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Original Research Paper

ERYTHROCYTE SURFACE SIALIC ACID DEPLETION AS PREDISPOSING FACTOR TO ERYTHROCYTE DESTRUCTION IN SHEEP EXPERIMENTAL MODEL OF AFRICAN TRYPANOSOMOSIS: A PRELIMINARY REPORT

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Summary: Changes in erythrocyte surface (ESSA) and free serum sialic (FSSA) profiles that could occur sequel to trypanosome infection with consequent destruction of red blood cells by mononuclear phagocytic system were investigated in an experiment in which 8 sheep were infected with *Trypanosoma congolense* (*T. congolense*), while six other sheep served as uninfected controls. The infection with *T. congolense* caused rapid decline in ESSA concentration and packed cell volume (PCV) in sheep. Concomitantly, rise in mean FSSA concentration was observed in the *T. congolense* infected sheep. Major periods of decline in ESSA concentration and greatest increase in FSSA concentration coincided with period of highest parasitaemia levels. The ESSA concentration in the infected sheep stabilized as the infection aged but the concentration of this parameter remained at comparatively lower levels than that in the control sheep and relative to the pre-infection value on day 0 of infection. This ESSA level in *T. congolense*-infected sheep was maintained with only minor fluctuations up to the termination of the experiment. PCV, ESSA and FSSA concentrations remained relatively unchanged in the control group throughout the course of the experiment that lasted for 53 days. The post-infection mean values of FSSA and ESSA in the *T. congolense*-infected and control sheep were 2.3±0.5mg/ml and1.7±0.9mg/ml, and 1.9±0.2mg/ml and 2.1±1.2mg/ml, respectively. The respective values of PCV, ESSA and FSSA concentrations in the infected sheep differed significantly ($P<0.05$) from those in the control sheep. Further investigations to elucidate the possible roles of sialyltransferase in the recovery of ESSA and, consequently, erythrocyte mass in trypanosome-infected animals are undoubtedly needed.

Key words: sialic acid; sialidase; trypanosomosis; *Trypanosoma congolense*; anaemia

Introduction

Anaemia is one of the principal features and, perhaps, a major cause of tissue pathology and death in the acute phase of trypanosomosis (trypanosomiasis) in livestock (1-8). Although varying reports on the mechanisms of its development in trypanosome-infected animals abound (9), the general consensus is that the anaemia in both human and animal trypanosomoses is predominantly the result of haemolytic crisis, in which the erythrocytes are being destroyed by cells of mononuclear phagocytic system (10,11). The role of trypanosomal sialidases is one of the most documented of such mechanisms (12-17). Sialidases hydrolyse the glycosidic linkage between sialic acids and the underlying sugars thereby cleaving off the sialic acids (neuraminic acids), which are found ubiquitously distributed on terminal positions of macromolecules and cell membranes in the body (18-20).

Occurrence of sialidases has been reported in *Trypanosoma vivax* (*T. vivax*), *T. congolense*, *T.*

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brucei, *T. rhodesiense, T. evansi, T. rangeli* and *T. cruzi* (12-17). These enzymes, when released by infecting trypanosomes, contribute to the development of anaemia by liberating the sialic acid on erythrocyte surface, an action that demasks galactose residues via which such desialylated erythrocytes bind to cells of the mononuclear phagocyte system through a specific receptor and are ultimately taken up and degraded (11,20-26).

A good understanding of pathophysiological mechanisms involved in the development of the anaemia in trypanosome-infected animals is pivotal to the identification of molecular targets that could be exploited biotechnologically to evolve the necessary panacea to the menacing effects of the infection in both humans and animals.

Sheep were reported to be the most suitable models in the study of erythropoiesis (27,28). We report in this paper some changes in the packed cell volume (PCV), erythrocyte surface (ESSA) and free serum sialic acid (FSSA) concentrations induced by experimental *T. congolense* infection of sheep.

Materials and methods

Experimental animals

Fifteen normal healthy sheep of the Yankasa breed with ages that ranged between 18 and 24 months were purchased from a livestock market within an area apparently free of tsetse, in Katsina State of northern Nigeria. The ages of these animals were confirmed using the dental eruption pattern described (29). Fourteen of these sheep were used in the experiment, while the remaining one served as donor animal. The animals were on arrival accommodated in a fly-proof experimental animal house and adequately fed on high plane of diet.

The sheep were also provided with water *ad-libitum*. Series of treatments were administered to the animals that include deworming using albendazole (Albendazole®, Pantex, Holland B.V.) at a dose rate of 5mg per kilogram body weight, antibiotic therapy with Oxytetracycline (Trodax®) at a dose rate of 20mg per kilogram body weight. The animals were also sprayed against external parasites using diazinon (Diazintol®, Animal Care, Nig. Ltd.) at a concentration of 2ml per litre of water (162mg/ml concentration). The individual animals were ear-tagged for proper identification. The sheep were allowed to acclimatize for a period of three months during which they were subjected to such routine handlings as collection of blood samples, which were used for parasite screening and establishment of baseline haematological values and determination of rectal temperature, each, twice a week. Prior to the commencement of the experiment, the sheep were certified to be free of trypanosomes based on weekly haematocrit (30).

Trypanosome Stock

The parasite *T. congolense* (NITRE-53624609) used in this experiment was donated by the Nigerian Institute for Trypanosomiasis Research, at Vom, Plateau, Nigeria. The trypanosome was isolated from a pregnant cow in Karu of Nasarawa State of Nigeria. The stabilate of this parasite was inoculated into two Sprague Dawley rats; one intraperitoneally and the other intradermally. The infected rats were immediately transported to Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. These rats were kept in separate cages and fed adequately with pelleted chicken feeds. Blood sample was collected from each of these rats everyday for determination of parasitaemia. When parasitaemia was at swarming degree in the rat that was inoculated intraperitoneally on day 6 post-inoculation, it was anaesthetised with chloroform and its jugular veins were then severed to collect sufficient blood. This blood, which was contained in a vacutainer and anticoagulated with heparin, was used to inoculate the donor sheep.

Detection of parasitaemia in the donor sheep

Beginning from day 1 post-infection, blood sample was collected from the donor sheep every day into vacutainer containing an anticoagulant, ethylenediaminetetraacetic acid (EDTA). This blood was used to detect the appearance of the parasites in the peripheral circulation in order to determine the prepatent period and parasitaemia level that would be sufficient to infect the experimental sheep using the modified method of Paris et al (31).

Animal allocation and infection with T. congolense

The parasites, *T. congolense*, were first detected in the blood of the donor sheep on day 11 post-infection. By day 15 post-infection, parasitaemia was

at its peak with a parasite count of $1x10^5$ trypanosomes per millilitre ($1x10^5$ tryps/ml) of blood. On this day of infection (tagged day 0 of infection), the experimental sheep were allocated to two groups. The first group comprised of 8 sheep and was infected with trypanosomes (and was therefore termed the infected group), while the second group, which consisted of 6

sheep served as the un-infected control group. These groups were closely matched on the basis of haematocrit index (infected group, 32.8±2.6%; control, 32.5±3.3%). On this day 0, each of the animals in the infected group was inoculated via the jugular vein with 2 ml of blood containing $1x10^6$ *T. congolense* organisms. Estimation of the number of trypanosomes was done using the modified method of Paris et al (31).

Haematological and parasitological analyses

Beginning from day 0 of infection and throughout the experimental period that lasted for 53 days, 0.5 ml of blood sample was collected every day from each of the animals in both the infected and control groups into a vacutainer containing EDTA as an anticoagulant. This blood was used for detection and estimation of parasitaemia level in the infected group using the modified method of Paris et al. (31) and estimation of packed cell volume using the standard microcapillary method.

Preparation of erythrocyte membranes (Ghosts) and determination of erythrocyte surface sialic acid (ESSA) concentration

Beginning from day 0 of infection and up to the termination of the experiment, 2 ml of blood was collected everyday from each of the animals in the two groups into a screw- capped test tube containing 0.3 ml of reconstituted acid citrate dextrose (an anticoagulant). This blood was used for the preparation of erythrocyte ghosts according to the method of Dodge *et al.* (32). ESSA concentration was determined using 50µl of the suspension of washed erythrocyte ghosts incubated with 100µl H_2SO_4 for 1hour at 80°C in order to

liberate bound sialic acid. Sialic acid concentration was subsequently measured in the mixture using thiobarbituric acid assay (TBA assay) (33,34).

Determination of free serum sialic acid (FSSA) concentration

Beginning from day 0 of infection and up to the end of the experiment, 1 ml of blood sample was collected everyday from each of the animals in the two groups into vacutainer without anticoagulant. The blood was allowed to clot at refrigeration temperature until serum became expressed. The serum was aspirated and dispensed into serum vials and stored at -20 °C until needed for the assay of sialic acid. FSSA concentration was determined by TBA assay (33,34).

Statistical analysis

The mean values of both ESSA and FSSA concentrations in the infected group were compared statistically with those in the control group using student's t-test (35).

Results

Parasitaemia

T. congolense organisms were first detected in the blood of some of the sheep in the infected group on day 8 post-infection. By day 11 post-infection, all sheep in the infected group were showing parasitaemia. Hence, mean prepatent period in this experimental infection was 9.5 ± 1.3 days. Parasitaemia was intermittent in individual animals of the infected group. Mean parasitaemia level rose on day 8 from $1.0x10^3$ trypanosomes per millilitre (tryps/ml) of blood (i.e. a 1+ parasitaemia score) to a peak value of $5x10^5$ tryps/ml (i.e. a 5+ parasitaemia score) on day 23 post-infection (Fig. 1). This was immediately followed by a slight drop in the mean parasitaemia level to values that ranged between 1.0×10^5 and 5.0×10^5 tryps /ml. This fluctuating parasitaemia level was maintained up to day 36 post-infection after which a sudden drop to a basal level of 1.0×10^3 tryps/ml was observed. This basal mean parasitaemia level was maintained, with only occasional spikes, until the termination of the experiment (Fig. 1). All the sheep in the control group remained aparasitaemic throughout the course of the experiment. Thus, the time interval days of 9 and 36 represents the major period of high parasitaemia levels.

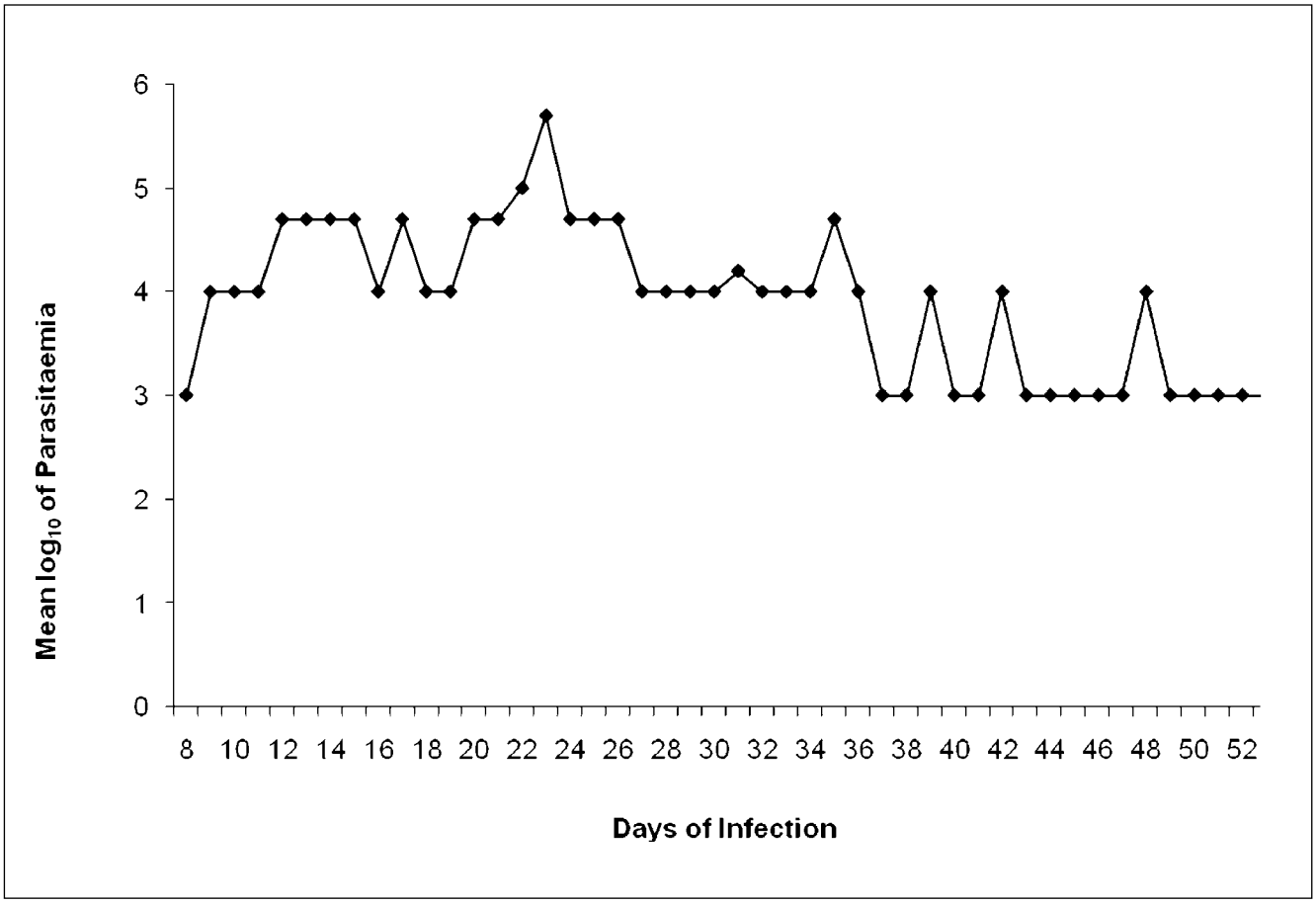

Figure 1: Mean $\log_{10}$ of Parasitaemia in *T. congolense*-infected sheep

Packed cell volume (PCV)

The mean pre-infection PCV in the *T. congolense*-infected and control sheep were 33.4 ± 3.3 and 33.5 ± 2.4 %, respectively. Following infection with the trypanosome, there was a gradual and progressive drop in the mean PCV value in the infected group until a minimum value of 16.7 ± 2.3 % was recorded on day 32 post-infection (Fig. 2). Thereafter, a staggering increase to a highest value of 21.3 % was observed on day 41 post-infection. The mean PCV then stabilized at values that fluctuated between 17 and 20 % up to the end of the experiment (Fig. 2). The post-infection mean PCV in the control group remained normal relative to the pre-infection one (Fig. 2). The difference between the post-infection mean PCV values in the *T. congolense*-infected and control groups (which were 23.9 ± 5.4 % and 31.5 ± 2.7 %, respectively) was significant ($P<0.05$).

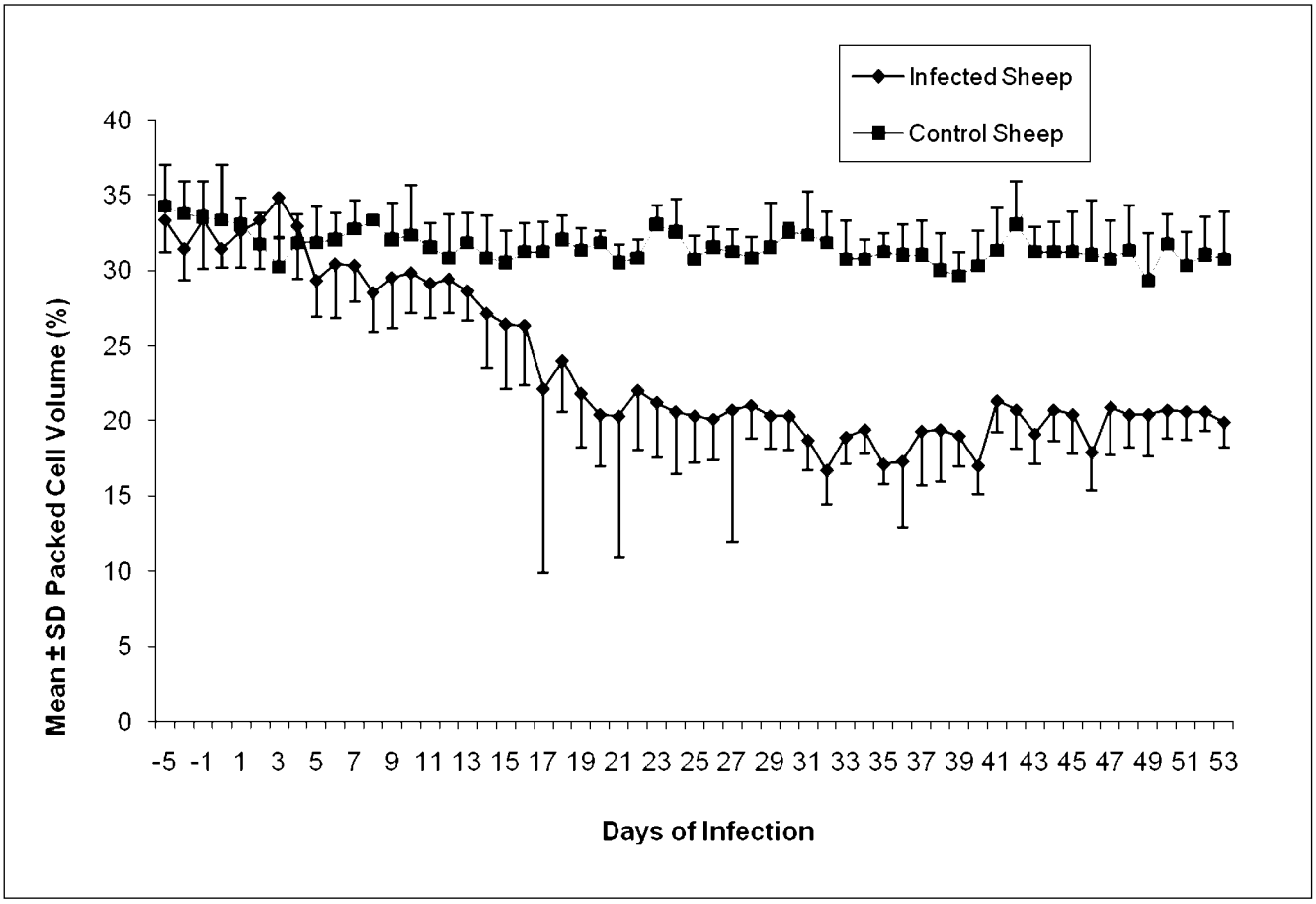

Figure 2: Mean packed cell volume in *T. congolense*-infected and control sheep

FSSA concentration

The pre-infection mean FSSA concentrations in the infected and control groups were 1.6 ± 0.7 mg/ml and 1.9 ± 1.0 mg/ml, respectively. Following infection with trypanosomes, a gradual rise in the value of this parameter was observed in the infected group beginning from day 5 of infection to reach a peak value of 4.0 ± 2.1 mg/ml on day 16 post-infection (Fig. 3). This peak value was then followed by a drop in the mean FSSA concentration to a level (1.9 ± 0.7 mg/ml) that was comparable with that in the control group on day 20 post-infection (Fig. 3). Thereafter, mean FSSA concentration stabilized with only some occasional surges up to the end of the experiment (Fig. 3). The mean FSSA concentration in the control sheep remained at the same level, with only minor fluctuations, relative to the pre-infection value on day 0 of infection. Mean FSSA level was highest between days 6 and 23 post-infection (Fig. 3). The mean post-infection FSSA concentration (2.3 ± 0.5 mg/ml) in the *T. congolense*-infected sheep was significantly higher ($P<0.05$) than that (1.9 ± 0.2 mg/ml) in the control sheep.

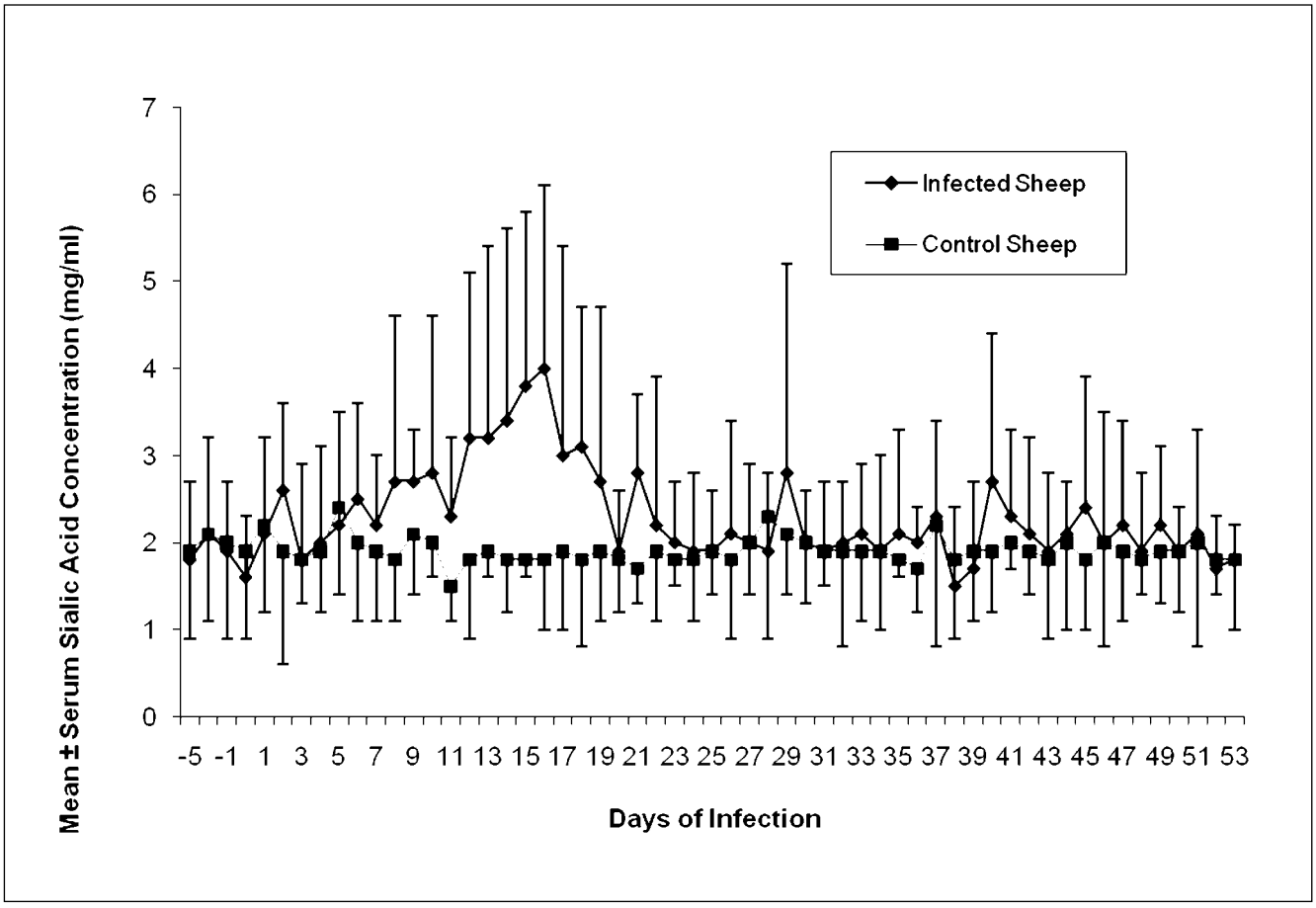

Figure 3: Mean serum sialic acid concentration in *T. congolense*-infected and control sheep

ESSA concentration

Mean ESSA concentration values in the *T. congolense*-infected and control sheep were comparable up to day 8 post infection. Following this period, a dramatic fall in ESSAconcentration was then observed in the infected group to reach the lowest level of 0.6±0.4 mg/ml on day 12 post-infection. Major decrease in ESSA concentration was observed between days 9 and 23 post-infection (Fig. 4). Thereafter, the value stabilized. Post- infection mean ESSA level was persistently lower than that in the control group up to day 53 when the experiment was terminated. The time interval between days 7 and 24 appears to be the period of greatest sialic acid loss from the erythrocyte surfaces (Fig. 4). Post-infection mean of ESSA level in the control sheep remained fairly unchanged during the course of the experiment (Fig. 4). Unlike with FSSA concentration, the post-infection mean ESSA concentration (1.7±0.9 mg/ml) in the infected group was significantly lower than that (2.1±1.2 mg/ml) in the control group.

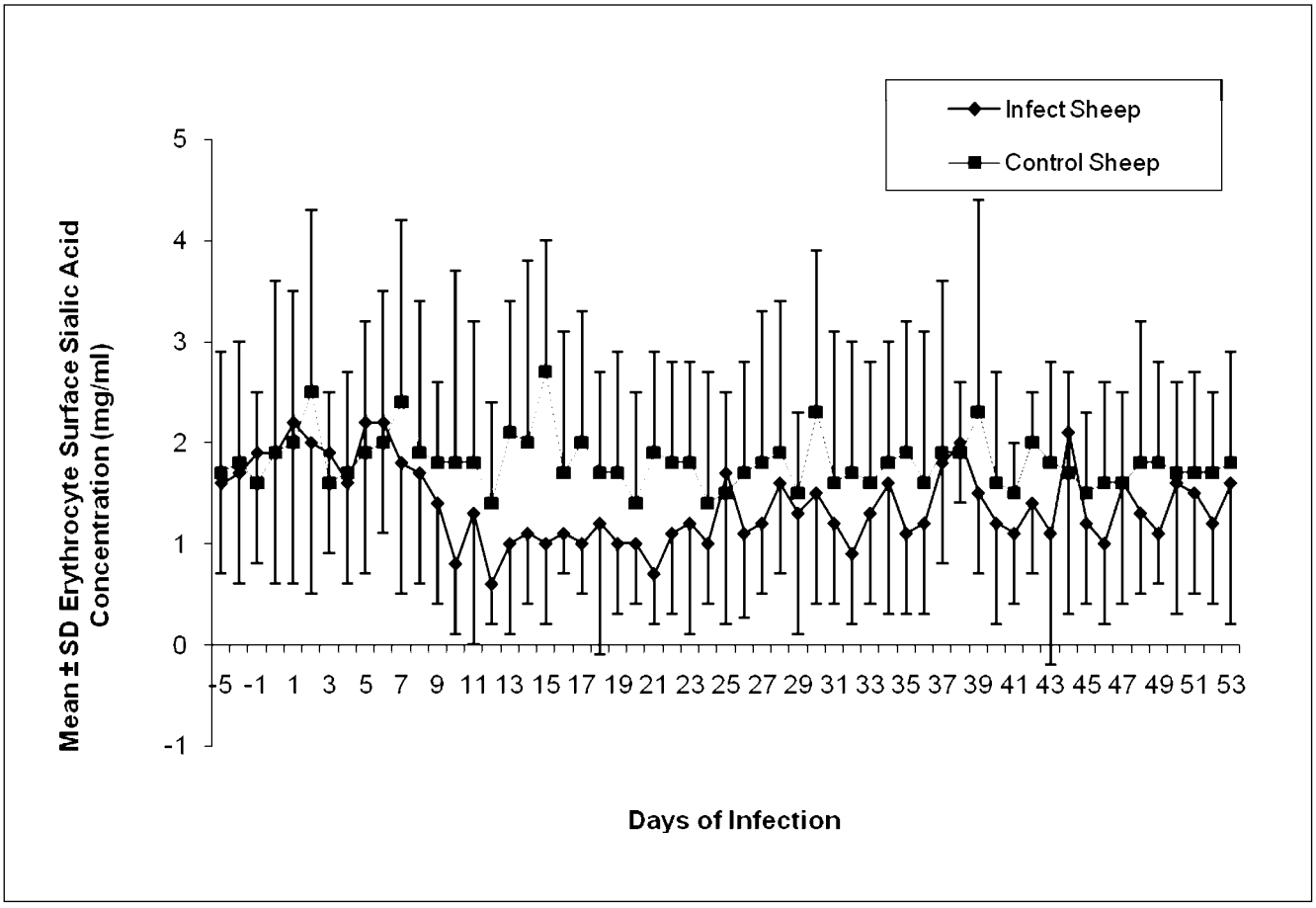

Figure 4: Mean erythrocyte surface sialic acid concentration in *T. congolense*-infected and control sheep

Discussion

The findings in this study re-affirm those in the previous reports (17,20,21). The significant reduction in ESSA concentration in the *T. congolense*-infected sheep, as observed in the present study, was most probably the result of elaboration and subsequent release of the sialic acid-hydrolyzing enzyme, sialidase, by the infecting trypanosomes (6,12-14,16,17,26). This is more so since bloodstream *T. congolense* sialidase was reported to play a contributory role in the development of anaemia in mice (6). The rise in the FSSA concentration, which accompanied reduction in ESSA concentration, suggests that erythrocytes were the major source of the FSSA in the infected sheep. The concurrence of decline in ESSA concentration with fall in packed cell volume, especially, in the major period of high parasitaemia levels as observed in the present study was earlier reported in *T. vivax*-infected cattle (Esievo et al., 1982), *T. evansi* sialidase hydrolysis of ghost red blood and brain cells (16) and *T. congolense*-infected mice (6) and is a strong basis on which trypanosomal sialidase could be implicated as being responsible for the reduction in ESSA and consequent decline in erythrocyte mass, which ultimately results in anaemia (6,20,21).

The pathophysiological mechanisms involved in the trypanosomal sialidase-induced destruction of red blood cells is that removal of sialic acid from the exposed epitopes of the erythrocytes causes some physicochemical alterations. These changes predispose them to bind to β-D-galactose-specific lectin on surfaces of macrophages and, consequently, resulting in the uptake and clearance of the desialylated erythrocytes (16,20,36). Indeed, the finding in numerous reports (24,26,37) that β-Glycosidically linked β-galactoside residues inhibited the rate-limiting binding step in the erythrophagocytic step is a strong evidence to aver that

anaemia development in trypanosome infected animals is consequential, at least in part, to loss of ESSA. This is because the terminal galactosyl residue of lactose enables it to bind to galactose-specific lectins of macrophages (6), thereby competitively inhibiting similar binding and subsequent destruction of desialylated erythrocytes. Thus, we may infer that anaemia developed in the *T. congolense*-infected sheep in the present study as a consequent effect of the parasite's sialidase-removal of sialic acid from the erythrocytes, which rendered them prone to erythrophagocytosis.

Even though ESSA concentration in the *T. congolense*-infected sheep stabilized at the later stages in the course of the infection as observed in this study, its failure to return to normality or levels that would be comparable with that in the control sheep would exacerbate the anaemic condition. This is because the red blood cells during this time would have reduced life span on account of their reduced ESSA (20,36). The perpetually reduced ESSA in the *T. congolense*-infected sheep may be attributable, among other factors, to the combined effect of trypanosomal sialidase, which continued to cleave off the sialic acid from the erythrocytes, and autoinduction of the activity of sialate-pyrvate-lyase (aldolase, EC 4.1.3.3). This latter enzyme, which is localized in the cytosol of mammalian cells, regulates the recycling of the sialic acid by hydrolyzing it to pyruvate and the corresponding acyl-mannosamine (20,38). Sialate-pyruvate-lyase occurs in bacteria but has never been reported in trypanosomes (20).

Although sialyltransferase activity was not investigated in this study, the return to normality in the FSSA concentration, following an initial rise, in the *T. congolense*-infected sheep may be the result of the activity of the enzyme, which depletes FSSA to resialylate cells, glycoconjugates and glycolipids (39-44). It may also be reasonable to attribute staggering increase and subsequent stabilization of PCV in the *T. congolense*-infected sheep to the activity of the sialyltransferase since resialylation of the erythrocytes would retard the rate of their binding to macrophages and therefore prolongs their lifespans. *T. congolense* infection of sheep resulted in depletion of ESSA with rapidly developing anaemia, thus, re-affirming the findings in previous reports (6,20,21) that the effect of trypanosome infection on ESSA may be a major mechanism of erythrocytes destruction. Further investigations to elucidate the possible roles of sialyltransferase in the recovery of ESSA and, consequently, erythrocyte mass in trypanosome-infected animals are undoubtedly needed. The revelations from previous, present and the recommended future studies could be exploited to biotechnologically develop an appropriate panacea to the detrimental effects of trypanosome infection in humans and animals.

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ZMANJŠANJE SIALIČNE KISLINE NA POVRŠINI ERITROCITOV KOT PREDISPONIRAJOČI DEJAVNIK ZA RAZPAD ERITROCITOV PRI OVCAH, POSKUSNO OKUŽENIH S TRIPANOSOMO: PRELIMINARNO POROČILO

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Povzetek: V poskusu, v katerega je bilo vključenih osem ovc, ki so bile okužene s *Trypanosomo congolense* (*T. congolense*), in šest ovc, ki so služile kot neokužena kontrola, smo ugotavljali spremembe površine eritrocitov (ESSA) in prostih serumskih sialnih profilov (FSSA), ki se lahko pojavijo po okužbi s tripanosomo, ter posledično uničenje rdečih krvniček, ki ga povzroči fagocitni sistem enojedrnih celic. Okužba s *T. congolense* je povzročila hitro znižanje koncentracije ESSA in hematokritske vrednosti pri ovcah. Poleg tega je bilo opaženo povečanje srednji vrednosti FSSA pri ovcah, inficiranih s *T. congolense*. Glavna obdobja, v katerih je prihajalo do znižanja vrednosti ESSA ter povišanja vrednosti FSSA, so sovpadala z obdobjem najvišjega nivoja parazitemije. Vrednost ESSA se je pri okuženih ovcah ustalila, ko je okužba zastarala, vendar pa je vrednost omenjenega parametra ostala na primerjalni ravni kot pri kontrolnih ovcah in sorazmerna z nivojem pred okužbo v začetku poskusa. Raven ESSA se je pri ovcah, okuženih s *T. congolense*, ohranila z manjšimi nestalnostmi do konca poskusa. Vrednosti hematokrita, ESSA in FSSA so ostale nespremenjene v kontrolni skupini med celotnim poskusom, ki je trajal 53 dni. Srednje vrednosti FSSA in ESSA po okužbi pri ovcah, okuženih s *T. congolense*, ter pri kontrolnih ovcah so bile 2.3 ± 0.5mg/ml in 1.7 ± 0.9mg/ml, ter 1.9 ± 0.2mg/ml in 1±1.2mg/ml. Posamezne vrednosti hematokrita, ESSA in FSSA so se značilno razlikovale med skupino okuženih ovc ter kontrolno skupino. Potrebne pa bodo še nadaljnje raziskave, ki bodo osvetlile možne vloge sialtransferaze v ponovnem normaliziranju ravni ESSA in posledično mase eritrocitov pri živalih, okuženih s tripanosomo.

Ključne besede: sialna kislina; sialidaza; tripanosomiaza; *Trypanosoma congolens*; anemija