Original Article

The effect of recurrent malaria infections on bone and cartilage at the distal femoral epiphysis of rats: A histological study

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Abstract

Objective: The aim of this study was to investigate the normal changes that occur at the distal femoral epiphysis of rats and determine whether recurrent bouts of malaria altered them in anyway.

Materials and Methods: The 1st phase of the study made use of 30 Sprague-Dawley rats aged 4 weeks. Two rats were culled each week for 15 weeks, the femoral bones of the rats were then harvested. Histological sections of the distal femora were prepared and studied. The 2nd phase involved 32 animals that were randomly assigned to four groups of 8 animals each. (Group A to D) Group A was given oral antimalarial drugs only, Group B was inoculated with *Plasmodium berghei* (NK65) only, Group C was inoculated with *P. berghei* (NK65) and treated with oral antimalarial drug, and Group D animals were neither inoculated nor given antimalarial drugs. At the end of the experiment histological sections of the femoral epiphysis of the rats were prepared and studied.

Results: The microscopic architecture of the epiphyseal cartilage changed significantly as the animals aged. Significant differences were observed in mean bone thickness of the various experimental groups. There was however no significant difference in the mean cartilage thickness when comparison was made within the various groups.

Conclusion: Although recurrent bouts of malaria infection appear not to alter the normal histological changes that occur in epiphyseal bone and cartilage layers, it most likely has an adverse effect on the rate of bone tissue deposition.

Keywords: Bone, epiphysis, malaria, ossification, stunting

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INTRODUCTION

Malaria is a disease of the tropics. In 2017 the World Health Organisation estimated that 216 million cases of malaria occurred worldwide. Majority of these cases (approximately 90%) were reported in Sub Saharan Africa.[1] Malaria is reported to affect many organs in the body. These include the brain, where it causes cerebral malaria

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with its associated neurological complications,[2] the heart, where it has been implicated in myocarditis and ischemic cardiomyopathy,[3] and the eye where it has been associated with retinal changes.[2,4] Renal injury has also been documented in malaria infection.[5] Malaria in pregnancy has been linked with intrauterine growth restriction as well as growth impairment during the 1st year of life.[6]

Many studies have been conducted to investigate the effect of malaria infection on the longitudinal growth of children in sub-Saharan Africa, almost all of these studies have been anthropometric in design.[7-12] While some studies have reported an association between malaria infection and stunting,[7,9,10,12,13] suggesting indirectly that bone formation may be affected by malaria infection, there are other studies that have found no such association between malaria infection and stunting in children.[8,11,14,15]

There appears however to be no histological studies in literature that have investigated the effect of malaria on the various tissues that are located at the epiphysis of bone. The aim of this study was therefore to investigate the effect that recurrent bout of murine malaria may have on bone and cartilage tissue that occur at the distal femoral epiphysis of rats.

MATERIALS AND METHODS

The study was conducted in 2 phases, the 1st phase investigated the changes that occurred at the distal femoral epiphysis of rats as they aged, while the 2nd phase examined the effect that malaria infection has on the bone and cartilage that are present at the epiphysis.

The 1st phase of the experiment involved 30 Sprague-Dawley rats aged 3 weeks. The animals were housed at the animal house, of the College of Agriculture and Natural Science, University of Cape Coast. They were all kept under the same laboratory conditions, fed with standard laboratory chow, and given autoclaved tap water in the morning and evening. The care and use of the animals conformed to our institutional guidelines in compliance with national and international laws and guidelines for the use of animals in biomedical research.

The rats were culled every week (two animals per week), starting from the age 4 weeks to 18 weeks. All animals were anesthetized with chloroform prior to being sacrificed. Both femurs were removed from the animals after careful dissection and the bones were preserved in 10% formalin solution and later decalcified in 8% formic acid solution over a 24 h period. The bones were transected and the distal portions were bisected in the coronal plane. Both halves were placed in a single cassette and embedded with paraffin wax. The blocks were then sectioned using a Leica RM 2125RT microtome at 4 μm thickness and placed on glass slides. Sections were stained using hematoxylin and eosin (H and E) staining.

Using AmScope MD35 640 × 480 Pixel Still and Video USB Digital Eyepiece Camera, microscopic images of the histological slides were captured and observed.

The 2nd phase of the study involved thirty two Sprague-Dawley rats aged 6 weeks. The rats were kept under conditions previously described for the 1st phase of the study.

Animals were randomly assigned to four groups (Group A–D) of 8 animals each.

- Group A: Rats in this group were given oral artemether/lumefantrine combination drug only (dosage 3.2 mg/kg/body weight) twice daily for 3 days
- Group B: Rats were inoculated with 0.2 ml of parasitized blood
- Group C: Rats were inoculated with 0.2 ml of parasitized blood and treated with oral artemether/lumefantrine (dosage 3.2 mg/kg/body weight) twice daily for 3 days
- Group D: Rats were neither inoculated with parasitized blood nor given artemether/lumefantrine therapy.

Thick film slides were prepared from tail vein blood to establish the presence of infection by calculating the parasite density, after which treatment was started. After treatment with artemether/lumefantrine, the animals were rested for a period of 5 weeks at which time the experimental protocol was repeated (2nd cycle). All animals were anesthetized with chloroform and subsequently sacrificed after the 2nd cycle of the experiment at which point they were 18-week-old. Both femurs were removed and were processed as earlier described in the 1st phase of the study.

Analysis

IBM Statistical Package for the Social Sciences (SPSS) version 21 (IBM Corp., Armonk, NY, USA). was used for data analysis. The mean thickness and standard deviation of the epiphyseal bone and cartilage for each group, as well as the mean bone to cartilage ratio for each group were calculated. Statistical significance of the difference between the group means were determined using a One-way analysis of variance (ANOVA) test. A least significant

difference *post hoc* (multiple comparison) test was then run to identify the group(s) that had statistically significant difference(s) in their mean bone to cartilage ratio at a significance of 0.05.

RESULTS

Phase One

The results from this study showed that generally epiphyseal bone layer increased with increasing age while the cartilaginous growth plate thinned out and reduced in thickness as the rats aged [Table 1]. From week 4 to week 8, the microscopic structure of the epiphyseal growth plate of the distal femur of the rats was organised into 6 distinct anatomic zones of reserve, proliferation, maturation, hypertrophy and calcification, degeneration and the osteogenic zone [Figure 1]. Starting from the 9th week, areas of reduced cellularity were noted in the first 4 zones but were most striking in the reserve zone. These areas of reduced cellularity alternated with regions of normal cellular composition [Figure 2]. The areas of reduced cellularity became more frequent with increasing age such that by week 18 the zones of reserve and proliferation appeared to be completely devoid of cells. The few chondrocytes that were present in the growth plate at this time were mainly observed in the areas previously regarded as maturation and hypertrophic zones. These cells had the semblance of hypertrophic chondrocytes and were seen either as solitary cells or grouped in small clusters [Figure 3].

A thin band of bone matrix which run parallel to, and was directly apposed to the metaphyseal surface of the cartilaginous plate was observed in older animals [Figure 3]. The presence of this metaphyseal bone matrix effectively sandwiched the cartilaginous growth plate between the epiphyseal and a newly formed metaphyseal bone plate [Figure 3].

The morphology of the epiphyseal bony layer also changed with increasing age. The epiphyseal bone plate was observed to be discontinuous in length in weeks 4 and 5 but became continuous by the 6 week. Scattered within the bone matrix were spaces filled with bone marrow cell [Figure 2]. The number of these spaces also increased with increasing age. By the 18th week the epiphyseal bone layer was observed as a continuous layer overlying the epiphyseal growth plate with spaces occupied with bone marrow cell, located at different depth within it [Figure 3].

Phase Two

All animals in Group B (rats infected with malaria only) died by post infection day 14 hence no results were obtained for Group B.

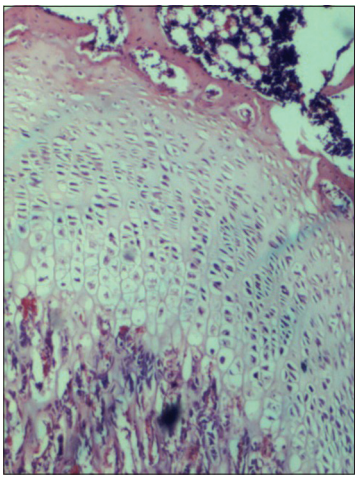

Figure 1: A light micrograph of the distal femoral epiphysis of a 5-week-old rat showing a thick cartilaginous growth plate showing the typical zones of reserve, proliferation, maturation, and hypertrophy with an overlying epiphyseal bone layer H and E Staining

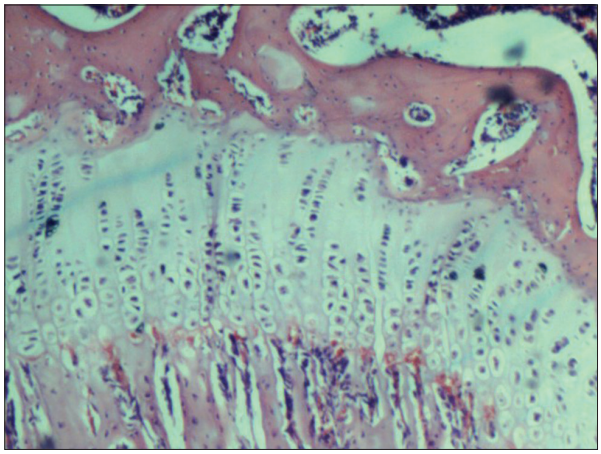

Figure 2: A light micrograph of the distal femoral epiphysis of an 11-week-old rat showing reduced cellularity with the epiphyseal cartilage and an overlying epiphyseal bone plate with bone marrow cell filled spaces scattered within it H and E Staining

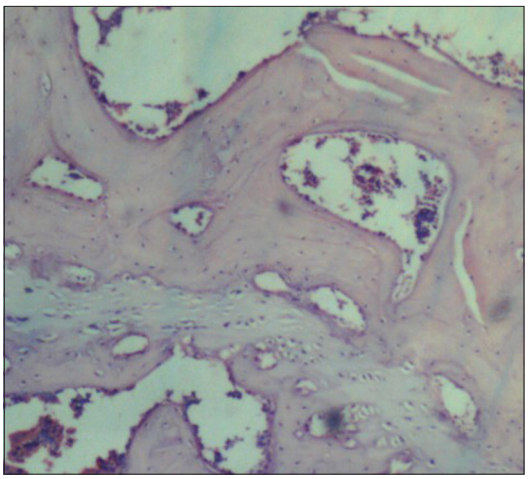

Figure 3: A light micrograph of the distal femoral epiphysis of an 18 week old rat showing a thinned out epiphyseal cartilage with very few cells sandwiched between an epiphyseal bone layer with bone marrow cell filled spaces and a metaphyseal bone layer. H and E Staining

The micrographs of epiphyseal bone and cartilage layer animals in the remaining groups (A, C and D) showed all the features previously described in phase 1 for week 18 old rats. No noticeable differences were observed when the epiphyseal growth plate of the animals in Groups A, C, and D were compared.

When the mean epiphyseal bone thickness was calculated it was found that the group with the greatest mean bone thickness was group D (control) while animals in group A were found to have the least [Table 2]. The mean cartilage thickness was also found to be lowest in group A animals and highest in group C animals [Table 2].

When the mean bone thickness and mean cartilage thickness of the epiphyseal bone matrix were analyzed using ANOVA, it was found that there was a significant difference in the mean bone thickness of the various groups ($P < 0.05$), there was however no significant difference in the mean cartilage thickness. Post Hoc analysis showed that there was statistically significant difference between the mean bone thickness of animals in group A and C as well as animals in group C and D. [Table 3].

Table 1: The Mean Bone Thickness, Cartilage Thickness and Bone: Cartilage Ratio of Rats of Different Ages

Age in weeks	Bone (μm)	Cartilage (μm)	Bone:Cartilage Ratio
4	43.38	306.50	1:7.31
5	52.65	262.11	1:5.20
6	59.17	276.66	1:4.74
7	79.05	187.53	1:2.38
8	96.66	208.80	1:2.19
9	128.51	244.33	1:1.99
10	130.22	231.66	1:1.81
11	130.83	247.81	1:1.98
12	128.99	231.93	1:1.84
13	157.56	220.89	1:1.43
14	156.35	205.46	1:1.32
15	154.72	141.93	1:0.95
16	176.08	148.72	1:0.87
17	182.75	130.45	1:0.72
18	192.38	140.73	1:0.73

Table 2: Descriptive analysis of the Bone thickness, Cartilage thickness and bone to Cartilage Ratio of animals in Experimental Groups

Group	Mean±SD		Ratio
	Bone (μm)	Cartilage (μm)	
A	121.98±26.99	81.66±24.47	1:0.69
C	104.02±32.46	90.02±25.64	1:0.94
D	131.41±38.17	88.76±30.38	1:0.72

SD: Standard deviation

Table 3: Least significant difference post hoc multiple comparison of the mean bone thickness of the different experimental group

Group	Group	Mean difference	Significant
A	C	17.96	0.000*
	D	−9.43	0.055
C	D	−27.39	0.000*

*Significant at 0.05

DISCUSSION

In our study, it was found that the histological features of the epiphyseal growth plate of older animals differed considerably from those of younger rats. From week 4 to 8, the histological features of the cartilaginous growth plate were organized into 6 distinct anatomic zones of proliferation, maturation, hypertrophy, degeneration, and osteogenic as have been previously reported.[16-19]

By the 9th week however, the epiphyseal plate showed alternating regions of reduced cellularity and normal cellularity, with the reserve zone being the area of greatest cell depletion among the six zones. The depletion of chondrocyte numbers was also found to progress with increasing age of experimental animals. These finding appears not to be limited to rats as similar findings have been reported in other mammals such as dogs and humans.[20] It has been suggested that cell depletion results from concurrent reduction in the rate of proliferation of chondrocytes as well as an increase in the rate of cell death within the cartilage.[21]

The formation of a metaphyseal bone which ran parallel to the epiphyseal growth plate and the appearance of large acellular areas have previously been reported by Roach *et al.* (2003).[22] They theorized that these changes may play a role in restricting linear growth within the epiphyseal plate of aging rats. The termination of longitudinal growth has been reported to occur between 11th and 13th week of life.[23,24] The fact that these changes were first observed at week 16 in our study makes it highly unlikely that the changes observed contribute to the cessation of linear growth in rats as was postulated. The growth plate continued to persist even after sexual maturation and the cessation of linear growth had occurred as has been reported by linear growth had occurred as has been reported by earlier studies.[22,25]

When the micrographs from the various experimental Groups (A, C, and D) were observed, there was no noticeable difference in their microscopic architecture of both cartilage and bone tissue from that described for 18-week-old rats. It thus appears that malaria infections may not alter the normal histological architecture of both bone and cartilage at the epiphyseal-metaphyseal junction. There however appears to be no literature to either support or refute this assertion and future research

using different microscopic techniques may be needed to further confirm this observation. Though the morphology of the epiphyseal bone plate appeared not to be altered by malaria infection, there was a significant difference in the mean epiphyseal bone thickness when comparison was made within the various groups ($P<0.05$). The disparity was observed when the mean bone thickness of animals in group C (infected with malaria parasite and treated with the antimalarial) were compared with those of group A and D. In both instances the mean bone thickness for group C was found to be significantly reduced ($P<0.05$) indicating that bone tissue formation had reduced in animals in group C.

It is worth noting that when the mean bone thickness of group A (given the anti-malarial only) was compared to that of the control group (D) is was found that there was no significant difference between them, thus point to the fact the anti-malaria drug, artemether/lumefantrine does not significantly reduce bone tissue formation.

The above observation therefore suggests that the reduction in formation of bone tissue that was observed in group C animals (infected with malaria and treated with anti-malarial) was most likely as a result of the presence of the malaria infection and not the antimalarial drug. This finding provides histological evidence that malaria infections most likely influences bone formation and deposition adversely.

Although this study measured epiphyseal bone thickness, it would not be misplaced to surmise that longitudinal bone growth may possibly be retarded by recurrent bouts of malaria, since the events that result in the formation of bone tissue at the epiphysis are analogous to those that bring about bone tissue formation at the diaphysis.[26] Almost all of the research that have been conducted to investigate the association between malaria and stunted growth have been anthropometric studies.[7-13] This therefore appears to be the first study that has provided some histological evidence that malaria infection may possibly retard bone deposition and consequently result in stunted growth.

It is also important to note that the mean bone thickness was reduced in the animals even though they have been treated with antimalarial and the malaria parasites had been subsequently eliminated. This suggests that the eradication of the *Plasmodium* parasite from the blood stream does not appear to prevent the delay in the rate of ossification. Thus, pointing to the fact that the phenomenon or mechanism that brings about the delay in bone deposition may not necessarily depend on the *Plasmodium* parasite being physically present in the blood stream, but perhaps on some factors and substances that may be released into the blood stream during the infection, and whose effect on bone tissue formation and deposition lingers on long after the parasite has been eradicated. During malaria infection, inflammatory cytokines are released as part of the body's natural response. Some of these cytokines such as tumor necrosis factor-alpha and interleukin-1 have already been found in other studies not related to malaria, to influence bone and cartilage forming processes within the body.[27] Based on all these findings, it would not be out of place to implicate inflammatory cytokines and other substance that are released as part of the body's response to the presence of the plasmodium parasite as possible causal agents of this delay in growth plate development. The mean cartilage thickness of the various groups did not differ significantly from each other, suggesting that malaria infections do not influence the activities of cells of cartilage tissue as much as those of bone tissue.

CONCLUSION

The findings from this study show that although recurrent bouts of malaria infection appear not to alter the normal histological changes that occur in epiphyseal bone and cartilage layers, it most likely has an adverse effect on the rate of bone tissue deposition. This gives histological evidence to support the finding of many anthropometric studies which have indirectly linked malaria infections with linear bone growth retardation and the development of stunted growth in children who live in malaria endemic areas.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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