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Correlation of acetylcholinesterase activity in the brain and blood of wistar rats acutely infected with *Trypanosoma congolense*

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ABSTRACT

Objective: To investigate the neurotransmitter enzyme Acetylcholinesterase (AChE) activity in the brain and blood of rats infected with *Trypanosoma congolense (T. congo)*. **Methods:** Presence and degree of parasitemia was determined daily for each rat by the rapid matching method. AChE activity was determined by preparing a reaction mixture of brain homogenate and whole blood with 5, 5-dithiobisnitrobenzioc acid (DTNB or Ellman's reagent) and Acetylthiocholine (ATC). The increase in absorbance was recorded at 436 nm over 10 min at 2 min intervals. Trypanosome species identification (before inoculation and on day 10 post infection) was done by Polymerase chain reaction using specific primers. **Results:** The AChE activity in the brain and blood decreased significantly as compared with the uninfected control. The AChE activity dropped to 0.32 from 2.20 μ mol ACTC min^{-1}mg $protein^{-1}$ in the brain and 4.57 to 0.76 μ mol ACTC min-1mg $protein^{-1}$ in the blood. The animals treated with Diminaveto at 3.5 mg/kg/d were observed to have recovered significantly from parasitemia and were able to regain AChE activity in the blood but not in the brain as compared to the control groups. We also observed, that progressive parasitemia resulted to alterations in PCV, Hb, RBC, WBC, neurophils, total protein, lymphocytes, monocytes and eosinophil in acute infections of *T. congo*. Polymerase chain reaction (PCR) of infected blood before inoculation and on day 10 post infection revealed 600 bp on agarose gel electrophoresis. **Conclusions:** This finding suggest that decrease in AChE activity increases acetylcholine concentration in the synaptic cleft resulting to neurological failures in impulse transfer in *T. congo* infection rats.

1. Introduction

African trypanosomosis is caused by the systemic infection of the host by the extracellular parasite, of which one of the most important species in this disease is *Trypanosoma congolense (T. congo)*[1]. It is the most common cause of Nagana in Africa resulting to problems in the animal industry[2,3]. The disease is characterized by very high parasitemias and serious pathological complications such as anemia, immune suppression and is usually lethal to the host[4]. Trypanosomes infections are associated with several clinical manifestations which extend to central nervous system (CNS) infiltration, progressive mental degeneration and psychiatric manifestations[5] in the latter stage of the disease.

Infection by *T. congo* has shown that the parasite causes severe clinical pathological effects in the host[6] and one of the challenging issues of trypanosomiasis is that trypanosomes have variant surface glycoprotein (VSG) which is the main antigenic determinant of the immune system[7]. This Scenario enhances the parasite ability to resist treatment[8,9] thus initiating the risk of drug toxicity leading to exacerbation of the disease in the host[10,11].

Acetylcholinesterase (AChE) is the neurtransimitter enzymes that terminate the neurotransmission at cholinergic synapses by splitting the neurotransmitter acetylcholine (ACh) to chloline and acetate[12]. ACh plays an important role in sending signals from one neuron to the next when it is released from vesicles in the axon terminus, across the synapse, and onto receptors in the dendrites of the next neuron. The appearance of trypanosomes in the CNS courses

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many neurological disorders[13] and this has raised many questions on how trypanosomes affect neurotransmission and the courses of paralysis in trypanosomosis.

An earlier report[14] have shown that cholinesterase activity in wistar rats acutely infected with *Trypanosoma evansi (T. evansi)* decreased with progressive increase in parasitemia but the advent of genetic variation existing between Trypanosoma species, have necessitated the need to study the AChE activity in *T. congo* infected rats and the effect of treatment of the disease during infection in rats. Therefore, we report here the correlation of AChE activity in the brain and blood of rats acutely infected with *T. congo* and the consequences of treatment on the activity of the enzyme.

2. Material and methods

2.1. Chemicals

Acetylthiocholine (ATC), Bovine serum Albumin (BSA), 5, 5-dithiobisnitrobenzioc acid (DTNB or Ellman's reagent), were purchases from Sigma Aldrich company (St Louis, M O, USA). Diminaveto (1.05 g of Diminazene diaceturate and 1.31 g Antipyrine) was from Drugs and pharmaceutical limited Ambermath India. All other reagents were of analytical grade.

2.2. Animals

Wistar Albino rats weighing between 180-210 g were housed in cages and fed with commercial rat pellets with free access to water. The rats were randomly divided into 3 groups of 5 animals each. Group A was infected with 1×10^6 trypanosomes, Group B was infected with 1×10^6 trypanosomes and treated with 3.5 mg/kg/d Diminaveto and Group C was uninfected and untreated but received 0.2 mL of normal saline by intraperitoneal injection.

2.3. Trypanosome

T. congo species (kilifi strain) number BS2/TC/SP28/P4 isolated from an infected goat was obtained from center for Biotechnology Research, Ahmadu Bello University Zaria-Nigeria. The parasite was isolated from a goat and had been serially passaged through mice five times before we received it.

2.4. Parasitemia estimation

The presence and degree of parasitemia was determined daily for each rat by the rapid matching method[15]. A drop of blood was collected from the tail and placed on a glass slide then a thin blood smear was prepared and examined under a light microscope×400 magnification.

2.5. Trypanosomes species identification by polymerase chain reaction

A nested polymerase chain reaction (PCR) was performed as described[16]. The infected blood from a donor rat was collected for trypanosome species identification before inoculation and on day 10 post infection for rats in groups A and B. Briefly, 25 μL reaction volume containing 0.5 μL of each primer, 0.5 μL of dNTPs, 0.5 μL of Dream Taq Polymerase, 2.5 μL of Dream Taq buffer and double distilled water to a final reaction volume of 25 μL. Cycling conditions were as follows: 1 cycle at 95 ℃ for 300 s followed by 30 cycles at 94 ℃ for 60 s, 56 ℃ for 60 s, 72 ℃ for 30 s and a final extension of 33 s. Two consecutive reactions were carried out: using a Phusion blood direct kit (enzymes) and a set of outer primers (OSK 1425 and 1426) was used following the manufacturer's instructions. This was followed by inner primers (OSK 1423 and 1424) in the second round of reactions in which 1 μL of PCR product from the first round reaction was added to 25 μL of the second round of reaction in a fresh PCR tube. The Sequences of PCR Primers are given as: OSK 1423 Forward Outer 5′TGC AAT TAT TGG TCG CGC 3′, OSK 1424 Reverse Outer 3′CTT TGC TGC GTT CTT 5′, OSK 1425 Forward Inner 5′AAG CCA AGT CAT CCA TCG 3′, OSK 1426 Reverse Inner 3′TAG AGG AAG CAA AAG 5′.

2.6. Hematology examination

This was carried out on day 20 post-infection. Bloods from the rats were drawn into the capillary tubes and sealed before centrifuging in a microhaematocrit for 5 min at 10 000 rpm. The packed cell volume (PCV) was calculated as percentage of the total blood volume occupied by red blood cell (RBC) mass in the haematocrit which depends mostly on the number of the RBCs. Differential white cell count was done by dropping blood from the edge of a glass slide then it was air dried before Leishman stain was added on the dry smear blood. Immersion oil was dropped at the edge of the slide before viewing in a light microscope × 100. The haemoglobin (Hb) concentration was evaluated by a spencer haemoglo-binometer chamber with a drop of blood and the colour was matched against the standard colour of the haemoglobino-meter. The corresponding value on the scale in g/dL was read.

2.7 Collection and preparation of brain and blood samples

Each rat was sacrificed under anesthesia and the brain was quickly removed, weighed and placed on an inverted Petri dish on ice on day 20 post-infection. The brain was homogenized in 10 mL of a medium containing 10mM Tris - HCl buffer (pH 7.2) and 160 mM sucrose. The total homogenate was centrifuged at 3 500 g in a refrigerated centrifuge for 5 min. The supernatant was used for AChE activity determination. The blood was earlier collected in tubes with sodium heparin and the samples were diluted 1:50 (v/v) in lysis solution (0.1 mmol/L potassium/sodium phosphate buffer containing 0.03% Triton X-100) to determine AChE activity in blood.

2.8. Enzyme assay

The activity of AChE in the brain was determined by the methods described[17,18]. The mixture was prepared by mixing 0.4 mL aliquot of the homogenate and added to 2.6 mL phosphate buffer (0.1 M, pH 8.0) and 100 μL

of DTNB (270 μM). This was pre-incubated for 2 min at 30 ℃ and the reaction was started with the addition of 20 μL ATC (30 mM). The product of thiocholine reaction with DTNB was determined at 412 nm for a period of 10 min at 2 min intervals for the absorbance per minute. The specific activity is expressed as μmol ACTC min^{-1} mg $protein^{-1}$. The AChE assay in whole blood was determined by the method described[17,19]. The incubation system was composed of sodium phosphate buffer 0.063 mM (pH 7.4), DTNB 0.316 mM and 0.5 mL of the hemolyzed blood. The increase in absorbance was recorded at 436 nm over 10 min at 2 min intervals. Protein concentrations of the brain and blood homogenates were determine by the Coomasie blue method[20] using BSA as the standard. The specific activity of whole blood AChE was calculated and expressed as μmol ACTC min^{-1} mg $protein^{-1}$.

3. Result

3.1. Degree of parasitemia

The levels of parasitemia in (Figure 1) revealed that *T. congo* parasites were observed on day 4 post-infection with clinical manifestations. A progressive increase in parasitemia was observed over time for group A and decrease in parasitemia level as treatment commenced from day 4 for 5 d regime. The peripheral blood of the animals in group B was clear of parasite as from day 16 post infection and clinical signs such as apathy and weakness were not observed.

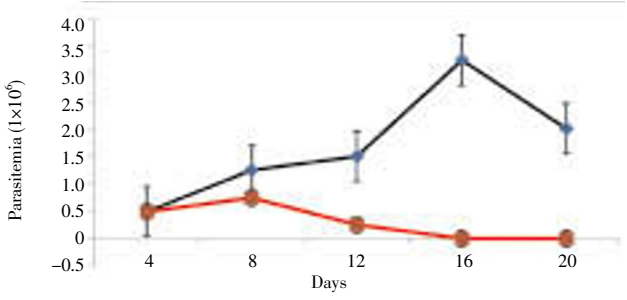

Figure 1. Levels of parasitemia in group A (Black = infected with *T. congo* and untreated) and group B (Red = infected with *T. congo* and treated with diminaveto from day 4 postinfection).

3.2. Trypanosome species identification

The infected blood of the rat that was used to inoculate the rats in groups A and B was first used for identification of the trypanosome species by PCR and agarose gel electrophoresis (AGE). This was also done for blood samples collected on day 10 post-infection for groups A and B. The band in Figure 2 runs at 600 bp this indicates that the parasite is *T. congo*. The group C animals did not show any band after PCR and AGE.

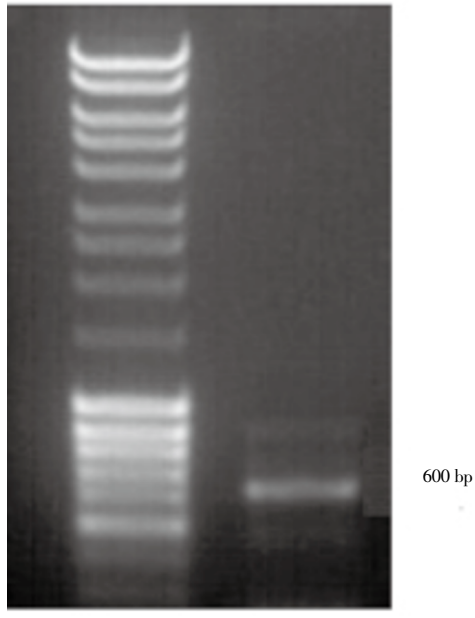

Figure 2: Ethidium bromide-stained Nested PCR products after agarose gel electrophoresis.
The band runs at about 600 bp which indicates the parasite is *Trypanosoma congolense*.

3.3. Hematological analysis

Blood was collected for hematological analysis to assess the progression of the disease on day 20 post infection for all the groups. Statistical analysis revealed a decrease in PCV, Hb, WBC, Total protein, neutrophils and lymphocytes as the disease progresses. The animals in group B that received

Table 1
Hematological parameters.

Parameter	Group A	Group B	Group C
PCV (%)	32.00 ± 0.44^{c}	34.00 ± 1.00^{c}	$46.00\pm0.00^{a,b}$
Hb (g/dL)	10.00 ± 0.40^{c}	11.90 ± 0.60	13.90 ± 0.30^{a}
WBC ($\times10^{9}$/L)	6.20 ± 1.70^{bc}	9.00 ± 0.11^{a}	10.30 ± 0.68^{a}
Total protein	4.80 ± 1.00^{c}	5.40 ± 0.10	6.80 ± 0.70^{a}
Neutrophils (%)	$17.00\pm2.00^{b,c}$	30.00 ± 2.00^{a}	32.30 ± 3.55^{a}
Lymphocytes (%)	$64.00\pm6.80^{b,c}$	68.00 ± 3.00^{a}	67.70 ± 7.50^{a}
Monocytes (%)	1.67 ± 0.29	3.00 ± 0.00	1.30 ± 2.30
Eosinophil (%)	$0.67\pm0.50^{b,c}$	0.34 ± 0.00^{a}	0.30 ± 0.50^{a}

Group A was infected with *T. congo* and untreated, group B was infected with *T. congo* and treated and group C was uninfected and untreated.

diminaveto showed a degree of hematological recovery (Table 1).

3.4. Acetylcholinesterase activity

The AChE analysis revealed a significant decrease of the enzyme activity in the brain and blood (Figure 3) as parasitemia progresses. The AChE activity dropped to 0.32 from 2.20 μ mol ACTC min^{-1}mg $protein^{-1}$ in the brain and 4.57 to 0.76 μ mol ACTC min^{-1}mg $protein^{-1}$ in the blood. It was also observed that group B animals which received treatment had a higher AChE activity than untreated but infected members in group A. This suggests that diminaveto was able to clear parasites in the blood and not in the brain. The parasite therefore continued the proliferation in the brain which accounts for the decrease in AChE in the brain.

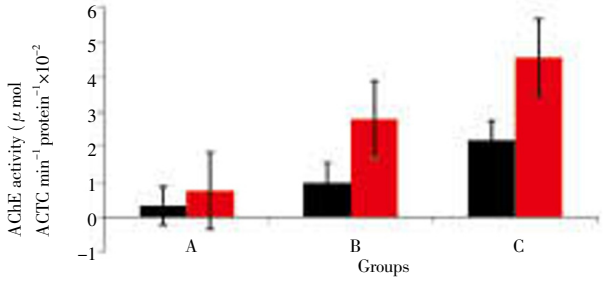

Figure 3. Acetylcholinesterase activity in the blood (red) and brain (black) in Group A (infected with *T. congo* and untreated), group B (infected with *T. congo* and treated) and group C (uninfected and untreated).

4. Discussion

The appearance of parasitemia was detected in the blood smears on day 4 post-infection with *T. congo* and with a sudden increase of the trypomastizotes in the blood and thereafter in the brain. This clinical situation was associated with decrease in PVC, Hb, WBC, Total protein, neutrophils and lymphocytes of animals in group A. The marked decrease of RBC indices indicates anemia[21,22]. The decrease in RBC indicates disorders in the blood due to *T. congo* parasites which will be responsible for decrease in immune system, increase in neurological disorder and lymphocytopemia[23]. Oxidative stress is also known to plays a significant role in the pathogenesis of *T. congo* infection[21].

From this study we observed that the trypanosomes have the ability to inhibit AChE activity and therefore prevent AChE from working normally leading to a high level of ACh to persist in the synapses[24]. Normally, it is a bad idea to always have Acetylcholine (ACh) hanging around in the synaptic cleft of which the next neuron is required to fire only when it is supposed to, and any ACh lingering around would cause neurons to misfire. To get rid of any excess ACh, AChE is employed for this task. AChE removes any of the ACh that is left over after the impulse has passed. The function of ACh when released is to enhance neuromuscular junction by initiating muscle contractions. AChE slows the heart rate when functioning as an inhibitory neurotransmitter and also behaves as an excitatory neurotransmitter at neuromuscular junctions[25].

The identification of *T. congo* specie in the blood of infected rats was authenticated by ethidium bromide stained AGE nested PCR product (Figure 2) which detects the parasite nucleic acids. This is known to be a very sensitive method that can detect Trypanosoma DNA in presence of host DNA[26]. This property gives credence to the PCR detection of *T. congo* for the pre-patent period and chronic phase of the disease.

Pharmacological manipulation of cholinergic function has been found useful in the treatment of CNS disorders[18]. The effect of treatment with diminazene aceturate in trypanosome infected rats on AChE did not restore the enzyme activity immediately as compared to the group infected with only *T. congo*. The parasitemia in the blood decreased with the administration of Diminaveto to members of group B and there was disappearance of parasitemia as from day 16 for group B. The AChE in the blood was higher than that in the brain after treatment with Diminaveto this was because the drug cleared the parasite in the peripheral blood but not in the brain. The free parasite in the brain therefore sustained there effect in the brain AChE. This observation superimposes that the parasite (or its metabolite) have the ability to bind to the active site of AChE therefore inhibiting its activity.

The *T. congo* effect on AChE in this study was observed to decrease significantly in acute infection and there was significant change with that of the control. The treatment of the disease in group B did not completely restore the AChE activity in the blood but it increased significantly above the untreated animals in group A. ACh is considered as the most important neutrotransmitter involved in regulation of cognitive functions[24]. This changes in AChE could explain the motor dis-coordination and paralysis seen in animals infected with *T. congo* in this study. AChE is an important regulatory enzyme that controls the cholinergic nervous impulses, both in brain cholinergic synapses and in the muscle[14].

We conclude that the acute increase in parasitemia accounts for the decrease in AChE activity in brain and blood which implies that ACh will be high in the motor division and autonomic ganglia. The excess ACh accumulates therefore making the neurons to constantly fire, causing the muscles in the animals to develop paralysis and

asphyxiation which is a common symptom observed at the latter stage of the disease.

Conflict of interest statement

We declare that we have no conflict of interest.

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