

Comparative studies on erythrocyte calcium, potassium, haemoglobin concentration, osmotic resistance and sedimentation rates in grey duiker (*Sylvicapra grimmia*), sheep and goats experimentally infected with *Trypanosoma congolense*

Ajibola Oluwole Ogunsanmi¹, and Victor Olusegun Taiwo^{2*}

¹Department of Wildlife and Fisheries Management, University of Ibadan, Ibadan, Nigeria

²Department of Veterinary Pathology, University of Ibadan, Ibadan, Nigeria

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ABSTRACT

A comparative study of the haemoglobin (Hb) concentration, erythrocyte sedimentation rates (ESR), osmotic resistance (EOR) and erythrocyte dynamics of calcium (Ca⁺⁺) and potassium (K⁺) ions was carried out on 10 grey duiker (*Sylvicapra grimmia*), 15 female West African dwarf sheep and 15 female Yankassa goats during the course of experimental *Trypanosoma congolense* infection. Grey duiker developed a transient parasitaemia and mild anaemia, while sheep and goats developed progressive parasitaemia, pyrexia, anaemia and loss of condition necessitating their treatment at 49 days post-infection to prevent imminent mortality. Grey duiker maintained consistently unchanged levels of Hb concentration, ESR and erythrocyte Ca⁺⁺ levels, and transiently reduced EOR and erythrocyte K⁺ levels throughout the course of *T. congolense* infection. On the other hand, infected sheep and goats developed progressive decreases in Hb concentration, EOR and erythrocyte K⁺ levels, progressive increases in ESR and erythrocyte Ca⁺⁺ levels. These changes were much more severe in infected goats and sheep. This study has shown that the relative trypanotolerance of infected grey duiker are based on their superior ability to control parasitaemia, maintain erythrocytic structural integrity and homeostasis, and hence limit anaemia and other deleterious effects of trypanosome

* Contact address:

Dr. Victor Olusegun Taiwo, Department of Veterinary Pathology, University of Ibadan, Ibadan, Nigeria, E-mail: victaiwo52@yahoo.com

infection than is the case with its domestic small ruminant counterparts. Thus, grey duiker may be a substitute for African buffalo in research efforts to unravel the mystery of trypanotolerance, especially in both wild and domesticated ruminants. These animals also offer valuable substitutes for animal protein sources for the teeming human population in tsetse and trypanosome endemic areas where their mass domestication and rearing is encouraged.

Key words: erythrocyte biochemistry, grey duiker, sheep, goats, *Trypanosoma congolense*, trypanotolerance

Introduction

Historically, blood has been regarded by man as the essence of life, the seat of the soul, and progenitor of psychic and physical strength. Basic knowledge of the normal physiology of the erythrocytic membrane provides an insight into the pathophysiologic mechanisms of cellular destruction in haemolytic diseases. Erythrokinetic studies in trypanosome-infected animals have shown that erythrocyte life-span is reduced as a result of various mechanisms which alter the erythrocyte cell membrane and predispose the erythrocyte to phagocytosis, lysis and fragmentation (SULIMAN and FELDMAN, 1989). The presence of trypanosomosis and tsetse infestation makes it impossible to productively keep livestock in many of the tropical regions of Africa (MURRAY and GRAY, 1984). At present, trypanosomosis is mainly controlled by suppression of the tsetse fly population and by the chemotherapeutic treatment of infected animals, as well as chemoprophylaxis (OLUBAYO et al., 1990). Due to drug resistance and toxicity (DIACK et al., 1997; EISLER et al., 1997), environmental pollution and chemical production costs, there is a need to find alternative means to control tsetse flies and trypanosomosis. One solution to this problem is the use of trypanotolerant cattle, sheep and goats. However, their trypanotolerance traits have not been adequately defined, mainly due to the lack of genetic markers to be used for selection of these traits. Previous reports suggest that indigenous sheep and goats in West Africa (ILCA, 1979) and East Africa (GRIFFIN and ALLONBY, 1979) possess greater innate tolerance to natural trypanosome infection than do exotic breeds. On the other hand, these small ruminants had been implicated as important reservoirs of trypanosome infection to other livestock (MULLA and RICKMAN, 1988).

Although there are some reports which have compared large ruminants such as African buffalo with Boran cattle (GROOTENHUIS et al.,

1990) and wildebeest with cattle (crosses between Zebu and Charolais breeds (RURANGIRWA et al., 1986) on their ability to control trypanosome infection, there is no report which has compared small ruminants, such as grey duiker, with sheep and goats.

The purpose of this study was to compare haemoglobin values, erythrocyte sedimentation rates, osmotic resistance, and erythrocytic intracellular kinetics of calcium and potassium ions in trypanotolerant grey duiker with more susceptible sheep and goats infected with *Trypanosoma congolense* by intravenous infection, and hence elucidate the role these factors play in the resistance of grey duiker to trypanosomosis.

Materials and methods

Experimental animals. Ten (6 male and 4 female) African grey duiker, 15 female West African dwarf goats and 10 female Yankassa sheep were used in this experiment. The grey duiker, aged 17-23 months and with an average weight of 12.75 ± 2.1 kg, were bred in captivity and housed in a semi-detached stall with adjacent paddocks at the University of Ibadan Zoological Garden. Grey duiker were fed on leaves of *Ipomea involucreta*, *Aztekia africana* and *Eucalyptus toroliana* supplemented with maize grain, millet and fruits of *Milletia thonningii*. The goats were aged 18-24 months, had a mean weight of 18.76 ± 1.12 kg; sheep aged 20-26 months had an average weight of 20.89 ± 1.36 kg. The sheep and goats were purchased from a private livestock farm located on the outskirts of Ibadan, Nigeria. They were housed in concrete-floored pens in the small ruminant unit of the Teaching and Research Farm, University of Ibadan. They were fed on commercial ration supplemented with *Cynodon plectostachyum*, *Centrosema pubescens*, maize and yam peels. The grey duiker, sheep and goats had free access to salt lick and water. Pre-infection conditioning and veterinary care were performed as previously described (OGUNSANMI et al., 1994b).

Infection with Trypanosomes. *Trypanosoma congolense* IL1180 was obtained from the International Livestock Research Institute (ILRI), Nairobi, Kenya. The parasite was derived from clone IL 968, a derivative of STIB 212 (NANTULYA et al., 1984), which was isolated from a lion in the

Serengeti region of Tanzania (GEIGY and KAUFMANN, 1973). The parasites were kept by 2-3 passages in albino mice until needed. The mice were subsequently bled by cardiac puncture into a tube containing normal saline. The trypanosomes were counted and concentration was adjusted by the addition of a buffered saline solution (WELLDE et al., 1989) to make 3.0×10^6 trypanosomes/ml. Each experimental animal was given 2 ml of the parasite suspension by slow intravenous (i.v.) injection through the jugular vein using a 21G needle.

Pooled data from three pre-infection samplings from these animals served as control data in this study. All infected sheep and goats were treated with a single dose of deep intramuscular (i.m.) injection with 3.5 mg/kg diminazene aceturate (Berenil[®], Hoechst, Germany) at 49 days post-infection (p.i.) in order to prevent them from dying. Rectal temperatures were taken daily (8.00 a.m.) throughout the course of infection, while parasitaemia was detected initially daily for 14 days and thereafter weekly (PARIS et al., 1982). The experiment lasted for 63 days.

Experimental protocols. Blood samples were collected weekly by venipuncture and processed within 30 minutes of collection. Hb concentration was determined by the cyanmethaemoglobin method (OGUNSANMI et al., 1994a) while ESR was determined by the Wintrobe's technique as described by JAIN (1986). Erythrocyte counts of packed cells (pellet) was estimated in improved Neubauer chamber (JAIN, 1986). Haemolysate of each pellet was obtained initially by diluting one trillion (10^9) erythrocytes five-fold with cold, deionised water. Final dilution (20-fold) of haemolysate with deionised water was used for the determination of K^+ , while haemolysate final dilution of 20-fold with Lanthanum chloride solution (0.7g La^{3+} in 100 ml of 4% TCA + 100 ml deionised water) was used for Ca^{++} determination. The lanthanum used is to reduce phosphorus interference and to break calcium-phosphate complex. Ca^{++} and K^+ were expressed in mmol/ 10^9 cells and mmol/ 10^9 cells, respectively. Ca^{++} concentration was determined using atomic absorption spectrophotometer (AAS, Model 603, Perkin Elmer, Norwalk, Connecticut, U.S.A.) while the K^+ was also determined by using Flame photometer (Corning 400, Corning Scientific Limited, England). Ca^{++} and K^+ were expressed in μ mol/ 10^9 cells and mmol/ 10^9 erythrocytes, respectively.

Erythrocyte osmotic resistance (EOR) was determined in each heparinised blood collected according to the method described by JAIN (1986). Briefly, 16 tubes containing various dilutions of a freshly prepared 1% phosphate buffered solution (pH 7.4) with distilled water were set up in such a way that the first tube had an NaCl concentration of 0.85%, while the fifteenth tube had 0.1%. The sixteenth tube contained only distilled water. Into each tube was added 20 ml of blood (for sheep and goats) or 10 ml of blood (for duiker, because of the high viscosity of its blood) which was mixed by inversion and incubated at room temperature for 30 minutes. The tubes were centrifuged at 2,000 rpm for 10 minutes, 3.5 ml of the supernatant of each tube was transferred into a cuvette and the optical density (O.D.) read on a spectrophotometer (Pye Unicam 6-500uv) at 540 nm, using distilled water as a blank. The percentage of haemolysis was calculated from O.D. value, with the assumption that a complete (100%) haemolysis occurred in the 16th tube. Cumulative osmotic resistance was obtained by plotting the percentage haemolysis against days p.i. at both isotonic (0.85%) and hypotonic (0.60%) saline (NaCl) concentrations.

Statistical analysis. Data for each animal species were subjected to 2-way analysis of variance (ANOVA) (Statistical Analysis System. Users' Guide, Ver. 6.03, SAS Institute Inc., Cary, North Carolina, USA., 1987) and Duncan's multiple range test (DUNCAN, 1959) for the determination of statistical differences within and between the pre-infection and post-infection data of the three species at 95% confidence interval ($P < 0.05$).

Results

Trypanosomes were detected in the peripheral blood of the infected sheep and goats between the 7th and 8th day p.i., while grey duiker became parasitaemic between the 11th to 14th day p.i. Peak parasitaemia occurred in duiker on day 21 p.i., while this occurred on 14 day p.i. in sheep and goats. These were correlated positively with peaks of temperature. Grey duiker exhibited shorter waves of parasitaemia and maintained their temperature to within 38.60 ± 0.2 °C. In contrast, sheep and goats exhibited persistent and undulating fever (40.8 ± 0.3 °C for sheep and 41.05 ± 0.5 °C

for goats). Grey duiker became non-parasitaemic from day 35 p.i. onwards, while sheep and goats continued to develop variable and consistent parasitaemia, severe anaemia and loss of condition, necessitating their treatment with Berenil® on day 49 p.i. to prevent them from dying. One week after treatment, the sheep and goats were aparasitaemic, apyrexia and had slightly improved body condition.

Results of Hb concentration and ESR for the three species are presented in Figs. 1 and 2. The pre-infection mean Hb concentration value of grey duiker doubled those of sheep and goats. In general, Hb concentration did not decrease significantly ($P>0.05$) with increasing number of days p.i. in grey duiker. However, there were significant decreases ($P<0.05$) in sheep and goats from day 14 p.i. until day 49 p.i., when they were treated. Hb concentration increased to pre-infection levels 2 weeks after treatment (Fig. 1).

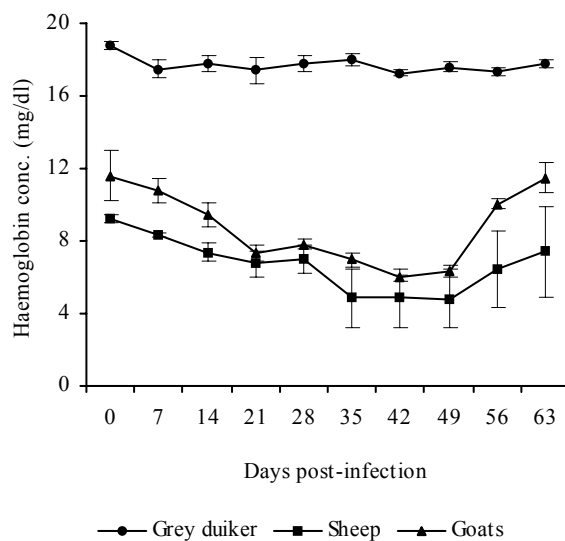


Fig. 1. Haemoglobin concentration values of grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

There were no significant changes ($P>0.05$) in ESR values for grey duiker throughout the period of the experiment, but in the infected sheep and goats, ESR values increased between 1000-1500-fold ($P<0.05$) above

pre-infection values from day 14 to day 49 p.i. (Fig. 2). These values returned to pre-infection levels on day 56 p.i.

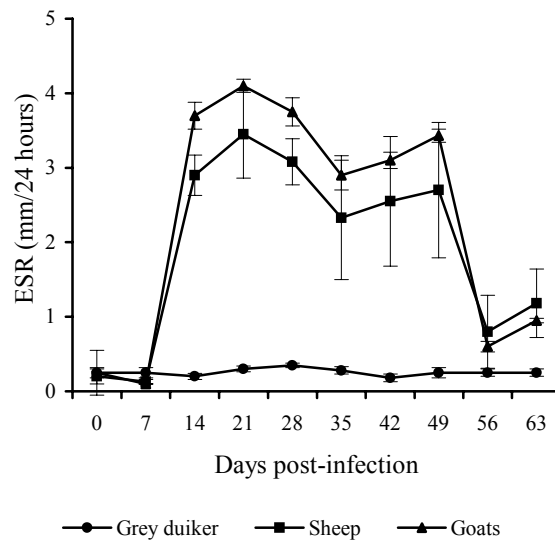


Fig. 2. Erythrocyte sedimentation rates (ESR) of grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

The EOR in isotonic saline concentration (0.85% NaCl) and hypotonic saline concentration (0.60% NaCl) in grey duiker, sheep and goats are illustrated in Figs. 3a and 3b, respectively. Using normal saline (0.85%), the EOR in grey duiker decreased transiently between day 14 and day 35 p.i. and thereafter increased, while it progressively decreased ($P<0.05$) in infected goats from day 14 until day 49 p.i., when they were treated. EOR values in infected sheep decreased inconsistently but were lower than those of pre-infection levels. A similar, but more pronounced EOR decrease trend was observed at the hypotonic saline concentration of 0.6% in all three species of animals. This was, however, more pronounced in infected goats (Fig. 3b).

A. O. Ogunsanmi and V. O. Taiwo: Comparative haemoglobin concentration and erythrocyte biochemistry of grey duiker, sheep and goats during trypanosomosis

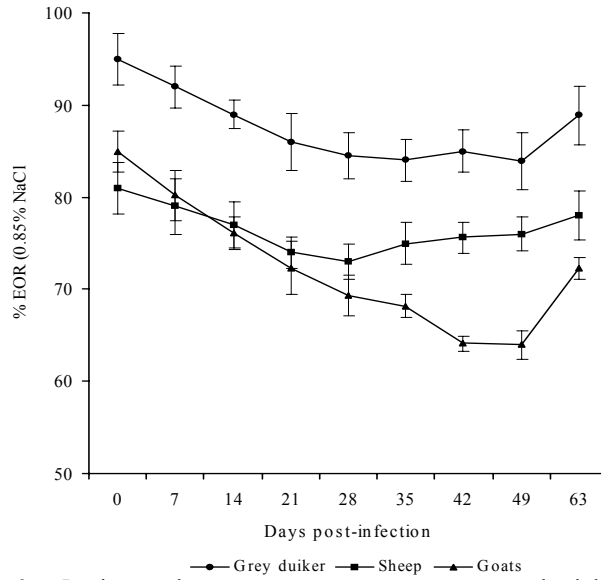


Fig. 3a. In vitro erythrocyte osmotic resistance (EOR) at physiological saline concentration (0.85%) in grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

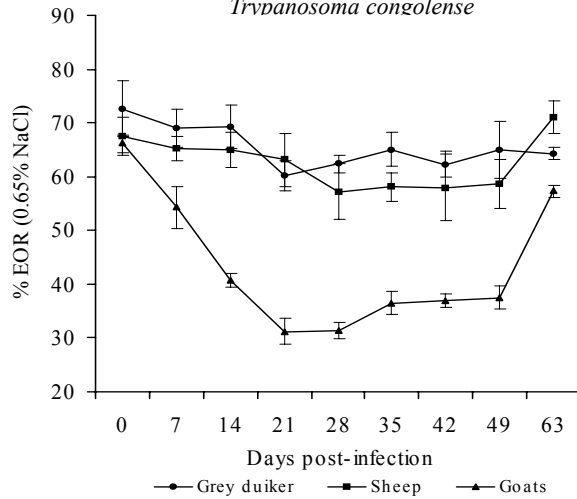


Fig. 3b. In vitro erythrocyte osmotic resistance (EOR) at hypotonic saline concentration (0.60%) in grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

A. O. Ogunsanmi and V. O. Taiwo: Comparative haemoglobin concentration and erythrocyte biochemistry of grey duiker, sheep and goats during trypanosomosis

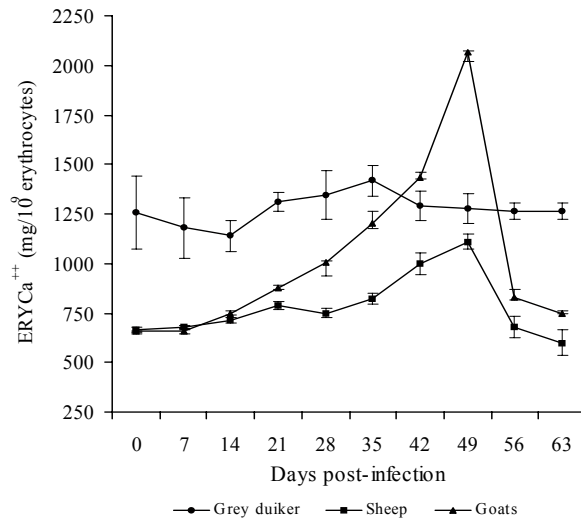


Fig. 4. Erythrocyte calcium (ERYCa⁺⁺) levels of grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

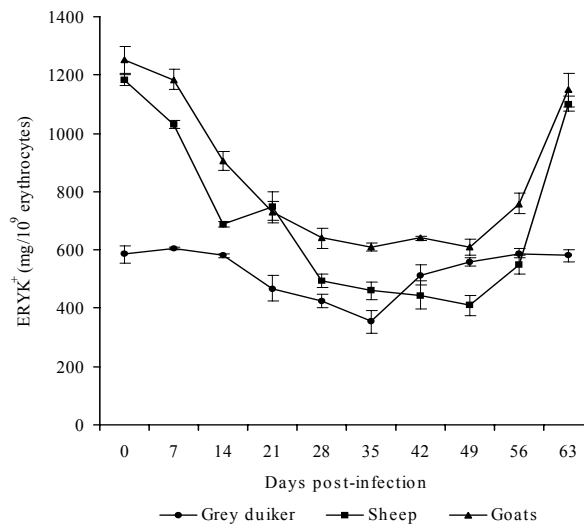


Fig. 5. Erythrocyte potassium (ERYK⁺) levels of grey duiker, sheep and goats experimentally infected with *Trypanosoma congolense*

Results of the erythrocyte Ca^{++} and K^{+} levels of grey duiker, sheep and goats infected with *T. congolense* are shown in Figs. 4 and 5, respectively. Pre-infection erythrocyte Ca^{++} and K^{+} levels of grey duiker doubled those of sheep and goats. There were no significant changes ($P < 0.05$) in Ca^{++} values in the grey duiker throughout the course of infection. Erythrocyte Ca^{++} levels increased ($P < 0.05$) in infected sheep from a pre-infection value of 662.8 ± 14.1 to 1108.0 ± 37.0 mg/ 10^9 erythrocytes on day 49 p.i., when they were treated (Fig. 4), with a subsequent decrease to pre-infection levels. A similar but more pronounced increase was recorded in infected goats (Fig. 4). There were significant decreases ($P < 0.05$) in K^{+} levels of grey duiker between day 21 and day 35 p.i., before returning to pre-infection levels thereafter (Fig. 5). Similar but more pronounced decreases ($P < 0.05$) in erythrocyte K^{+} occurred in infected sheep and goats throughout the period of study, returning to near pre-infection levels on day 63 p.i. (Fig. 5).

Discussion

Anaemia is a major clinical feature of African trypanosomosis in man and animals (ANOSA, 1988; OGUNSANMI et al., 1994a). The factor(s) and mechanism(s) by which wild animals such as buffalo, waterbuck and wildebeest resist and/or control anaemia during trypanosome infections are still not clearly understood (GROOTENHUIS et al., 1990; OLUBAYO et al., 1991). The present study showed that infected grey duiker developed mild and shorter duration anaemia, as typified by the development of little or no reduction in Hb concentrations. In contrast, sheep and goats developed severe and more prolonged anaemia, as typified by highly significant decreases in Hb concentrations during infection, most especially between the 14th and 49th days p.i. when they had to be treated to prevent imminent mortality. These findings are in agreement with those of previous workers (GROOTENHUIS et al., 1990; OLUBAYO et al., 1990, OLUBAYO et al., 1991) who reported that buffaloes and waterbuck experimentally infected with *T. congolense* developed less severe, or no anaemia and were found to control parasitaemia much better than the Boran (an East African Zebu) cattle. It is noteworthy that grey duiker had a higher mean pre-infection Hb concentration than sheep and goats in this study. This phenomenon may indicate either a better health status in the grey duiker or an indication of

pure physiological advantage (OLUSANYA, 1975; JAIN, 1986; OYEWALE, 1991) of grey duiker over sheep and goats prior to infection with *T. congolense*.

There was no significant elevation of ESR in infected grey duiker in this study. However, ESR values were significantly elevated in infected sheep and goats, with the effect being more serious in goats than in sheep. Increased ESR values have long been associated with anaemia, increased serum fibrinogen and/or globulin, specifically gammaglobulins (IKEJANI, 1946a; JAIN, 1986) and these have been reported in African animal trypanosomosis (ANOSA, 1988). Therefore, the increased levels of ESR in sheep and goats during trypanosome infection in this study signified a correlation with the severity of disease observed in these animals compared to those observed in infected grey duiker.

Potassium is largely an intracellular cation with over 98% of the exchangeable K^+ located intracellularly. Its distribution across the cell membrane plays a critical role in the maintenance of cardiac and neuromuscular excitability (CARLSON, 1989; OGUNSANMI et al., 1994b). The results of the present study showed that the grey duiker was more able to curtail loss of intracellular K^+ during trypanosome infection, which is complimentary to the little or no reduction in haemoglobin levels and the subsequently the concomitant, less severe anaemia exhibited by this species of animal. On the other hand, the prolonged duration and severe significant intracellular loss of K^+ correlated with the degree of hypohaemoglobinaemia and the devastating anaemia suffered by sheep and goats during *T. congolense* infection in this study. Decreased values in erythrocyte K^+ had been linked with increased erythrocyte fragility, and therefore anaemia, in *T. brucei* infected rats (IKEJANI, 1946a; OYEWALE, 1987).

The results of this study showed normal erythrocyte Ca^{++} levels increased in infected grey duiker throughout infection, while erythrocyte Ca^{++} levels increased by 67.3%, and by 213.5% in infected sheep and goats, respectively. Excessive intracellular calcium is deleterious to erythrocytes (LINMAN, 1975). Depletion of ATP in erythrocytes *in vitro* decreases calcium pump activity, thus resulting in an increase in intracellular calcium, increased membrane rigidity and decreased erythrocyte deformability (CLARK et al., 1981; KRETCHMAN and ROGERS, 1981;

AMINOFF, 1988). Thus, erythrocytes with these features are more prone to phagocytosis by “filtering” activity of activated macrophages of the mononuclear phagocytic system (MPS), which have been widely recognised as active participants in the pathogenesis of anaemia in African animal trypanosomosis (MURRAY and DEXTER, 1988; TAIWO and ANOSA, 2000). The increased erythrocyte Ca^{++} observed in *T. congolense* infections of sheep and goats in this study is consistent with the findings of EKANEM et al. (1996), who reported elevated erythrocyte calcium and calcium ATPase activity in *T. brucei* -infected mice.

The present study showed that erythrocytes of grey duiker had greater *in-vitro* osmotic resistance than those of sheep, and especially goats, using a physiological saline concentration (0.85% NaCl) during *T. congolense* infection. However, the ability to withstand lower saline concentration (0.60% NaCl) decreased with decreasing saline concentrations, with erythrocytes of infected grey duiker and sheep showing more superior osmotic resistance capability than those of goats. The implication of these findings is that if, during trypanosome infection, a situation arises and there is a reduction in the osmolality of circulating plasma, goat erythrocytes will be more prone to spontaneous osmotic lysis and hence develop a more severe haemolytic crisis. Also, goat erythrocytes, to a lesser degree than sheep and duiker erythrocytes, will have more leakage of their cytosol constituents into the plasma, thus making them age and be destroyed faster during trypanosome infection. The decrease in osmotic resistance in *T. congolense* infections of sheep and goats can be attributed to a decrease in erythrocyte potassium (IKEJANI, 1946a; IKEJANI, 1946b; OYEWALE, 1987). This physiological mechanism of superiority in erythrocyte osmotic resistance in the grey duiker would lead to reduced pathogenic effects in the tissues of trypanosome-infected animals as a result of hypoxia sequel to severe anaemia.

The relative trypanotolerance of the infected grey duiker based on their superior abilities to relatively limit anaemia and control parasitaemia as judged by consistent erythrocytic values of Hb concentration, erythrocyte sedimentation rate, osmotic resistance and erythrocyte K^+ , could be directly, although partly, related to their efficient erythrocyte calcium metabolism during *T. congolense* infection. In contrast,

the severe, devastating and prolonged duration anaemia suffered by the *T. congolense*-infected sheep, and especially goats, in this study could be closely related to the observed pronounced decreases in Hb concentration, ESR, erythrocyte osmotic resistance and erythrocyte K⁺, as well as to the increase in intraerythrocytic calcium levels. It is therefore suggested that these mechanisms exhibited by *T. congolense*-infected grey duiker could be partly responsible for the trypanoresistance reported in this species and other wild ruminants, as well as in trypanotolerant cattle. Thus, grey duiker may be a less costly substitute for African buffalo in research efforts designed to unravel the mystery of trypanotolerance, especially in wild, and some domesticated, ruminants.

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A. O. OGUNSANMI, V. O. TAIWO: Usporedno istraživanje koncentracije kalcija, kalija i hemoglobina u eritrocitima te njihova osmotska otpornost i brzina sedimentacije u sive dujker antilope (*Sylvicapra grimmia*), ovaca i koza pokusno invadiranih protozoonom *Trypanosoma congolense*. *Vet. arhiv* 74, 201-216, 2004.

SAŽETAK

Istraživana je koncentracija hemoglobina (Hb), brzina sedimentacije, osmotska otpornost i dinamika kalcijevih i kalijevih iona u eritrocitima na deset sivih dujker antilopa (*Sylvicapra grimmia*),

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15 afričkih patuljastih ovaca i 15 Yankassa koza pokusno invadiranih protozoonom *Trypanosoma congolense*. U sive dujker antilope razvila se prolazna parazitemija te blaga anemija, za razliku od ovaca i koza u kojih je zabilježena progresivna parazitemija, pireksija, anemija, te gubitak kondicije što je iziskivalo liječenje 49. dana nakon invazije. U sive dujker antilope nisu primijećene nikakve promjene u koncentraciji hemoglobina, brzini sedimentacije eritrocita i razini kalcijevih iona u eritrocitima. Manje promjene su primijećene na razini osmotske otpornosti i razini kalijevih iona. Suprotno tome, u ovaca i koza utvrđen je nagli pad koncentracije hemoglobina, smanjena osmotska otpornost i smanjena razina kalijevih iona, te ubrzana sedimentacija i povećana razina kalcijevih iona. Zbog toga je, za razliku od ostalih preživaca, došlo samo do ograničenog razvoja anemije i drugih štetnih učinaka tripanosomske invazije. Zaključeno je da je relativna otpornost prema tripanosomama u sive dujker antilope vjerojatno uvjetovana boljom kontrolom parazitemije, boljim održavanjem građe eritrocita i homeostaze. Radi toga bi dujker antilopa mogla poslužiti u istraživanjima tripanotolerancije umjesto afričkog bivola. Osim toga ta vrsta može biti izvor životinjskih bjelančevina u endemskim područjima tripanosoma i muhe ce-ce te se potiče njezino pripitomljavanje.

Ključne riječi: eritrociti, siva dujker antilopa, ovca, koza, *Trypanosoma congolense*, tripanotolerancija
