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Original Research Article

Effects of Calcitriol Supplementation on the Hematological Parameters of Sleep Deprived Wistar Rats

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ABSTRACT

The present study investigates the hematologic effect of Calcitriol on rats undergoing sleep deprivation. Male Wistar rats were treated with Calcitriol 120 ng/kg and subjected to sleep deprivation for four successive days. Twenty four hour after last injection, animals were sacrificed and blood was collected for haematological analysis. A four-day sleep restriction caused a decline in total white cell count and increased mean cell hemoglobin and mean cell volume. Furthermore, red blood cell count, packed cell volume, hemoglobin concentration and mean cell hemoglobin concentration also declined in sleep deprived rats. Peripheral blood cell examination revealed that these effects were mild in rats treated with calcitriol. Our findings showed that a four-day paradoxical sleep restriction altered the biochemical integrity of erythrocytes. The observed immunosuppressive effects of sleep deprivation were reversed by exogenous vitamin D supplement, calcitriol. However, only the functional haemoglobin component of red cells was enhanced by a high dose of calcitriol which appears unbeneficial for other units of the erythroid-forming processes. It is therefore possible that the erythrocytic enhancing power of calcitriol is dose dependent and we suggest that lower doses (<120ng/kg) may be required to produce beneficial effects on erythropoiesis.

Keywords: Paradoxical sleep deprivation, Calcium homeostasis, blood parameters, Immune functions.

INTRODUCTION

Sleep is a vital process of life and serves many important functions such as preservation, restoration, and memory processing. [1] It is recommended that adults should get between seven and nine hours of sleep each night. Repeated disruption of the natural sleep cycle or failure to initiate sleep can lead to a sleep deficit, which in turn causes physical, mental, emotional fatigue and pathological changes. [2]

Sleep deprivation consists either in a complete lack of sleep during a certain period of time or a shorter than optimal sleep time. Inadequate sleep is widely viewed as a health threat that affects normal development and healthful aging. [3] The most common causes of sleep deprivation are those related to contemporary lifestyle and work-related factors; these conditions affect a considerable number of people across world. A chronic reduction in the sleep time or fragmentation of sleep results in the disruption of sleep cycle. [4]

The first report on the total chronic sleep deprivation in rats dates back to 1962 and since then various researchers have examined the consequences of sleep

deprivation on health using human and animal models. [2,5] Everson *et al.* [3] opined that chronically inadequate sleep results in abnormal bone formation and abnormal bone marrow in rats. [3] The bone marrow is the major site of hemopoiesis in adults; hence the products of hemopoiesis will be affected by bone marrow defects when calcium metabolisms become altered.

Under a normal sleep/wake schedule, diurnal rhythms have been identified in levels of circulating blood cell populations, with highest lymphocyte levels observed at night-time. [6] A number of researchers have theorized sleep induced cerebral anaemia [7] while others postulated that sleep disorders may be associated with a raised haematocrit. [8,9]

The effect of sleep deprivation on observed rhythmicity in circulating blood cell populations is currently controversial. [6,10] It is not clear the role that dietary calcium plays in the modulation of sleep cycle and hematopoiesis. It has been reported that increasing dietary calcium might simultaneously reduce iron absorption and erythropoiesis. [11]

Calcitriol is a synthetic vitamin D analog which is active in the regulation of the absorption of calcium from the gastrointestinal tract and its utilization in the body. Calcitriol is approximately 99.9% bound in blood. Several studies have examined the impact of sleep deprivation (SD) on biological systems including calcium which interacts with vitamin D. Sleep deprivation is known to be a form of stress, and it can alter behavioral, physiological, as well as cellular functioning; therefore, stressor like sleep deprivation may affect the levels of Calcium in the body. [12]

Calcitriol and other vitamin D metabolites are transported in blood, by an alpha-globulin vitamin D binding protein. Studies have established a direct relationship between elevated endogenous Calcitriol levels and abnormalities of calcium metabolism with potential for vascular calcification [28,29] Ca^{2+} uptake is of key importance for promoting differentiation and proliferation of erythroid precursors at the stages of burst-forming units erythroid (BFU-E) and colony-forming units erythroid (CFU-E). [13,14]

It is clear that sleep deprivation is associated with inflammation [15] and vitamin D is an underlying factor in sleep-related issues. [16] Association between Vitamin D deficiency and anaemia as well as inflammation has been established. [17] Ca^{2+} is a universal signaling molecule involved in the regulation of cell cycle, fat metabolism, structural integrity, motility and volume. Like other cells, red blood cells (RBCs) rely on Ca^{2+} dependent signaling during differentiation from precursor cells. [18]

In view of the association of sleep disturbance and disorders with various pathologies, there is the need to establish its independent effects on haematopoietic cells which are often evaluated as prognostic or diagnostic markers. Also, Vitamin D may suppress inflammatory pathways, and studies to determine whether vitamin D supplementation ameliorates Anaemia of inflammation are warranted. It is also important to determine the role of calcium, which is 99% deposited in the bones (major site of hemopoiesis) on these cells.

The broad objective of this work is to determine the effects of Calcitriol supplementation on the cellular components of blood in sleep deprived Wistar rats and establish associative effects of sleep disturbance on immune functions and structural integrity of haematopoietic cells.

MATERIALS AND METHODS

Animals and Facilities: Eighteen (18) male Wistar rats (150-200g) were used for the study. They were housed at the College of Medicine animal care unit of Afe Babalola University under standard conditions of temperature (23±2 ^{0}C), humidity (55±15%) and 12 hour light (7.00am - 7.00pm). The cages were constantly cleaned in order to prevent the animals from contracting diseases. They were fed with standard

commercial rat pellets and allowed water ad-libitum. All procedures in the animal handling were according to the guidelines of Animal use Ethical Committee of the National Institute of Health Guide for Care and Use of animals. The animal care committee of Afe Babalola University approved the experimental protocols.

Experimental Protocol

Treatment/Sacrifice: The Animals were divided into three treatment groups as shown in Table 1. Animals in groups II and III were pretreated daily with phosphate buffer saline (1ml/kg) and Calcitriol (120ng/kg), respectively, after which they were sleep deprived. Whereas, group I rats only received intraperitoneal injection of normal saline (1ml/kg). The treatment and sleep deprivation lasted four (4) days across all groups. Note that the test drug calcitriol was dissolved in Phosphate buffer saline, PBS (vehicle) [which contains 100mM NaH_2PO_4 dibasic -28.392gm, 0.9% NaCl - 18.0g, 0.1% NaN_3 - 2.0g, PH = 7.6]. Thereafter, the animals were sacrificed by cervical dislocation and blood was obtained via cardiac puncture. Blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) tubes. A Full blood count including peripheral blood film examination, white blood cell count, differential leukocyte count, red blood cell count, haemoglobin estimation and packed cell volume was carried out manually on the samples according to previously described methods. [19] Blood smears for differential counts were prepared at the same time, air dried and later stained with Giemsa. About 200 cells were counted and identified on each smear. The photographs of composite blood cells were prominent and carefully taking at x400 magnification.

***Sleep Deprivation*:** A modified multiple platform sleep deprivation tank was used for this study. The animals were placed within a large aquarium netted tank containing ten multiple platforms projected from the base. The top of the platforms are relatively small to the animal's body size. The platforms are distant from the walls of the tank to prevent the animals from leaning on the walls. The tank is filled with water to a level that is 2cm below the top of the platforms. With this design, the animals could only get NREM (non-rapid eye movement) sleep, but at the onset of REM (rapid eye movement) sleep with ensuing muscular relaxation, they will either fall into the water and climb back to their platforms or will get their nose wet enough to awaken them. [20] Food baskets were suspended from the netted cover to ensure the animals have access to pelletized feed ad-libitum.

Table 1.0: Treatment design

Groups	Sample size	Treatment
I, NS	n=6	Normal saline (1ml/kg), i.p
II, PBS+SD	n=6	Phosphate buffer saline (1ml /kg), i.p + SD
III, Calcitriol+SD	n=6	Calcitriol (120ng/kg), i.p + SD

NS=Normal saline, PBS=phosphate buffer saline, SD=sleep deprived, i.p=intra-peritoneally

Statistical Analysis: Data were compared by means of Welch's *t*-tests that do not assume equal variances. The family-wise type I error was set at $P<0.05$ for all comparisons. Holm's adjustment was applied to correct for multiple comparisons, in which each null hypothesis is tested for rejection according to the sequential decreasing strength of ordered P values. Graphs were plotted using Graph Pad Prism (Version 5.04) and values are expressed as means ± standard deviation (SD).

RESULTS

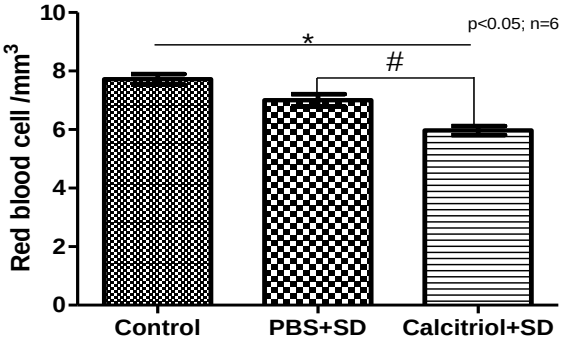

Figure 1: Bar chart illustrating variations in the population of Red blood cells of Control, Sleep deprived and SD+Calcitriol supplemeted Wistar Rats. The chart depicted significant ($p<0.05$) falls in RBC count of PBS+SD and Calcitriol+SD compared to control and with further decreases in Calcitriol+SD group when compared with PBS+SD rats. *$p<0.05$ (compared to control), #$p<0.05$ (compared within treatment group); n=6.

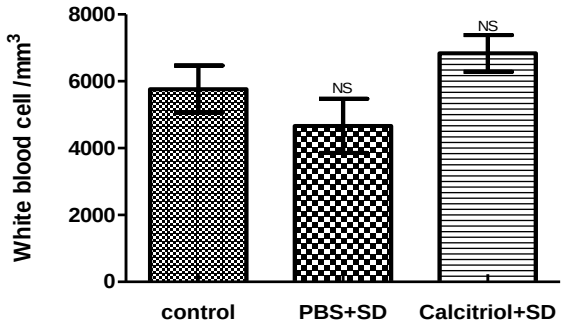

Figure 2: WBC of Control, Sleep deprived and SD+Calcitriol supplemented Wistar Rats. A non-significant (p>0.05) decrease was observed in PBS+SD group when compared with the control. Also, there was a non-significant (p>0.05) increase in Calcitriol+SD group when compared to control and PBS+SD. *p<0.05, NS=p>0.05; n=6.

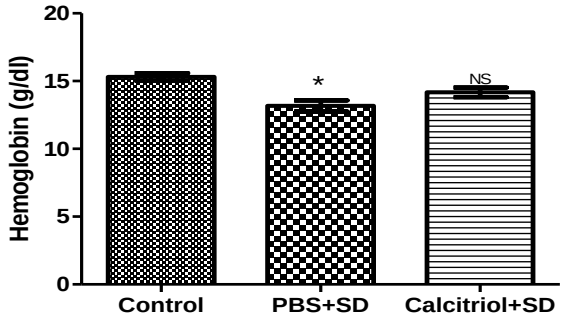

Figure 3: Blood hemoglobin concentration of Control, Sleep deprived and SD+Calcitriol supplemeted Wistar Rats. The figure shows significant (p<0.05) decrease in hemoglobin concentration of PBS+SD group compared to control. Calcitriol supplementation has no significant (p>0.05) effect on the parameter. *p<0.05, NS=p>0.05; n=6.

RBC, WBC, PCV and Haemoglobin Concentration indices of Sleep Deprived Rats: Quantitative estimation of red blood cells (RBC), white blood cells (WBC) and haemoglobin concentration (Hb) show a main effect of treatment (p<0.05). Four days of sleep deprivation induce a significant decrease in the number of RBC and WBC and in Hb (p<0.05). Administration of calcitriol to sleep deprived animals also induce a significant decrease in the number of RBC in comparison to control group (p<0.05) and to sleep deprived group (p<0.05), but have no significant effect on WBC and Hb (p>0.05) (Figure 1,2 and 3, respectively). However statistical analysis of the fraction packed cell volume show no significant differences in both treatment groups in comparison to control group (p>0.05) (Figure 4)

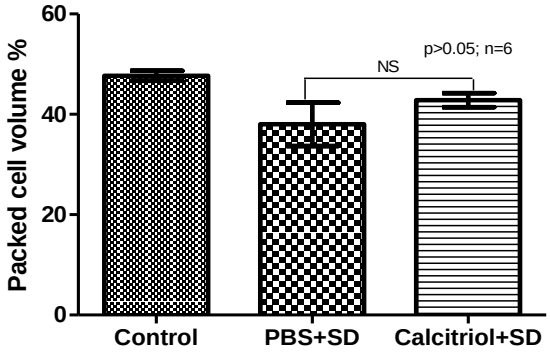

Figure 4: PCV of Control, Sleep deprived and SD+Calcitriol supplemeted Wistar Rats. % volume of red cells was not significant in both treatment groups compared to the control. *p<0.05, NS=p>0.05, n=6.

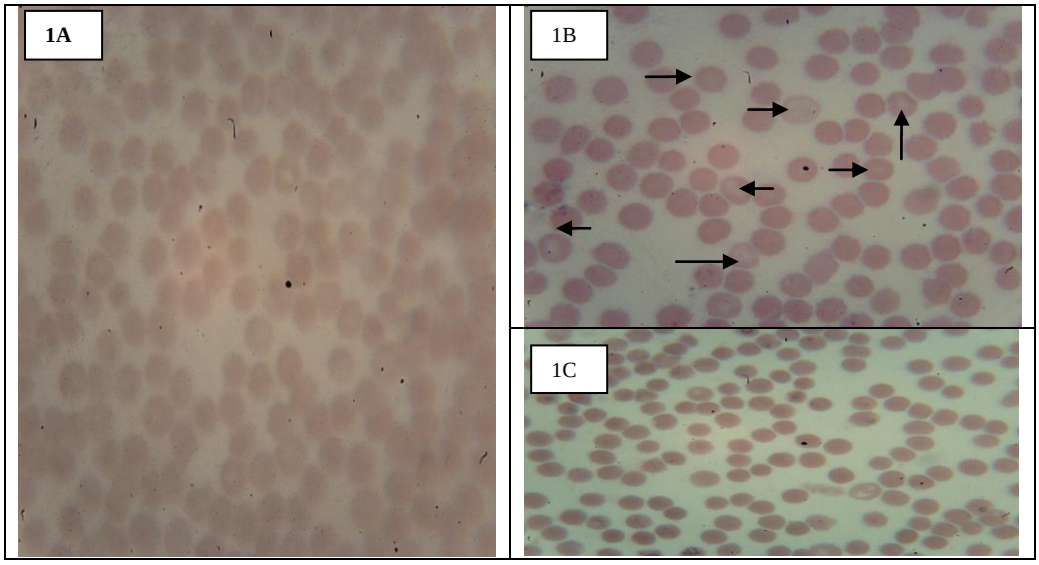

Figure 5: Photomicrographs showing composite blood cells of NS (A), PBS+SD (B) and Calcitriol+SD (C) groups. NS group shows normal blood cell morphology with adequate platelet distribution. B indicates the presence of poikilocytes and also shows that the size and colour of RBC in the blood film of sleep restricted rats were severely affected (microcytic and hypochromic). Platelet count in this group was also reduced. Polychromatophilic cells were prominent in the blood of caltriol-supplemented rats. [X400 magnifications]

Effects of Sleep Restriction on the Morphology of Blood Cells

As shown in figure 5a, the blood picture of control rats indicates the presence of normocytic and normochromic cells with adequate platelet distribution. The photomicrographs also revealed that cells in the PBS+SD group were microcytic and hypochromic, undergo poikilocytosis and a left shift Neutrophil leukocytosis and scanty platelets are presents (Figure 5b). Polychromasia, reticulocytosis and scanty platelet are prominent features of the Calcitriol+SD group (Figure 5c). The blood cells in this group are also macrocytic.

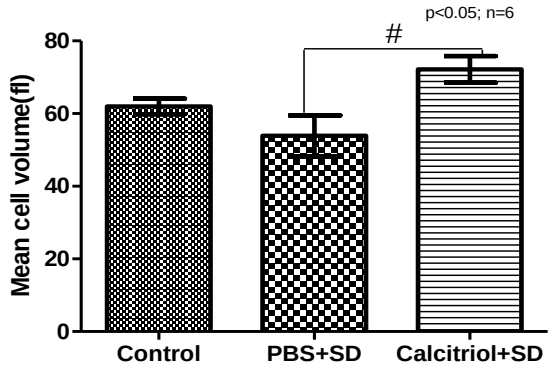

Figure 6: Bar chart showing the Mean cell volume (MCV) of Control, Sleep deprived and SD+Calcitriol supplemeted Wistar Rats. A non-significant ($p>0.05$) decline in MCV of PBS+SD was observed as compared to control and reversedly increased in Calcitriol+SD group. MCV increase in Calcitriol+SD group over PBS+SD was statistically significant ($p<0.05$). *$p<0.05$ (compared to control), $^{\#}p<0.05$ (compared within treatment group); n=6.

Effects of Sleep Restriction on Mean Corpuscular Volume (MCV) and Mean Cell Hemoglobin (MCH) Concentration

The red cell indices (Absolute Values) presented in figure 6 & 7 reflects the observation made on the blood picture. The results showed that MCV was non-significantly ($P>0.05$) decreased or increased in PBS+SD and Calcitriol+SD, respectively, when compared with the control. Comparison between the treated groups showed a significant ($p<0.05$) increase in MCV of Calcitriol-supplemented group. Mean corpuscular haemoglobin was increased in both treated groups when compared with the control; significant ($p<0.05$) values were recorded at highest for Calcitriol+SD.

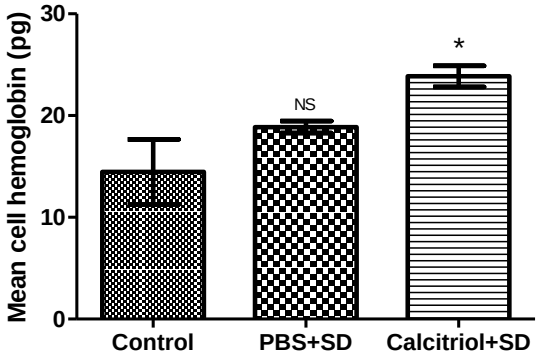

Figure 7: Illustration of Mean cell hemoglobin (MCH) of Control, Sleep deprived and SD+Calcitriol supplemeted Wistar Rats. MCH was increased in both treatment groups as compared to control. But values were only statistically significant ($p<0.05$) for Calcitriol+SD. *$p<0.05$, NS=$p>0.05$; n=6.

DISCUSSION AND CONCLUSION

Despite advances in scientific investigations over the past years, the precise link between calcium homeostasis and haemopoiesis is yet to be fully established, so also is the understanding of intertwined sleep deprivation induced flunctuations in blood parameters in associated health conditions. Therefore, the effects of 96 hours of wakefulness (paradoxical sleep restriction) on hematologic parameters of Wistar rats and the potential role of calcium modulator (Calcitriol) were investigated.

In this study, we observed that sleep deprivation increased WBC counts, but decreased RBC count, PCV, haemoglobin concentration (Hb), MCV and MCHC. Also, parameters measured from rats that received Calcitrol in addition to sleep deprivation differ statistically. According to our results, sleep deprivation supressed erythropoiesis and enhanced leukopoiesis in a manner that was altered by Calcitriol supplementation. The potential roles of Vitamin D and its derivatives in erythrocyte formation, functions and clinical implications have been critically argued and previously theorized. In a previous demonstration, association between vitamin D deficiency and increased risk of anaemia, decline in

mean hemoglobin concentration and intake of erythrocyte-stimulating agents have been established. [21]

The reduced PCV and Hb observed in this study may be due in part to stress induced vitamin D deficiency which is associated with sleep disorders. [22] This is possible because hypovitaminosis D has been reported to induce hypophosphatemia which contributes to the severity of haemolysis. [23] Increased in vitro haemolysis caused a reduction in red blood cell count as observed in this study.

A study examining the effect of calcitriol on erythropoiesis in 33 patients with chronic uremia suggested that calcitriol promoted increased erythroid colony formation. [24] However, our finding using normal Wistar rats does not support increased proliferation of red blood cell precursors following Calcitrol administration. This is because the red blood cell count in the Calcitrol+SD was lower than PBS+SD and the Control group. Therefore, it is possible that erythrocytic enhancing power of calcitriol is dose-dependent. To support this obvervation, previous *in vitro* demonstration has shown that high doses of Vitamin D inhibited angiogenesis in trangenic murine retinoblastoma. [25] Hence, a lower dose (<120ng/kg) of Calcitriol may be required to produce beneficial effect on erythropoiesis. In contrast, Calcitriol supplement increased mean corpuscular haemoglobin suggesting that it promotes functional haemoglobin in the erythrocytes. It is believed that sleep is a form of stress and the observed increase in MCH in the peripheral blood of Calcitriol+SD treated rats could be an indication of pharmacological response of the supplement to low oxygen tension-induced by sleep deprived stress.

Experimental study involving up to five days and nights of wakefulness reported increased WBCs, most notably neutrophils, in response to sleep deprivation. An increase in leukocytes has been confirmed in subsequent sleep deprivation research involving continuous wakefulness greater than forty hours. [26,27] Therefore, significant increases in neutrophils and monocytes leukocyte subset recorded in this study agree with the findings of Born et al. [26] However, the total leucocyte count did not significantly differ from values obtained from the control rats. A moderate increase was observed in the group which received Calcitriol in addition to sleep deprivation.

CONCLUSION

In conclusion, the data obtained reveals that sleep deprivation alters the cellular composition of peripheral blood, that is, it caused a decline in red blood cell count, packed cell volume, hemoglobin concentration and mean cell hemoglobin concentration in rats. Peripheral blood cell examination revealed that these effects were mild in rats administered Calcitrol.

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Disclosure Statement

All the authors have indicated no financial conflicts of interest associated with this study or any of the procedures and materials used for the purpose of the study.

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