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Original Article

Experimental African trypanosomiasis: Effects on plasma melatonin concentration and pineal gland histology in rodents

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Abstract

Trypanosomiasis remains a major public health problem to man over much of tropical Africa. The disease is caused by protozoan parasites of the genus *Trypanosoma* and is fatal if untreated. The effects of *T.b.brucei* infection on plasma melatonin concentration and pineal gland histopathology was investigated in male albino rats. Twelve rats were each infected intraperitoneally with 0.2ml of infected blood containing approximately 1.0 x 10^4 live *T.b.brucei* parasites. Twelve other rats served as uninfected controls. Trypanosomes were detected in the blood of infected rats 5-8 days post-infection. There was a significant difference (P=0.0382) in plasma melatonin concentration between control and experimental rats. Histopathological changes in the pineal gland of experimental rats included tissue degeneration and pinealocytes with pyknotic nuclei. These histopathological changes were responsible for the decrease in plasma melatonin concentration in the experimental rats.

Keywords: Trypanosomiasis, Melatonin, Pineal gland, Histopathology

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1. Introduction

Human African trypanosomiasis, commonly known as sleeping sickness, is one of the neglected and re-emerging infectious diseases in Africa that has been accorded little attention and priority [1]. The disease is caused by flagellate protozoan parasites of the genus *Trypanosoma* and is transmitted by the bite of an infected tsetse fly of the genus *Glossina.* It is estimated that over 66 million people in 37 African countries are at risk of contracting the disease, 300,000-500,000 people are currently affected, and 48,000 deaths can be attributed to the disease each year [2]. The disease continues to be a major health problem throughout sub-Saharan Africa and is

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likely to be so for the foreseeable future [1].

There are two distinct forms of human African trypanosomiasis; the chronic West African form which occurs in west and central Africa, and the acute East African form, which occurs in east and southern Africa. West African trypanosomiasis is caused by *Trypanosoma brucei gambiense* and accounts for about 95% of the reported cases of sleeping sickness [3]. East African trypanosomiasis is caused by *T.b. rhodesiense* and accounts for about 5% of the reported cases of sleeping sickness in Africa [3]. There is an early or haemolymphatic stage, when the trypanosomes are restricted to the blood and lymph system, and a late or encephalitic stage, when the parasites cross the blood-brain barrier to invade the central nervous system [3]. In the absence of treatment, sleeping sickness patients die within months when infected with *T. b. rhodesiense* or within years when infected with *T. b. gambiense*.

The most characteristic symptom of human African trypanosomiasis is the severe disturbance in the sleep/wake cycle with typical diurnal somnolence (which gave the disease its other name, sleeping sickness) and in many cases nocturnal insomnia [4]. Melatonin (5-methoxy-N-acetyltryptamine), the principle hormone secreted by the pineal gland, is a key regulator of the sleep/wake cycle, and other circadian rhythms [5]. Melatonin functions as a synchronizer of the biological clock by providing information about night-length [6,7]. It is apparent that melatonin and the pineal gland have widespread effects and optimal functioning of the pineal gland is essential to our well-being.

The production and secretion of melatonin follows a circadian rhythm, with the levels being higher during the night and lower during the day [8,9]. Research into melatonin secretion patterns in the vertebrates, as well as in humans, has shown that melatonin levels rise with onset of darkness and fall with the onset of daylight [10. 11, 12, 13, 14]. As daylight approaches, light perceived by the retina is encoded, and this information is relayed to the pineal gland. Light serves to suppress pineal activity, and subsequently, melatonin levels decrease to very low levels and remain there until production and secretion are once again stimulated by the onset of darkness [15]. Although the circadian pattern of melatonin secretion has been established, little has been reported on the changes of melatonin secretion in various pathological conditions. This study was, therefore, carried out to investigate the effects of *T.b.brucei* infection on the plasma concentration of melatonin, and pineal gland histology in rats.

2. Materials and Methods

Experimental Setup

Twenty four male albino rats, aged 3-3½ months and weighing 200-220g, were used in this study. The rats were randomly divided into two groups of twelve rats each. The first group of twelve rats constituted the uninfected controls while the other twelve were inoculated with a *T.b. brucei* (ILTat1.4) isolate and constituted the infected experimental group. These laboratory rodents were chosen because they allow easy handling and present an acute infection that has strong similarities with the human disease [16].

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The rats were housed at room temperature (25.2±3.6 ^{0}C) in the mini-laboratory animal house of the Department of Biological Sciences, University of Eldoret, Kenya. They were housed three per cage and were exposed to 12/12hours of light/dark cycle throughout the study period. The rats were provided with food (Mice Pencil, Unifeed Millers Ltd, Kisumu, Kenya) and water *ad libitum.*

Two weeks prior to baseline data collection, the rats were observed and accustomed to routine handling. They were also screened for ectoparasites besides being injected subcutaneously with 0.02ml of Ivermectin (Ivermin®, Sinochem Ningbo Ltd., China), a broad-spectrum parasiticide that effectively controls both endoparasites and ectoparasites. The research committee of the Department of Biological Sciences, University of Eldoret, Kenya, approved this study and the animals were handled in accordance with internationally accepted principles for laboratory animal care and use [17].

Inoculation of Experimental Rats

An isolate of the parasite *Trypanosoma brucei brucei* (ILTat1.4) was obtained from the International Livestock Research Institute (ILRI), Nairobi, Kenya. This strain was used because it allows a long disease course (30 days on the average) with central nervous system involvement by around twenty one days in mice [18]. The parasite was originally obtained from the blood of a naturally infected cow in Uhombo village, Kenya. The isolate was inoculated intraperitoneally to a donor rat for the purpose of harvesting enough parasites for subsequent inoculation into experimental rats. The donor rat was put in a cage and transported to the University of Eldoret, where the study was carried out.

The donor rat was monitored for the presence of parasites daily by direct microscope observation of trypanosomes in wet smears of blood samples obtained from tail bleeds. When parasitaemia was established, the donor rat was anaesthetised with ether and 2ml of blood obtained from it through cardiac puncture. One millilitre (1ml) of this blood was diluted with 2ml of phosphate buffered saline (PBS) solution (pH 7.4). Then, 0.2ml of this blood, containing approximately 1.0 x 10^4 live *T.b. brucei* parasites was injected intraperitoneally to each of the twelve rats in the experimental group. The number of parasites was determined using the Neubauer haemocytometer method [19]. Tail blood samples were collected daily from the infected rats and screened for trypanosomes using wet and thin blood smear films [20]. The rats were also observed for any abnormal behaviour and/or any clinical signs of infection. The rats were infected after four weeks of pre-infection baseline data collection. Control rats were, concurrently, injected intraperitoneally with 0.2ml normal saline.

Determination of Plasma Concentration of Melatonin

Tail blood samples were collected weekly for the determination of plasma concentration of melatonin. Blood samples were collected from all the twenty four rats at night, between 10.00pm and 2.00am. This time was chosen because melatonin is a hormone of darkness; its blood levels are essentially undetectable during the day, but rise sharply during the night [21].

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Using the tail snip method, about 1ml of blood from each of the twenty four rats was collected in a vacutainer coated with ethylene diamine tetra acetate (EDTA). The blood sample was promptly centrifuged for 5 minutes at 12,000 revolutions per second. The plasma was quickly decanted into a test tube and immediately stored in a chest freezer (Haier Electrical Appliances Inc., Philippines) at -40°C. The concentration of melatonin in the plasma was determined the following morning using an automated immunoanalyzer machine (VIDAS®, bioMerieux SA, France).

Histopathological evaluations

The infected animals were allowed to go through the full course of infection and sacrificed when they were *in extremis* (32-36 days post-infection). For every experimental rat sacrificed, a control rat was sacrificed too. Each rat was anaesthetised with ether, decapitated and the brain immediately extracted from the skull. The brain was immediately put in 10% buffered neutral formalin and fixed for at least 48 hours. Thereafter, the brain was removed from the formalin solution and the pineal gland extracted using a sharp scapel blade. The pineal gland was processed histologically using an automated tissue processor (Global Medical Instrumentation Inc., USA). The processed gland was embedded in paraffin wax and sectioned with a manual rotary microtome (Leica Biosystems, Germany) at 5µm thickness. The thin sections were stained using the haematoxylin and eosin method [22], examined under a microscope, and photomicrographs taken.

Data Analysis

Data collected from this study was summarized as mean ± standard deviation and the difference between the means analyzed using Student's t-test (SPSS v19 for Windows) at 5% significance level.

3. Results

Parasite Detection

Parasites were detected in the tail blood of experimental rats five to eight days post-infection. The experimental rats showed no signs of disease for the first fifteen days post-infection. Thereafter, they showed apparent fatigue, decreased activity, sleep disturbances, lack of appetite, discharge from the eyes and nose, and paralysis of limbs and tail. The rats became emaciated too. On the other hand, the control rats showed no signs of infection throughout the study period. They were also of normal behaviour, appetite, and general activity.

Effect of *Trypanosoma brucei brucei* infection on plasma melatonin concentration

The concentration of melatonin in the plasma of control rats ranged between 81.40 ± 0.56 pg/ml and 82.44 ± 0.13 pg/ml throughout the study period (Figure 1). In experimental rats, plasma melatonin concentration remained within normal range (81.98 – 82.10 pg/ml) during the pre-infection period. The concentration decreased to70.48 ±0.24 pg/ml two weeks after infection, and then continued decreasing up to 42.81 ± 0.08 pg/ml by the time the study was terminated. The difference in plasma concentration of melatonin between the control and experimental rats was significant (P = 0.0382).

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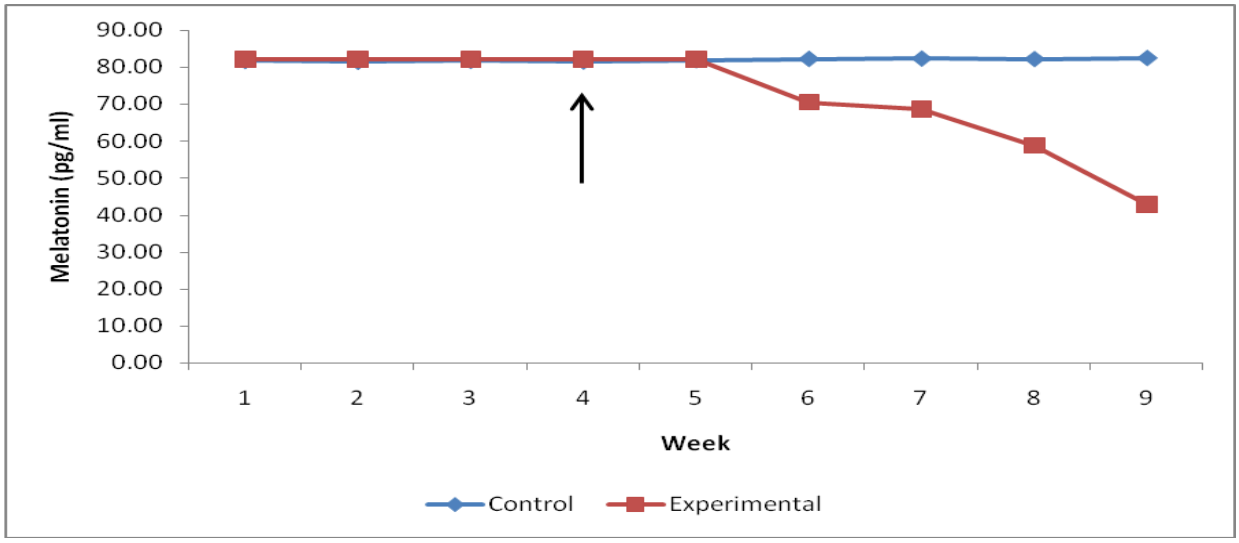


Figure 1. Pre- and post-infection plasma melatonin concentration in control and experimental rats. Arrow indicates time when experimental rats were inoculated with the *T.b.brucei* parasite.

Pineal Gland Histological changes

Mg x400Microscopic examination of transverse sections through the pineal gland of control rats showed normal pinealocytes and neuroglial cells (Figure 2a). The typical stringy appearance of the gland, which is due to criss-crossing of processes from both pinealocytes and neuroglial cells, was evident in these sections. On the other hand, transverse sections of pineal gland from infected rats showed pinealocytes with smaller and shrunken (pyknotic) nuclei, as compared to control rats. The typical stringy appearance was lacking and patches of tissue degeneration were quite evident in these glands (Figure 2b).

4. Discussion

In this study, trypanosomes were detected in the tail blood of experimental rats 5-8 days post-infection. This finding concurs with those of other investigators [4, 16, 23] who reported similar results in rats inoculated with *T.b.brucei.* Once inside the body, the host's immune system responds and kills subpopulations of the parasite, but because of the variable surface glycoprotein (VSG) coat on the trypanosome, a proportion of the trypanosome population escapes the immune response, proliferates and another relapse of parasites is observed in the blood [24]. The experimental rat's immune system was, thus, at first able to control the trypanosome population. This could explain the lack of any clinical signs of infection in these animals for the first two weeks after infection. Thereafter, the immune system became overwhelmed, setting the stage for the emergence of the observed signs of infection which ranged from apparent fatigue and reduced activity, to paralysis of limbs and tail at the terminal stages of the disease

The pineal gland is an end organ of the visual system and production of melatonin by the pineal is stimulated by darkness and inhibited by light [25, 26]. Photosensitive cells in the retina detect light and directly signal the

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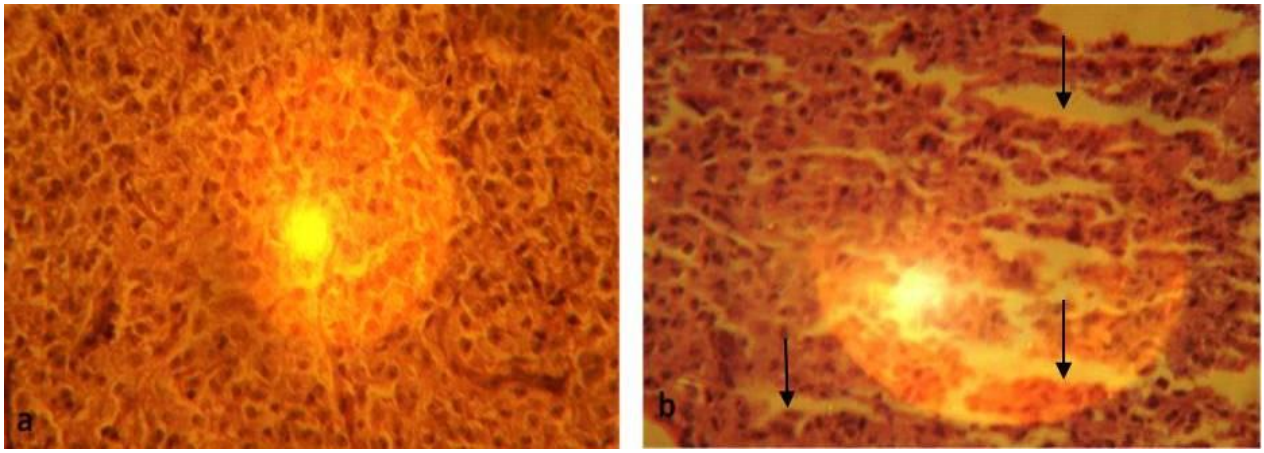


Figure 2: Photomicrographs of transverse sections through the pineal gland of a) control, and b) *T.b.brucei*-infected rats. Arrows indicate patches of tissue degeneration.

suprachiasmatic nucleus (SCN), via the retinohypothalamic tract. Fibers project from the SCN to the paraventricular nucleus (PVN), which relay the circadian signals to the spinal cord and out via the sympathetic system to superior cervical ganglia, and from there into the pineal gland. The main and physiological role of melatonin is the mediation of photoperiodic signals which determines circadian rhythms in animals.

In the present study, the concentration of melatonin in the plasma of *T.b. brucei*-infected rats remained normal during the pre-infection period. The concentration, however, decreased two weeks after infection and then continued decreasing further for the rest of the study period. These results concur with earlier findings by Kristensson *et al*. [27] who found decreased melatonin levels in the urine of trypanosome-infected rats. The decrease in plasma melatonin concentration coincided with the time when the infected rats started showing clinical signs of infection. This could have signaled the entry of trypanosomes into the rat's central nervous system. The decrease in melatonin concentration may also be linked to histological changes in the pineal gland induced by the trypanosome infection.

Microscopically, pineal gland is composed of pinealocytes and neuroglial cells amongst which ramifies a rich network of capillaries and postganglionic nerve fibres [28]. Pinealocytes, the majority cells, are the ones responsible for the production and secretion of melatonin. Pinealectomy in humans actually removes virtually all plasma melatonin and there is good evidence that the neural and biochemical pathways known to control pineal function in rats are similar to those in humans [29]. The histological changes that occurred in the pineal gland of experimental rats in this study included tissue degeneration and pinealocytes with pyknotic nuclei. These alterations point to a functional dysregulation in melatonin production and secretion by the pineal. This could explain the decrease in concentration of plasma melatonin recorded in the experimental rats.

It is pertinent to suggest that the low concentration of plasma melatonin in the experimental rats compromised many of the physiological functions of melatonin

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in these animals. Dysregulation in melatonin secretion has actually been associated with disturbances in the sleep/wake cycle in mammals [27, 30], as was noted with the experimental rats in this study. Studies carried out by Schultzberg *et al*. [31] and Schultzberg *et al*. [32] showed that *T. b. brucei* invasion of the brain was confined to circumventricular organs at six days post-infection, while penetration of the parenchyma commenced around thirteen days after infection in mice and rats. The parasites showed an early invasion in the choroid plexus and other circumventricular organs including the pineal gland, area postrema , and median eminence that lack a blood-brain barrier. At later stages, the parasites were shown to penetrate the blood-brain barrier and enter the brain parenchyma, as revealed by double immunohistochemical labeling of parasites and brain endothelial cells in a rat model of the chronic disease [33]. The invasion of the brain by the parasites may mark the beginning of symptoms of the disease which include disturbed circadian rhythms, sensory disturbances, and neuro-endocrinological dysfunctions [34].

The results of this study demonstrate that *T.b.brucei* infection causes marked histological changes in the pineal gland of infected rats. These changes could be the likely cause of reduced plasma melatonin concentration, and disrupted sleep/wake cycle, in the infected rats. With fewer pinealocytes, tissue degeneration, and, hence, a disrupted neural network in the pineal gland of infected rats, less melatonin was secreted. It is likely too, that the changes in plasma melatonin concentration would be an indication of invasion of the pineal gland by the trypanosomes.

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