International Scholars Journals

African Journal of Malaria and Tropical Diseases ISSN 4123-0981 Vol. 3 (8), pp. 204-208, August, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research paper

A study of the relationship between micronutrient status and malaria infection among children in Douala town, Cameroon

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Accepted 9 July, 2015

Malaria is an endemic parasitic disease that prevails particularly in warm tropical regions of the world. Micronutrient malnutrition such as vitamin A and iron deficiencies which is a public health problem in Cameroon is usually highly prevalent in malaria endemic areas. Characterizing the relationship between micronutrient status (vitamin A, zinc and calcium) and malaria infection among children in Douala town (Cameroon), serum levels of zinc, calcium and vitamin A, were assayed in a total of 116 Cameroonian children (62 controls and 54 malaria patients infected by *Plasmodium falciparum*) less than six years old by colorimetric and high pressure liquid chromatography (HPLC) techniques respectively showed a significantly lower vitamin A and calcium concentrations (P < 0.01) among malaria patients (0.8 ± 0.4 µmol/l and 81.3 ± 23.7 mg/l) as compared to the controls (1.1 ± 0.6 µmol/l and 96.3 ± 16.7 mg/ml). Vitamin A, calcium and zinc status were lower in 51.85%, 51.85% and 27.27% of malaria patients respectively. Significant correlations (P<0.01) were observed when the following parameters were coupled: Vitamin A/zinc among infected children (r = 0.01), and vitamin A and zinc among uninfected children (r = 0.415). This study suggests that P. *falciparum* use vitamin A and calcium of its host for its proper metabolism, leading to a decrease in serum levels of these nutrients.

Key words: Malaria, vitamin A, zinc, calcium, parasitaemia.

INTRODUCTION

Malaria is a prevalent disease in tropical and subtropical areas affecting about 300 - 500 million people a year (Hoffman et al., 2002). It is estimated that one to three million deaths occurs worldwide, mostly involving children under the age of five. In tropical Africa, close to 90% of morbidity and mortality is attributed to malaria. This disease is often link to changes in climate, poverty, malnutrition and the double resistance of the malaria parasite to usual anti-malaria drugs and insecticides (Müller and Garenne, 1999). Infection by malaria can cause to children, serious health problems and this often leads to death (Merger et al., 1979). The clinical state can be increased due to poor nutritional status more especially due to micronutrients deficiency.

Micronutrients deficiency such as vitamin A, zinc, iron, and calcium are more frequent amongst children in developing countries (Gibson and Ferguson, 1998). These deficiencies are often associated to increased susceptibility to infections such as malaria (Shankar and Prasad, 1998). Vitamin A is an essential micronutrient for vision, cell growth, normal immune function, haematopoietic system and reproduction. Vitamin A deficiency (VAD) as a public health problem is caused by a dietary pattern providing too little bioavailable vitamin A to support physiological needs under the prevailing circumstances. Physiolo-

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gical needs vary with growth rates and may be conditioned by environmental factors such as malaria infections.

Zinc is a micronutrient that intervenes in growth, cell differentiation and immune function. All of which are involved in resistance to malaria. It is also a cofactor for several enzymes notably those that regulate storage and metabolism of vitamin A (Macdonald, 2000). Its deficiency hampers the functioning of the immune system by lowering lymphocytes T response and the production of cytokines. This can eventually contribute to the increase risk of infection (Steketee, 2003). Calcium is an essential nutrient required during the earlier stage of life for strengthening of bone and teeth. It plays a vital role in the maintenance of health and nutritional wellbeing at all stages in life. In addition, calcium plays parallel roles in impulse transmission, catalytic activation of proteins, blood coagulation, optimal functioning of the neuromuscular and in the intracellular systems where it intervenes as secondary messenger (Nordin, 1997). It has been observed in the entire world that the deficiency of calcium from food is responsible for serious diseases such as osteoporosis, cardio-vascular diseases, diabetes, obesity and colon cancer (Miller, 1997). This has motivated research on calcium, with much emphasis on its complex interaction with the different physiological state of man. This occurs in association with infectious diseases like malaria.

This study has as objective to characterize the relationship between micronutrient status (vitamin A, zinc and calcium) and malaria infection among children in Douala town (Cameroon).

METHOD

Collection and preparation of sera

After informed consent of the parents of children that came for consultation (for malaria) or vaccination (control) in the catholic medical centre of SIC Douala (From December 2004 – March 2005), volunteers were received in the blood collection room. After some preliminary routine questioning on the exact health and nutrition status, parameters such as age, sex, weight and height were collected. The blood was then extracted by venous puncture and collected into sterile tubes. The serum was obtained by centrifugation at 3000 rev/min for 5 min using a clinical centrifuge "Heraus labofuge" and aliquots stored in three portions. The first two were used the same day for the quantification of zinc and calcium. The third was stored frozen at – 20^{o}C to be used later for analysis of vitamin A by HPLC.

Analysis of vitamin A

This was done by a method initially described by Bieri et al. (1979). Briefly, 200 l of serum was introduced into a tube and 500 l of retinyl acetate in ethanol added as internal standard, and homoge-nised using a vortex, then 1 ml of hexane added, homogenised using a vortex and centrifuged at 2000 rev/min for 5 min at –5°C. The supernatant was removed using a pipette (single use) and introduced into a second tube, and the residue was extracted again using 1 ml hexane. All the hexane extracts obtained were pooled together and evaporated under nitrogen. The residue obtained was collected into 200 l of methanol, after passing through a sonicator, 60 l were injected into the HPLC system comprising: A column: supelco 58298 (supelcosil LC-18) 250 x 4.6 mm diameters, 5 µm particle size, a pre column, a pomp: Alltech 426, an integrator HP 3395, and a lamp (Diode Array detector) Linear UVIS 200. The elution was done at a mobile phase made of methanol: acetonitrile: water; (93:5:2; v:v:v) at a speed of 2 ml per min, average pressure: 1650, wave length of detection: 325 nm, range: 0.01 AUFS; rise time: 0.3 s. All analysis was done using yellow light to prevent the destruction of vitamin A.

Addition of the exact quantity and the constant of the internal standard to a known volume of the sample were used in the calculation of the vitamin A in serum, taking into consideration that the ratio of the heights of the peaks was proportional to the ratio of concentrations:

$$\frac{h_{RO}}{h_{RAC}} = \frac{C_{RO}}{C_{RAC}}$$

Where h_{RO}: height of peak of serum retinol, h_{RAC}: height of peak of retinyl acetate, C_{RAC}: concentration of retinyl acetate, C_{RO}: concentration of serum retinol. C_{RAC} is calculated from the coefficient of extinction $E^{1\%}_{1cm}$:

$$E^{1\%}_{1cm} = \frac{A_{328}}{C_{RAC}} \times b = 1795 \; ; (A_{328} = \text{Absorbance of retinyl acetate at 328 nm})$$

b = length of UV reading tube in cm, and $E^{1\%}_{1cm}$ = coefficient of extinction of retinyl acetate.

Diagnosis of malaria

This was done successively by thick smear and thin smear respectively according to these steps; spreading of blood on the blade, drying, coloration with Giemsa and May- Grünwall, washing, reading and using a microscope. Quantification of the parasites in the blood was done by relating the number of parasites in the blood to that of the white blood cells or red blood cells and adjusting the value to millimetre cube of blood (Moretti and Mandoul, 1977)

Analysis of serum calcium and zinc

Determination of serum zinc and calcium was done by colorimetric method using 5- Br-PAPS and methyl-thymol blue (Biosystem), respectively. The optical densities of the complexes formed were read using a spectrophotometer at 560 and 570 nm, respectively. The concentrations were calculated using the formulae:

$$\textit{Sample} \text{ conc.} = \frac{\textit{Absorbance of} \text{ sample}}{\textit{Absorbance} \text{ of standard}} \times \text{Standard conc.}$$

The protocol for the study was approved by the clinical ethics committee of the University of Douala. The statistical analysis was done using Statistica version 4.5, the normality of the results verified using Kolmogorov–smirnov test, comparisons between the non parametric variables done by using Non parametric test (whitney). The relationships between the variables were evaluated using Spearman correlation coefficient. Multiple comparisons of the different age groups were done by Analysis of variance. Compari - sons of the proportions were done by Fischers test using the statis-

Table 1. Range and mean values of results obtained for the sample population based on the clinical state of the subjects.

	1. Control			2. Malaria cases			t	P
	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD		
A. Female								
Age (years)	0.41	5.00	2.3 ± 1.4	0.66	5.00	2.3 ± 1.5	1.322	0.193
$[Ca^{2+}]$ (mg/l)	69.30	127.40	92.9 ± 14.7	8.60	115.00	78.6 ± 23.0	1.451	0.154
$[Zn^{2+}]$ (µmol/l)	1.00	36.64	15.2 ± 9.2	2.01	32.01	13.6 ± 8.7	0.543	0.590
Vitamin A (µmol/l)	0.37	2.15	1.2 ± 0.6	0.21	1.80	0.8 ± 0.4	2.123	0.040
Parasitemia (TPF/mm^3)	-	-	-	100	145000	12372.9±36798.1	-	-
B. male								
Age (years)	0.41	4.00	2.1 ± 1.3	0.66	5.00	2.3 ± 1.5	0.573	0.569
$[Ca^{2+}]$ (mg/l)	71.9	147. 7	98.9 ± 17.9	8.60	115.00	78.6 ± 23.0	3.582	<0.001
$[Zn^{2+}]$ (µmol/l)	2.00	24.42	13.9 ± 5.9	2.01	32.01	13.6 ± 8.7	0.138	0.891
Vitamin A (µmol/l)	0.24	3.20	1.0 ± 0.6	0.21	1.80	0.8 ± 0.4	1.404	0.166
Parasitemia (TPF/mm^3)	-	-	-	50.00	120000	9175.2 ± 26751.1	-	-
C. Global								
Age (years)	0.41	5.00	2.2 ± 1.3	0.33	5.00	2.6 ± 1.6	1.388	0.168
$[Ca^{2+}]$ (mg/l)	69.30	147.70	96.3 ± 16.7	8.60	132.20	81.3 ± 23.7	3.631	<0.001
$[Zn^{2+}]$ (µmol/l)	1.00	36.64	14.5 ± 7.5	2.00	32.01	13.7 ± 8.4	0.485	0.629
Vitamin A (µmol/l)	0.24	3.21	1.1 ± 0.6	0.21	2.06	0.8 ± 0.4	2.461	0.016
Parasitemia (TPF/mm^3)	-	-	-	50.00	145000.0	10701.4 ± 31599.9	-	-

Comparisons A versus B

Case of controls		Malaria cases	
Age (years)	t = 0.588 ; P = 0.559	Age (years)	t = 1.241 ; P = 0.222
$[Ca^{2+}]$ (mg/l)	t = 1.304 ; P = 0.198	$[Ca^{2+}]$ (mg/l)	t = 0.763 ; P = 0.450
$[Zn^{2+}]$ (µmol/l)	t = 0.651 ; P = 0.518	$[Zn^{2+}]$ (µmol/l)	t = 0.078 ; P = 0.938
Vitamin A (µmol/l)	t = 0.717 ; P = 0.476	Vitamin A (µmol/l)	t = 0.032 ; P = 0.975
		Parasitemia (TPF/mm^3)	t = 0.332 ; P = 0.742

TPF: Trophozoïtes of P. *falciparum*

tical program Stat Xact 3.

RESULTS

The results of the clinical state of the sample population are presented in Table 1. The difference in age between the control (2.2 ± 1.3 years) and malaria patients (2.6 ± 1.6 years) were not significant, but on the average blood calcium levels for malaria patients (81.3 ± 23.7 mg/ml) were significantly lower (P<0.001) than that of the controls (96.3 ± 16.7 mg/ml). This same phenomenon was observed for the cases of vitamin A (P = 0.016) with 1.1 ± 0.6 µmol/l and 0.8 ± 0.4 µmol/l for the controls and malaria cases, respectively.

Analysis of results based on sex reveals two major points: the female malaria patients have a mean serum vitamin A levels (0.8 ± 0.4 µmol/l) which were lower (P = 0,040) than those of the control cases (1.2 ± 0.6 µmol/l) of the same sex, the male malaria patients instead had a mean calcermia level (78.6 ± 23.0 mg/l) which were lower (P<0.001) than the controls. From results presented in Table 2, malaria patients that were deficient in calcium (51.85%) were significantly (P = 0.0064) higher than those of the control (20.96%). A similar situation was observed for the retinol (P = 0.0388) where 51.85 and 30.64% were observed for malaria patients and control, respectively. Distribution of subjects based on age group reveals a similarity between the two age groups: below 1 year and between 1 and 5 years.

Analysis of population based on the parasite in the blood (Table 3) reveals that increase in parasite level significantly reduce calcemia (P<0.001). This is even more with the cases of low parasitemia (1) and moderate parasitemia (2) on one hand (P = 0.003), and low parasitemia (1) and high parasitemia (3) on the other hand (P = 0.006). This phenomenon was also observed with vitamin A (P = 0.029), and was more important between (1) and (2), (P = 0.022). Two correlations were observed between vitamin A and zinc on one hand for the controls (r = 0.415; P<0.01) and on the other hand for the malaria cases (r = 0.501; P<0.001).

DISCUSSION

Results show that the proportions of malaria patients deficient in calcium (51.85%) or vitamin A (51.85 %) were

Table 2. Frequency distribution of parameters analysed.

Parameters	Subjects	Controls (n = 62)		Malaria cases (n = 54)		Fishers Test
		N	%	N	%	
Age	Less than 1 year	13	20.96	11	20.37	P = 1.0000
	between 1 and 5 years	49	79.03	43	79.62	
$[Ca^{2+}]$	Deficient (less than 81 mg/ml)	13	20.96	28	51.85	P = 0.0064
	Normal (between 81 and 104 mg/ml)	27	43.54	16	29.62	
	High (more than104 mg/ml)	22	35.48	10	18.51	
$[Zn^{2+}]$	Deficient (less than 7.6 µmol/l)	10	16.12	15	27.27	P = 0.3525
	Normal (between 7.6 and 15.3 µmol/l)	21	33.87	15	27.27	
	High (more than15.6 µmol/l)	31	50.00	24	44.44	
Vitamin A	Deficient (less than 0.70 µmol/l)	19	30.64	28	51.85	P = 0.0388
	Normal (more than or equal to 0.70 µmol/l)	43	69.35	26	48.14	

Table 3. Variation of parameters studied based on the parasitemia groups.

Parameters	Groups of parasitemia			ANOVA Test
	1. low (n = 32)	2. Moderate (n = 16)	3. High (n = 6)	
Age (years)	2.3 ± 1.5	2.9 ± 1.7	3.3 ± 1.9	F = 1.131 ; P = 0.332
$[Ca^{2+}]$ (mg/l)	91.6 ± 18.3	69.2 ± 20.9	58.9 ± 29.0	F = 8.588; P < 0.001
$[Zn^{2+}]$ (µmol/l)	16.1 ± 7.8	10.1 ± 8.6	10.6 ± 7.6	F = 2.816 ; P = 0.071
Vitamin A (µmol/l)	1.0 ± 0.5	0.6 ± 0.4	0.6 ± 0.1	F = 3.871 ; P = 0.029

Low parasitemia: 70 – 999 TPF/mm^3; Moderate parasitemia: 1000 – 10 000 TPF/mm^3;

High parasitemia: higher than 10 000 TPF/mm^3.

Analytic Test:

Case of Ca^{2} (Student-Newman-Keuls procedure)

1 *versus* 2: P = 0.003 2 *versus* 3: P = 0.344

1 *versus* 3: P = 0.006

Case of Vitamin A (Least Square Difference procedure)

1 *versus* 2: P = 0.022 2 *versus* 3: P = 0.778

1 *versus* 3: P = 0.057

significantly higher than that of their corresponding control group. This translates the negative effect of malaria on the levels of these two micronutrients. In fact, micronutrient deficiencies have been associated with increased morbidity and mortality from malaria, and malaria, in turn may contribute to poor nutritional status, reflecting the classic, vicious cycle of malnutrition and infection (Scrimshaw et al., 1968). This is justified by the fact that in the course of infection, nutrients move from circulation to the tissues causing a reduction from circulation (Keusch, 1998).

Reduction in calcium observed in malaria cases is caused by the clinical manifestation of malaria: fever, increase in pulse rate, sweat, shivering (Golvan, 1983) which affects neuromuscular excitability, nerve conduction and muscular contraction. These physiological phenomena require calcium for their functioning (Nestec, 1989). Thus half of the cases of tetany, neonatal convulsion, and low birth weight are due to hypocalcemia (Pettifor, 1994). Gazarini et al. (2003) found that trophozoites concentrate calcium in their internal compartment for metabolism. Losses in calcium can also be caused by losses during digestive and renal problems following malaria. Macpherson et al. (1985) showed that in malaria cases, erythrocytes parasites cytoadherents in the glomerular capillaries could cause renal insufficiency. This situation can cause an increase in urinary excretion of minerals such as calcium. Kramer and Ginsburg (1991) showed that changes in the permeability of the cells infected by the mature parasite, increases the intracellular concentration of calcium. The entry of calcium in the infected cells caused by changes in the form and fluidity of the erythrocytes membrane infected by P. *falciparum* could also cause the reduction of calcium in circulation (Kon et al., 1993).

The low level of vitamin A observed in malaria patients is probably due to the fact that vitamin A is an anti-infective vitamin, which plays an important role in immunity to infectious diseases. Thus, during malaria infection, vitamin A may enhance both antibody-mediated immunity

and cellmediated immunity. Thurnham and Singkamani (1991) hypothesized that decreases in serum vitamin A concentrations during infection, are indicative of a rapid redistribution of vitamin A into extravascular fluids, where they can more efficiently maintain tissues being exposed to reactive oxygen species resulting from the infection. In addition, the synthesis of acute phase reactants may increase the need for retinol uptake, since it may help incorporate mannose into glycoprotein during synthesis (Wolf, 1977). Fluctuations in vitamin A metabolism during acute infection was shown by Filteau (1999) . Similar obs-ervations were found in malaria infants that were malno-urished in Ghana (Filteau et al., 1993). Malaria thus have a variable effect on serum retinol probably due to the fact that metabolic stress is more due to the parasite-imm-unity relationship than to the density of the parasite alone (Adelekan et al., 1997). No difference on the means of the blood zinc levels were observed ($p = 0.90$) between the malaria and the control cases. These results are in conformity with those of Brown et al. (1993), where infection did not affected the serum zinc levels. But, according to Shankar (2000), during the acute phase response, zinc is redistributed from plasma to lymphocytes and to the liver, causing decreased zinc plasma concentrations and a microbio-static environment (Beisel et al., 1995). Serum zinc concentrations vary inversely with malaria parasitemia and may preferentially protect against more severe malaria with high levels of parasitemia. Deficiency of zinc will there-fore be beneficial for malaria cases as reduction of zinc in circulation reduces the zinc available for metabolism of microrganisms during infection creating the same advantage as in iron (Isaksen and Fagerhol, 2001). Significant correlation between zinc and vitamin A is explained by the fact that zinc intervenes in the syntheses and liberation of plasma retinol binding protein. It is also an important factor in the regulation of hepatic reserves of vitamin A (Sundaresan et al., 1977). The results has shown that the levels of serum calcium in children of Douala are generally lower than normal. About 35% of the subjects were deficient in calcium as against 21.55% deficient in zinc. This is generally due to hypocalcemia, and hypozincemia in a portion of the pop-ulation. The staple food for children in this region is made principally of soya beans and cereals. Phytate in soya beans and cereal have a negative effect on the absor-ption of calcium and zinc (Lönnerdal et al., 1984) . These results suggest that calcemia is more affected by malaria than zincemia, the later had just a slight variation. Malaria affects both zincemia and calcemia independently of sex. This is more evident as mosquito bites individuals' indep-endent of their sex. This study was done in the case of simple malaria (parasitemia < 200 000 TPF / mm^3) and has revealed that other factors besides the increase in the blood parasite causes the reduction of calcium. Malaria has a negative effect on the serum vitamin A and calcium levels in children, a situation which is not the same with zinc. It is therefore recommended to study the effect of repeated malaria infection on children. This work provides the evidence that a good nutritional status can render indivi-dual more resistant to malaria symptoms.

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