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Original Article

Microscopic detection of hemozoin in peripheral leukocytes fails to indicate plasmodial placental infection in pregnant women

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Abstract

Introduction: Malaria in pregnancy very often includes gestational (parasites in maternal peripheral blood) and placental (parasites in placental blood) infection, but the later condition can only be detected after delivery. High frequency of placental plasmodial infection has been confirmed in many countries and is associated with negative birth outcomes. With the hypothesis that placental infection is accompanied by hemozoin circulation in maternal peripheral blood, an exploratory study was conducted to evaluate the association between peripheral leukocytes with hemozoin and placental infection by *Plasmodium vivax* or *Plasmodium falciparum* in parturient women.

Methodology: A descriptive, transversal and exploratory (pilot type) study was carried out with women from two malaria-endemic localities of northwest Colombia. A total of 25 parturient women with confirmed placental infection and 25 without placental infection were included. Two independent readers measured the number of leukocytes with hemozoin in thick smears of maternal peripheral blood. Plasmodial infection in maternal peripheral blood and placental blood was detected by thick smear and quantitative polymerase chain reaction (qPCR).

Results: Four parturient women had leukocytes with hemozoin in peripheral blood; three of them had placental plasmodial infection and one was negative for placental infection. No statistically significant association between leukocytes with hemozoin in peripheral blood and placental infection was observed.

Conclusions: With this limited sample size, detection of leukocytes with hemozoin by thick smear of maternal peripheral blood did not indicate presence of placental infection.

Key words: pregnancy; placenta; malaria; hemozoin; leukocytes; Colombia

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Introduction

Gestational malaria is defined as the presence of asexual erythrocytic plasmodial parasites in peripheral blood of pregnant women, associated with malaria-related symptoms and signs. Often, gestational malaria is accompanied by placental infection (parasites and/or hemozoin in placenta), which may represent an additional threat to the mother and fetus [1,2]. Gestational malaria is commonly observed in the antenatal in the antenatal care facilities in endemic areas. A systematic review and meta-analysis of malaria prevalence confirmed the presence of peripheral and placental malaria parasites in one-third of more than 300,000 pregnant women in sub-Saharan Africa [3]; however, the prevalence of gestational and placental malaria is strongly influenced by the region, endemicity level, predominant plasmodial species, and the sensitivity of the diagnostic test [4-6].

In Colombia, the northwest region of Urabá-Altos Sinú/San Jorge-Bajo Cauca contributes over 60% of malaria cases in the country [7]. The burden of malaria in pregnant women is likely understimated since antenatal services are rarely accessed and around 60% of women attend fewer than four visits during pregnancy [8]. This highlights the need to optimize the contact between the pregnant women and the health system to evaluate and deal with diverse conditions, including presence of infectious diseases. In line with this, thick smear examination of peripheral blood is recommended at each antenatal checkup in endemic areas [9]. There are no official reports on the frequency of malaria during pregnancy in Colombia; some research studies carried out in the region Urabá-Altos Sinú/San Jorge-Bajo Cauca reported a frequency of gestational and placental infection detected by thick smear of 10% and 11%, respectively [8], while studies using a very sensitive molecular technique (quantitative polymerase chain reaction [qPCR]) confirmed a frequency of gestational and placental infection of 49% and 57%, respectively [10]. Therefore, submicroscopic plasmodial infection during pregnancy is very common in this region, and even submicroscopic infections were

associated with inflammation [1,10]. In addition, many cases of plasmodial infection during pregnancy were detected exclusively in the placenta [1,10]; in consequence, the thick smear examination of maternal peripheral blood does not necessarily correlate with placental infection.

Other diagnostic alternatives, such as immunochromatographic tests (rapid diagnostic test), have been used for detection of gestational and placental infection mainly in high-transmission areas such as sub-Saharan Africa where *Plasmodium falciparum* is the predominant species, but those tests have important limitations with low parasitemias [11-13] and cases of *Plasmodium vivax*. In addition, some rapid diagnostic tests have poor sensitivity for *P. vivax*, which is the predominant species in Colombia [14].

Plasmodium spp. degrade hemoglobin and convert the resulting free heme into hemozoin, an insoluble crystal that is highly reactive. Hemozoin can be phagocyted by neutrophils and monocytes and can be observed inside leukocytes or deposited in tissues for several months after the blood infection clears [15,16]. Hemozoin has been well characterized as a potential as predictor of severe malaria [17-24], and automated equipment, such as Cell-Dyn, has been used with 95% efficacy and 88% sensitivity to diagnose malaria infection [17,25-28]. In placental samples, polarized microscopy allowed detection of low placental parasitemia [29], and laser desorption mass spectrometry has been used as a tool for diagnosis of placental infection [30]. All these high technological approaches are impractical for field diagnosis of placental infection, where a reliable, simple, and low-cost test is required, with low risk to the mother and fetus. Hence, a non-invasive evaluation of peripheral blood of the mother with the ability to predict with relatively high accuracy the presence of placental infection is highly desirable. Thick blood smear is the diagnostic test available in most malaria-endemic areas and it is usually applied during routine antenatal checkups [9]. This study evaluated whether the presence of leukocytes with hemozoin in thick smears of maternal peripheral blood is an indicator of placental infection by either *P. falciparum* or *P. vivax*.

Methodology

Study site and population

Parturient women were recruited at the obstetric facility of the official hospitals in the municipalities of Puerto Libertador (basic level hospital) and Monteria (referral hospital), in the department of Córdoba (northwest Colombia). All women resided in the malaria transmission region termed Urabá-Altos Sinú/San Jorge-Bajo Cauca, which accounts for 60% of all malaria cases in Colombia. *P. vivax* is reported in approximately 60%–70% of cases and *P. falciparum* in 30%–40%. Epidemiologic characteristics of this region are described elsewhere [7,31,32]; briefly, the transmission intensity is low and stable, with no marked fluctuations in the number of malaria cases during the year. The entomological inoculation rate ranges from 3.5 to 4.8 infective bites per person per year [33], and the mean annual parasitic index (API: malaria cases/1,000 inhabitants) during 2000–2009 in Puerto Libertador was 23.4.

Study and sample design

A retrospective cross-sectional study was carried out to evaluate whether the presence of leukocytes with hemozoin in thick smears of maternal peripheral blood is associated with placental plasmodial infection, as a pilot study. A total of 50 parturient women were included in this study: 25 positive for placental plasmodial infection detected by qPCR or thick smear of placental blood, and 25 negative for placental plasmodial infection. This pilot study was part of a larger project exploring the epidemiology, clinical characteristics, and immunopathology of pregnancy-associated malaria in the region Urabá-Altos Sinú/San Jorge-Bajo Cauca of Colombia during 2008–2014, in which nearly of 500 pregnant women during labor were recruited.

The sample size for this pilot study was calculated based on the following parameters: $n = NZ^2\ p\ (1\text{-}p) / [(Ne^2) + (Z^2\ p\ (1\text{-}p))]$ [34]; where N is the population (N = 500); p is prevalence of hemozoin in leukocytes reported previously in same region [24] (p = 15%); Z corresponds to a confidence interval of 95% (Z = 1.96); and e is a sampling error estimated at 10% (e = 0.1). This resulted in a sample size (n) of 45, which was increased to 50 (25 with placental plasmodial infection and 25 without placental plasmodial infection). From the total 500 women, those who met the following inclusion criteria were selected: 1) availability of paired thick blood smears of maternal peripheral blood and placental blood; 2) good quality of thick smears (adequate staining, absence of precipitates and good contrast); 3) availability of complete clinical and epidemiological records.

Methods for collection of maternal peripheral blood and placental blood are described elsewhere [1,4,10]. The plasmodial infection in maternal and placental blood was diagnosed using thick blood smear and qPCR, as described previously [1,4,10]. Briefly, the

Field-stained thick films were read by an experienced microscopist, and were defined as negative if 200 fields (1, 000 × magnification) were free of parasites. DNA was extracted from blood spots in filter paper Whatman #3 using the Saponin-Chelex method and tested for *Plasmodium* using genus-specific primers and a hydrolysis probe. Samples with a cycle threshold (Ct) < 45 were tested in duplex species-specific reactions for *P. falciparum* and *P. vivax.*

Identification of leukocytes with hemozoin

Two independent trained microscopists read, under oil immersion objective, the thick smears of maternal peripheral blood and placental blood, looking for intracellular hemozoin in mononuclear and polymorphonuclear cells. The thick smears were stained with Field's, and intracellular hemozoin was recognized as brown aggregates in leukocytes (Figure 1). The thick smears were read in a zig-zag motion and once a leukocyte with hemozoin was detected, a count of 200 leukocytes was performed to determine the proportion of mononuclear and polymorphonuclear cells with hemozoin. Extracellular hemozoin was not recorded. A thick smear was considered negative for hemozoin after evaluation of all fields. Since the two readers had an optimal concordance (Kappa 0.918; $p < 0.001$), the two readings were considered as one.

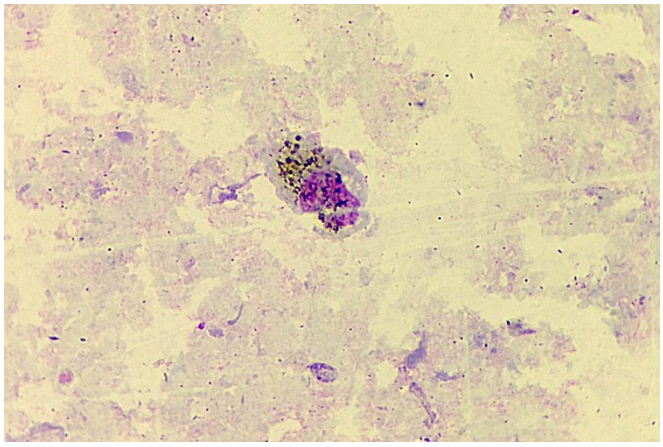
Data analysis

Data were analyzed using Epi Info 6.04 (CDC, Atlanta, USA) and SPSS 10.0 (IBM, Armonk, USA). Concordance between readers of leukocytes with hemozoin was evaluated with the Kappa index. Kruskal-Wallis and Chi-squared (χ^2) tests were used for comparison of continuous and categorical variables on independent groups, respectively. Significance was set at $p < 0.05$.

Ethical aspects

The project received ethical clearance from the ethics committee of Instituto de Investigaciones Medicas, Universidad de Antioquia (Act 889), and anonymity was guaranteed throughout the study. Every pregnant woman signed a voluntary consent form.

Figure 1. Hemozoin in a thick smear from peripheral blood was recognized as a brown pigment in the cytoplasm of a leukocyte (400 ×).



Results

The demographic characteristics were similar in all the studied women, regardless of the presence of placental plasmodial infection, except the history of malaria during pregnancy (Table 1). Leukocytes with hemozoin were detected in 4 of the 50 thick smears of maternal peripheral blood: 3 corresponded to the group of women with placental infection and 1 corresponded to the group negative for placental plasmodial infection (Table 2).

No statistically significant association was identified between the presence of leukocytes with hemozoin in peripheral blood and the presence of placental infection ($p = 0.297$) (Table 2). In contrast, there was a statistically significant association between placental plasmodial infection and plasmodial infection in maternal peripheral blood detected by thick smear (detection of erythrocytic parasites) ($p = 0.022$) as well as by qPCR (detection of parasite DNA) ($p < 0.001$) (Table 2). Of 25 women with placental infection, 17 (68%) had simultaneous infection in peripheral blood according to qPCR, while 7 (28%) had infection

Table 1. Demographic characteristics of the study women based on placental malaria.

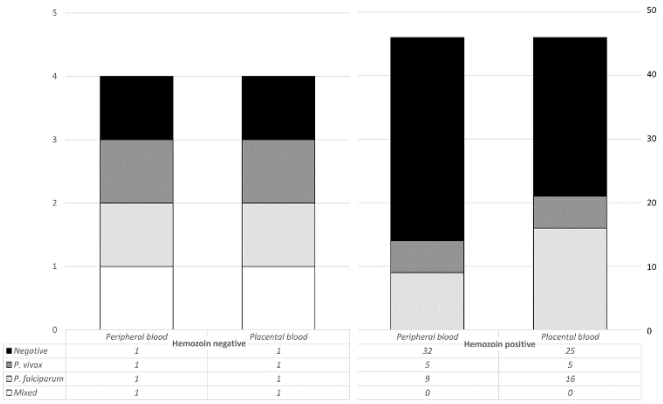
Variable	Placental infection		p*
	Positive (n = 25)	Negative (n = 25)	
Age [years] (mean ± SD)	23.0 ± 5.3	23.3 ± 5.9	0.848
Parity (mean ± SD)	2.0 ± 1.9	1.6 ± 2.0	0.386
Gestational age [weeks] (mean ± SD)	38.3 ± 3.2	37.5 ± 2.6	0.118
Hemoglobin at delivery [g/dL] (mean ± SD)	10.8 ± 1.1	11.3 ± 2.2	0.347
Birth weight of the newborn [g] (mean ± SD)	2823.8 ± 315.0	2969.2 ± 417.7	0.386
History of malaria during pregnancy (%[n])	76% (19)	32% (8)	**0.001**

* Continuous and categorical variables were compared with Kruskal-Wallis and Chi-squared (χ^2) tests, respectively

exclusively in placenta and 1 (4%) had infection exclusively in peripheral blood.

P. falciparum was detected in 64% of cases with placental infection diagnosed by qPCR (16/25), *P. vivax* in 24% (6/25), and both species (mixed infection) were detected in 12% (3/25) of cases. The distribution of plasmodial species detected by qPCR in peripheral and placental blood, according to the presence or absence of leukocytes with hemozoin in peripheral blood, is shown in Figure 2. Among those negative for leukocytes with hemozoin, 10 and 5 had peripheral infection by *P. falciparum* and *P. vivax*, respectively, and 16 and 5 had placental infection by *P. falciparum* and *P. vivax*, respectively (Figure 2). In addition, among the 4 cases positive for leukocytes with hemozoin in peripheral blood, 3 had simultaneous infection in placenta and peripheral blood according to qPCR (1 *P. vivax* and 2 mixed infection) and 1 case was negative for plasmodial infection in both compartments at delivery, but that woman had had a previous malaria episode during pregnancy.

Figure 2. Plasmodial species distribution detected by qPCR in peripheral blood and placenta, based on the status of leukocytes with hemozoin in peripheral blood detected by thick smear.



Discussion

This pilot study evaluated whether the presence of leukocytes with hemozoin in thick smears of maternal peripheral blood was associated with placental infection, in order to determine if detection of hemozoin by microscopy during pregnancy could be used as an indicator of placental malaria. Leukocytes with hemozoin were detected in four smears (8%, 4/50): three from the group with placental infection and one from the group without placental infection. Therefore, 12% of women with placental infection had leukocytes with hemozoin in peripheral blood. These findings indicate low sensitivity of the proposed test (leukocytes with hemozoin detected by thick smear of maternal peripheral blood) to detect placental infection, but adequate specificity since all women, except one, without placental infection also were negative for leukocytes with hemozoin in peripheral blood.

It is important to remark that the woman positive for hemozoin-containing leucocytes in peripheral blood by microscopy but negative at delivery for placental infection by qPCR had a previous malaria episode during pregnancy, which was treated adequately according to the national health guidelines [9]. Therefore, that case could correspond to a past infection with hemozoin phagocytated or deposited in tissues such as the placenta, which has been reported by other authors [35-37].

This study had a limited sample size and association measures such as odds ratio or relative risk could not be obtained; moreover, this small sample size could restrict the possibility to find statistical differences between the groups (type II error). However, this is the first study that quantifies leukocytes with hemozoin in thick blood smears, and the almost perfect concordance between two readers reflects the precision of the measurements and the possibility of its application in field. Other authors who have used microscopy for quantifying leukocytes with hemozoin used only thin blood smears [18,22-24,38], which have lower sensitivity than thick blood smears for malaria diagnosis [39,40]. In addition, methylene blue staining of thick smears to detect hemozoin, as recently reported

Table 2. Detection of hemozoin or malarial parasites (thick smear and qPCR) in peripheral blood infection and presence of placental infection, at delivery.

Result in peripheral blood		Placental infection [n (%)]			p
		Positive (n = 25)	Negative (n = 25)	Total (n = 50)	
Leukocytes with hemozoin (thick smear)	Positive	3 (12)	1 (4)	4 (8)	0.297
	Negative	24 (96)	22 (88)	46 (92)	
Plasmodial infection by thick smear	Positive	6 (24)	0 (0)	6 (12)	**0.022**
	Negative	19 (76)	25 (100)	44 (88)	
Plasmodial infection by qPCR	Positive	17 (68)	1 (4)	18 (36)	**0.000004**
	Negative	8 (32)	24 (96)	32 (64)	

by Mohopatra *et al.* [41], is worthwhile to explore in the context of placental infection in a large series of patients. Other more sophisticated techniques such us flow cytometry [26] and fluorescence microscopy [42] have been used to detect hemozoin containing leukocytes, but their application in the field is far more limited than microscopic reading of thick blood smears as proposed in this study.

Similar to other reports, this study confirms that the presence of peripheral plasmodial parasitemia in a pregnant woman is usually accompanied by the presence of parasites in placenta (placental infection) [43,44]. However, several infections are detected exclusively in the placenta, which is known as occult placental malaria and is associated with adverse pregnancy outcomes [45]. Specifically in this study, 14% (7/50) of women had placental infection in the absence of peripheral parasitemia. Other non-parasite-derived biological markers have been evaluated as potential indicators of occult placental infection; for example, decreases in soluble fms-like tyrosine kinase-1 and leptin and increases in C-reactive protein were associated with placental infection and correlated with placental parasitemia [45]. A test or marker to detect placental plasmodial infection before delivery could contribute to reducing the adverse outcomes associated with malaria in pregnancy.

Conclusions

Detection of leukocytes with hemozoin by thick smear of maternal peripheral blood appeared to have low ability to indicate presence of placental infection; however, further studies to confirm this finding require a larger sample size.

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