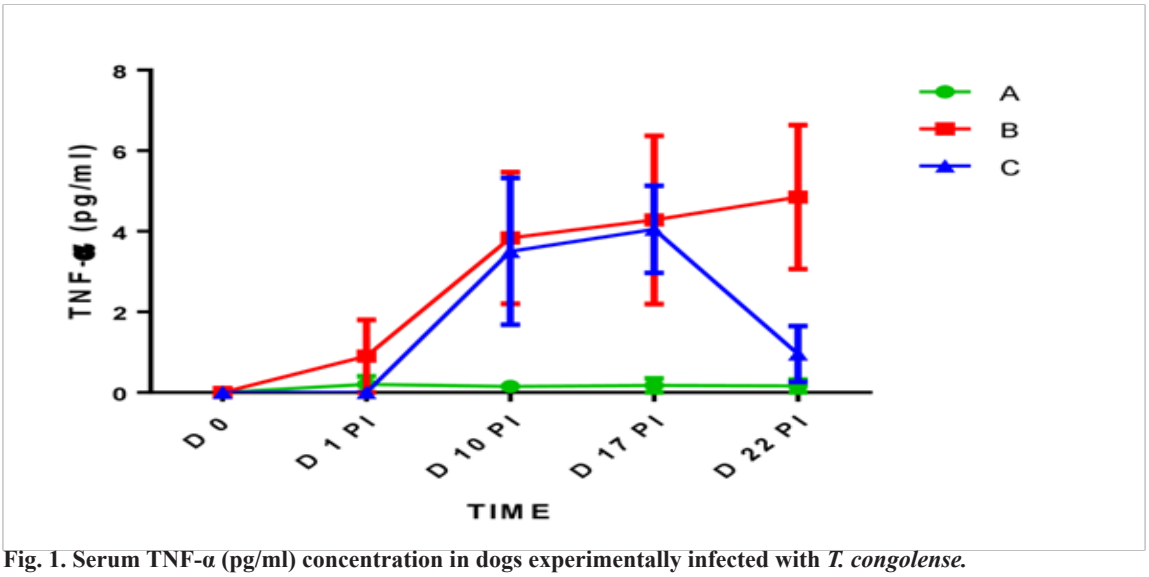

Fig. 1. Serum TNF-α (pg/ml) concentration in dogs experimentally infected with *T. congolense.*

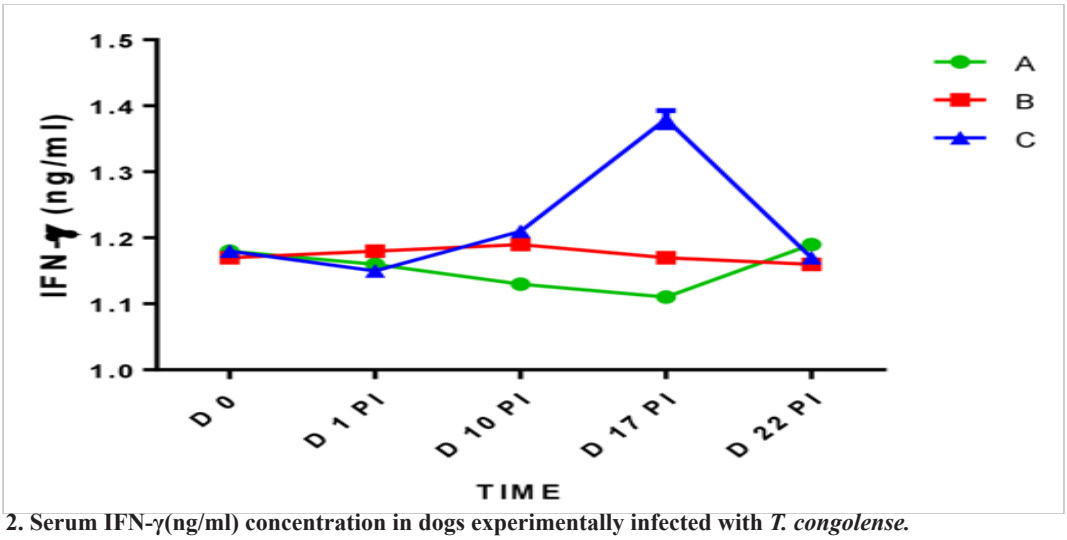

Fig. 2. Serum IFN-γ(ng/ml) concentration in dogs experimentally infected with *T. congolense.*

Serum IL-6 concentration (ng/ml) in dogs experimentally infected with T. congolense.

The serum concentration of IL-6 was detectable in both infected and uninfected groups while that of group A dogs exhibited fluctuations throughout the period of observations. In group B dogs, the serum concentration of IL-6rose gradually from D1 to D22 pi with a significant difference ($P < 0.05$) between the different days and that of the uninfected group A. Similar effect was observed in group C dogs with D10 and D22 pi being significantly ($P < 0.05$) higher than D0, D1 or D17. The effect between the groups was such that the serum levels of IL-6 of the infected groups B and C increased on days 1, 10 and 17 without a significant difference ($P > 0.05$) between the groups except on D22 pi. The concentration in the uninfected group A dogs remained at detectable level (fluctuating) all through the study period (Fig. 3).

Haematological and biochemical changes

All the haematological parameters (PCV, RBC, WBC, and lymphocytes count) in the infected groups decreased significantly ($p < 0.05$) compared to the uninfected except for the eosinophil, neutrophil and monocyte counts that were significantly ($p < 0.05$) increased. The biochemical indices (creatinine and urea)

increased significantly in the infected groups pi compared to the uninfected group and decreased in the treated group post treatment. Total protein, albumin and globulin decreased significantly ($p < 0.05$) in the infected groups pi. Globulin concentration later increased in the treated group post treatment.

Pearson correlation between serum TNF-α, IL-6 and IFN-γ with some haematobiochemical indices

There was a negative correlation between the serum TNF-α and that of PCV,RBC, Hb or albumin, but positively correlated with monocyte counts, creatinine or urea. However, it neither has significant correlation ($p > 0.05$) with the neutrophil nor lymphocyte counts. Serum IL– 6 had negative correlation with the PCV ($p < 0.05$), Hb ($p < 0.05$) or albumin ($p < 0.05$), but positively correlated with monocyte counts ($p < 0.05$), creatinine ($p < 0.05$) or urea ($p < 0.05$. There was no correlation between serum IL–6 and that of RBC, neutrophil and lymphocyte counts. Serum IFN-γ had no correlation with PCV, RBC, Hb, neutrophil, lymphocytes and monocytes count, albumin, creatinine or urea concentrations (Table 1).

Discussion

Interferon gamma is a proinflammatory cytokine which plays a role in innate immunity and also induces adaptive immune response. It has been observed that IFN-γ is one the proinflammatory cytokines elevated in the early stage of infection and that, it plays a role in the survival of murine patients with AT [31]. In this study, IFN-γ was detected in all the dogs on day 0 and became elevated in all the infected groups. This observation was similar to the findings of MacLean *et al*. [26], who reported significant elevation at both early and late stages compared to the control in human patients. The decrease in the concentration close to the baseline observed in group C on D5 pt might be probably due the clearance of the parasites from the blood.

Tumor necrosis factor alpha plays a significant role in parasitic infections [28]. The cytokine influence the out-come of an infection by mediating a range of systemic effects such as fever and modulation of haematopoiesis. The plasma concentration increased steadily from early stage to late stage of the infection in the infected groups B and C compared to the uninfected with that of group C decreasing post treatment. O'Gorman *et al*. [32] reported similar experience in cattle experimentally infected with *T. congolense*. In this study, the serum concentration of TNF-α negatively correlated with PCV, RBC, albumin and Hb concentration. The more the concentration of TNF-α increased, the more these parameters decreased.

This finding corroborates the reports that TNF-α contributes to anaemia of inflammatory diseases by inhibiting erythropoietin secretion and /or response along with direct toxicity on the erythroid precursor cells [33, 34]. It can be speculated that the continuous elevation TNF-α in this study contributed to the decreased in PCV, RBC, albumin and Hb concentration resulting anaemia observed which is a mark of severity of AT. Apart from the negative correlation of TNF-α with PCV, RBC, albumin and haemoglobin concentration, TNF-α positively correlated with monocytes count, serum creatinine and urea concentration. This relationship is suggestive of involvement of the cytokine in the pathogenesis of AT since increase in these parameters portends pathology.

Interleukin - 6 was undetectable in the sera of the dogs on days 0 and 1 pi. The serum concentration of IL-6 increased in the infected groups B and C throughout period of observation despite the treatment of group C dogs. O'Gorman *et al*. [32] and Kitani *et al*. [35], reported similar observations in their studies using cattle and mice, respectively. Different studies [36, 37] have correlated IL-6 elevation with disease severity and mortality. Tamayo *et al.* [38], reported that persistent elevation of IL-6 has been associated with development of multiple organ failure and poor prognosis. In this study, there was persistent elevation of serum IL-6 in all the infected dogs with negative correlation with the PCV, albumin and Hb concentration. Studies have shown that IL-6 plays a role in anaemia by inducing production of hepcidin (a bioactive form of type II acute phase protein) which binds to ferroportin (a cellular exporter) thereby blocking intestinal absorption of iron [39]. The correlation of IL-6 with PCV, albumin and Hb concentration was suggestive of its role in the severity of anaemia observed in the disease. Increased levels of creatinine and urea are indications of organ damage [40,41]. The positive correlation of IL-6 with creatinine

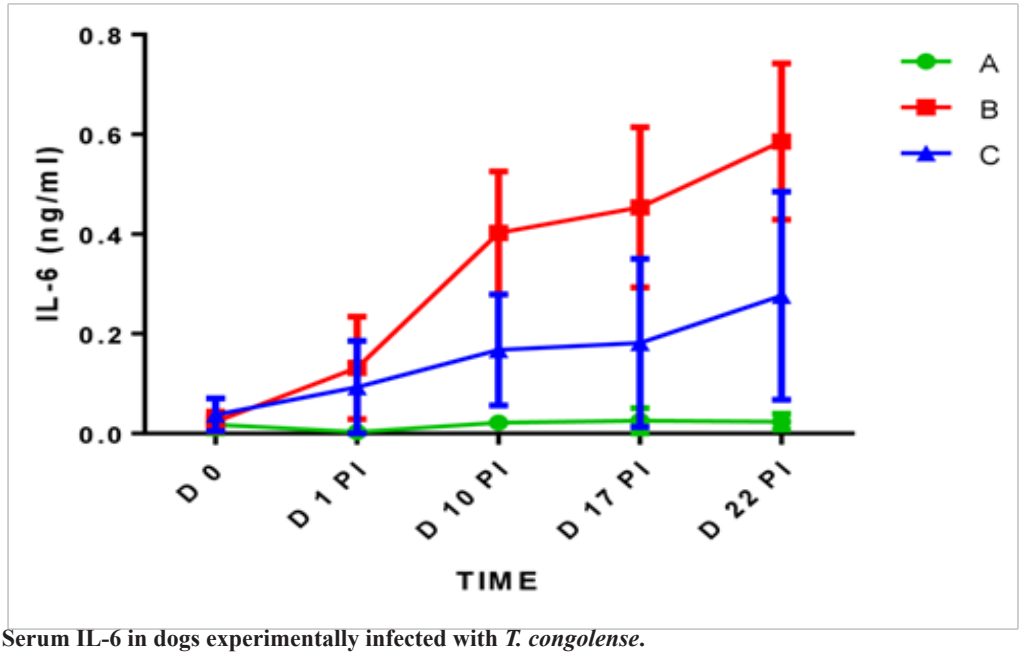

Fig. 3. Serum IL-6 in dogs experimentally infected with *T. congolense*.

TABLE 1. Correlation between serum proinflammatory cytokines and some health indices in dogs experimentally infected with *T. congolense*

Parameters	Correlation	TNF-α	IL-6	IFN-γ
PCV	P - values	0.01	0.03	0.96
	Pearson (r)	0.99*	0.97*	0.04
RBC	P - values	0.05	0.13	0.89
	Pearson (r)	0.95*	- 0.88	- 0.11
Hb	P - values	0.03	0.004	0.66
	Pearson (r)	0.97*	1.00*	0.34
Albumin	P - values	0.003	0.02	0.91
	Pearson (r)	1.00*	0.98*	0.09
Monocytes	P - values	0.10	0.03	0.56
	Pearson (r)	0.90	0.97**	- 0.44
BUN	P - values	0.04	0.004	0.66
	Pearson (r)	0.96**	1.00**	- 0.34
Creatinine	P - values	0.04	0.01	0.61
	Pearson (r)	0.96**	0.99**	- 0.39
Neutrophil	P - values	0.25	0.39	0.61
	Pearson (r)	0.75	0.61	0.39
Lymphocytes	P - values	0.23	0.36	0.67
	Pearson (r)	- 0.77	- 0.64	- 0.33

Key: * indicates significant negative correlation between the cytokines and the health indicators. ** indicates significant positive correlation between the cytokines and the health indicators.

and urea, and the continuous elevation of this cytokine in this study even after clearance of the parasites, it can be speculated that IL-6 played an important role in the pathophysiology of the disease.

Conclusion

Proinflammatory cytokines (TNF-α and IL-6) are elevated in NID experimentally infected with *T. congolense*. The elevation of TNF-α and IL-6 played an important role in the pathology of African trypanosomiasis.

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Conflict of interest

The authors declare no conflict of interest.

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