Effects of Anticoagulants on \textit{Plasmodium vivax} Oocyst Development in \textit{Anopheles albimanus} Mosquitoes

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\textbf{Abstract.} Artificial membrane feeding (AMF) assays are used to determine malaria transmission-blocking activity in \textit{Anopheles}. The purpose of this study was to determine the effect of the most widely used anticoagulants, EDTA and heparin, on development of the \textit{Plasmodium vivax} sporogonic cycle. Blood samples collected from 60 patients carrying \textit{P. vivax} infections were used to feed \textit{An. albimanus} using AMF. Seven days after feeding, mosquitoes were dissected to assess mosquito infection. Mosquitoes fed with blood containing EDTA showed a lower mean oocyst number as compared with those fed blood with heparin. However, this effect was minimized upon reduction of EDTA concentrations in the serum. This result may be explained by the fact that microgametocytes require Ca$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ to activate enzymes important for exflagellation process and motility of ookinetes. We therefore recommend that heparin be used as the anticoagulant of choice for blood used in AMF assays.

\section*{INTRODUCTION}

Infection of mosquitoes with malaria parasites is dependent on multiple characteristics of the parasite, vertebrate host, and mosquito vector.\textsuperscript{1,2} Parasite development in the mosquito can be inhibited by factors inherent to each \textit{Anopheles} species, such as physiologic or physical barriers in midgut and salivary gland cells and mosquito survival, distribution, and feeding behavior.\textsuperscript{3} Additionally, host immune responses, including antibodies, cytokines, and complement, may significantly modify parasite transmission rates in the mosquito.\textsuperscript{4,6} One must also consider various environmental factors, such as temperature and rainfall, which are known to modulate parasite development.\textsuperscript{7} The manner in which blood samples are collected and handled during AMF assay procedures may not completely reproduce natural transmission conditions. In particular, use of anticoagulants could interfere with exflagellation, cellular invasion, and parasite survival inside the mosquito midgut.

Sodium heparin and ethylenediaminetetraacetic acid (EDTA-K3) are anticoagulants frequently used in blood collection, including for AMF. Each anticoagulant has different effects on the coagulation cascade. Heparin binds to antithrombin, thereby accelerating the inhibition of proteases (principally factors Xa and IXa) involved in the coagulation cascade.\textsuperscript{8} EDTA affects the coagulation cascade by chelating calcium ions that are required for the activation of factor IX in the intrinsic pathway, and factor VIIa–Ca$^{2+}$–Xa complex in the common pathway.\textsuperscript{8} EDTA further chelates Mg$^{2+}$ and Mn$^{2+}$ ions required for the exflagellation process in the sexual life-cycle stage of \textit{Plasmodium}. Because ookinetes also need Ca$^{2+}$ for motility and invasion of the midgut,\textsuperscript{9} we speculate that after chelation by EDTA these ions would not be available to sustain microgametocyte viability and ookinete motility, thereby inhibiting fertilization and further progression of the parasite life cycle.

Herein, we aimed to investigate the effect of two common anticoagulants, EDTA and sodium heparin, on \textit{P. vivax} oocyst production to determine optimal blood-collection procedures for studies on the sporogonic cycle of the malaria parasite in laboratory-reared \textit{An. albimanus} mosquitoes. This mosquito species is one of the main malaria vectors in the Latin American subcontinent and is particularly abundant in Buenaventura region on the Pacific coast of Colombia.\textsuperscript{10} This knowledge is essential for standardization of AMF assays used in studies on parasite–mosquito interactions, mechanisms of transmission-blocking activity and mosquito infectivity, as well as for routine production of sporozoites.\textsuperscript{11,12}

\section*{MATERIALS AND METHODS}

\textbf{Study population and blood sample collection.} \textit{P. vivax}-infected patients (18–60 years old, males and non-pregnant females) from rural and urban areas of Buenaventura, a malaria-endemic city on the Pacific coast of Colombia, participated in the study. Parasite diagnosis was confirmed microscopically by Giemsa-stained thick smear. The study protocol was approved by the Ethics Committee of the Universidad del Valle. After written informed consent was obtained from each donor, a blood sample (total 22 mL volume) was collected by venipuncture into three Vacutainer tubes (5 mL in EDTA; 10 mL in heparin; 7 mL without any anticoagulant; Becton Dickinson, Franklin Lakes, NJ). Patients were then treated immediately with curative doses of anti-malarial drugs ( primaquine 15 mg/day for 14 days, plus chloroquine phosphate, 600 mg on the first day and 450 mg/day for 3 days), according to the standard therapeutic guidelines of the Colombian Ministry of Social Protection.\textsuperscript{13}

\textbf{Preparation of parasite samples for AMF assays.} Blood samples were placed in Vacutainer tubes and maintained at 37°C during the assay to prevent parasite exflagellation. The \textit{P. vivax}-infected red blood cells (iRBC) were then pelleted by centrifugation at 500g for 5 min at 37°C, and then washed with two volumes of serum-free RPMI 1640 medium (Sigma, St. Louis, MO). Plasma was removed from the packed cells, and the \textit{P. vivax}–iRBC was stored for \textasciitilde 15 min at 37°C until mosquito feeding.

To assess the effect of anticoagulant on \textit{P. vivax} infectivity after AMF, 30 samples of donors’ packed iRBC (200 \mu L) were mixed with an equal volume (200 \mu L) of either serum or...
plasma fractions (all at a hematocrit of 50%) and used for mosquito feeding. In addition, normal human serum consisted of a pool of human AB sera obtained from a Red Cross blood bank that was determined to be negative for malaria blood stage-specific antibodies by immunofluorescence. Control sera were aliquoted and stored frozen at −70°C until use. For TB assays, this sera was heat-inactivated for 30 min at 56°C to inactivate complement. The above procedures resulted in the following eight combinations for study: plasma−EDTA; plasma−heparin; serum alone; and AB control serum, each mixed with either iRBC−EDTA or iRBC−heparin from the same donor.

To study whether the action of EDTA diminishes with decreasing concentration of EDTA, we used an additional 30 iRBC samples prepared in normal AB serum: 3.6 mM (standard EDTA content in Vacutainer tubes), 1.8 mM, 0.9 mM, 0.5 mM, and 0.3 mM.

Artificial membrane feeding assays. Immediately after re-suspension, iRBC preparations were placed in the AMF device (constant 37°C temperature). Blood was then offered to batches of 110 laboratory-reared, 3- to 4-day-old female An. albimanus mosquitoes. After 30 min, nonfeeding mosquitoes were removed from the cages. Seven to eight days after feeding, 40 mosquitoes per batch were dissected and midguts were stained with 2% mercuriochrome. The number of oocysts per mosquito midgut was counted using a stereomicroscope at 400× magnification.

Statistical analysis. Batches of mosquitoes were assessed using two outcome measures: the geometric mean number of oocysts and the proportion of infected mosquitoes per batch. Transmission blocking was determined by (1) the percent reduction in the proportion of infected mosquitoes and (2) the geometric mean number of oocysts and the proportion of infected mosquitoes per batch. The Wilcoxon signed-rank test was used to compare mean estimates between batches fed on reconstituted iRBC−EDTA or iRBC−heparin blood samples. A paired analysis was used because each donor contributed an iRBC sample for each EDTA and heparin comparison. Each test was two-sided and evaluated at the 0.05 significance level. The same two outcomes were used to evaluate the effect of different concentrations of EDTA.

RESULTS

A total of 1,200 mosquitoes (30 batches) were dissected in each independent assay (anticoagulants and EDTA anticoagulant diluents) to estimate the EDTA effect in the proportion of infected mosquitoes and the percent reduction of geometric mean oocyst counts. The presence of EDTA resulted in a lower geometric mean oocyst number and percentage of infected mosquitoes compared with heparin in all four sera/plasma comparison groups. The largest reduction in geometric mean oocyst number and percentage of infected mosquitoes compared with control occurred with iRBC−EDTA plus plasma EDTA. Only sera did not have statistically significant differences between the iRBC−EDTA and iRBC−heparin groups in terms of the percentage of infected mosquitoes (Table 1). In addition, no statistical differences were observed when comparing geometric mean oocyst numbers between autologous sera and AB control serum with both iRBC−EDTA and iRBC−heparin.

When the concentration of EDTA in iRBC plus AB serum was reduced from 3.6 to 0.03 mM, this reversed the level of oocyst inhibition and percentage of infected mosquitoes, most notably in the iRBC−heparin group. As shown in Table 2, the iRBC−EDTA group showed a lower geometric mean oocyst number and percentage of infected mosquitoes than the iRBC−heparin group in all concentrations tested. Trends in the two groups of iRBC were similar. iRBC−heparin and iRBC−EDTA showed an increase in the number of oocysts with decreasing anticoagulant concentrations. Oocyst numbers increased from 4.14 to 6.94 oocyst/mosquito and from 1.44 to 5.80 oocyst/mosquito, respectively. A similar effect was observed when the number of infected mosquitoes was assessed. Feeding of mosquitoes with iRBC−EDTA in the presence of the highest EDTA plasma concentration (3.6 mM) produced significantly lower numbers of infected mosquitoes (11%).

DISCUSSION

The purpose of this study was to investigate the effect of anticoagulants EDTA-K3 and heparin on P. vivax oocyst production. We demonstrated that EDTA-K3 suppressed the development of gametocytes to oocysts in the sexual phase of the malaria life cycle. In a series of experiments, combinations of iRBC in heparin yielded a greater mean oocyst number and percentage of infected mosquitoes than iRBC supplemented with EDTA-K3.

The negative effect of EDTA-K3 on oocyst development and mosquito infectivity was reversed by anticoagulant dilution. At the lowest EDTA-K3 concentration (0.3 mM), the mean oocyst number and percentage of infected mosquitoes approximated that of the AB control. Enhancement of oocyst development was observed at 0.9 mM, but decreases in oocyst development were found at both lowest (0.3 mM) and highest (3.6 mM) EDTA-K3 concentrations, suggesting that ion concentration affects gametocyte exflagellation. More research is needed to confirm this result.

### Table 1

<table>
<thead>
<tr>
<th>Sample iRBC†</th>
<th>Mean oocyst number (SD)*</th>
<th>% Infected mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>iRBC−Heparin</td>
<td>iRBC−EDTA</td>
</tr>
<tr>
<td>Sera</td>
<td>10.1 (15.5)</td>
<td>3.4 (3.4)</td>
</tr>
<tr>
<td>Plasma−heparin</td>
<td>11.3 (13.3)</td>
<td>6.5 (11.49)</td>
</tr>
<tr>
<td>Plasma−EDTA</td>
<td>4.0 (8.6)</td>
<td>0.5 (1.0)</td>
</tr>
<tr>
<td>AB control serum</td>
<td>9.5 (7.0)</td>
<td>6.5 (8.4)</td>
</tr>
</tbody>
</table>

*Geometric mean number of oocysts was observed in batches of 40 mosquitoes each (SD, standard deviation); a total of 30 batches were dissected (1,200 mosquitoes).
† iRBC, infected red blood cells.
Several factors are known to influence gametocyte eXflagellation: pH, xanthurenic acid concentration, temperature, and the presence of host antibodies. Observations differ in mean oocyst number in the presence of EDTA and heparin may be explained by EDTA chelation of Ca\(^{2+}\) and, to a lesser extent, Mn\(^{2+}\) (the EDTA–Ca\(^{2+}\) complex is more stable). Calcium ions are found in the endoplasmic reticulum and extracellular medium and bind to calmodulin, an important mediator of calcium signaling, which is required for regulation of a wide variety of Ca\(^{2+}\)-dependent enzymes. With *Plasmodium berghei*, the capacity of xanthurenic acid to trigger eXflagellation does not require free extracellular Ca\(^{2+}\) but depends on Ca\(^{2+}\) stored within the gametocyte or the infected host cell. In studies with *P. falciparum*, xanthurenic acid was found to stimulate transformation of *P. falciparum* male gametocytes into motile male gametes by increasing guanyl cyclase activity in the presence of Mn\(^{2+}/Mg\(^{2+}\) ions. However, eXflagellation was shown to be blocked completely after chelation of intracellular Ca\(^{2+}\) by BAPTA-AM, a membrane-permeable Ca\(^{2+}\) chelator. Cofactors such as Mn\(^{2+}\) and Mg\(^{2+}\) are required for cGMP activity and are regulated by Ca\(^{2+}\). By affecting function of protein kinases, EDTA could inhibit motility. Members of the family of calcium-dependent protein kinases, such as CDPK4 and CDPK3, are known to influence gametocyte exflagellation: pH, xanthurenic acid concentration, temperature, and the presence of host antibodies.

### Table 2

<table>
<thead>
<tr>
<th>EDTA (mM)</th>
<th>Mean oocyst number (SD)*</th>
<th>% Infected mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>iRBC–heparin</td>
<td>iRBC–EDTA</td>
<td>iRBC–heparin</td>
</tr>
<tr>
<td>3.6</td>
<td>3.02 (1.75)</td>
<td>1.52 (1.24)</td>
</tr>
<tr>
<td>1.8</td>
<td>3.45 (2.39)</td>
<td>2.73 (2.06)</td>
</tr>
<tr>
<td>0.9</td>
<td>4.15 (3.17)</td>
<td>3.43 (2.07)</td>
</tr>
<tr>
<td>0.5</td>
<td>5.40 (4.82)</td>
<td>5.32 (3.55)</td>
</tr>
<tr>
<td>0.3</td>
<td>8.31 (5.35)</td>
<td>6.71 (5.82)</td>
</tr>
<tr>
<td>AB control serum</td>
<td>10.70 (9.14)</td>
<td>6.13 (3.53)</td>
</tr>
</tbody>
</table>

* Standard deviation.
† iRBC, infected red blood cells; n = 30 batches (1,200 mosquitoes dissected per concentration).

The role of Ca\(^{2+}\) ions in parasite development in the mosquito is not clear. EDTA could affect interaction with calcium-binding proteins present in the ookinete surface and with receptors in the mosquito midgut. As occurs with the p24 protein of *Toxoplasma gondii*, EDTA may affect an integral membrane protein, Pf340, a sexual stage-specific surface antigen of *P. falciparum*, considered to be a potential target of transmission-blocking immunity and known to contain motifs shared among calcium-binding proteins.

In conclusion, this study has shown that EDTA interferes with *P. vivax* development in *An. albimanus* mosquitoes injected artificially by bloodmeal. Therefore, it is recommended that only heparin be used for blood collection with studies of the sporogonic cycle of the malaria parasite. Further assessment of the role of Ca\(^{2+}\), Mn\(^{2+}\), and Mg\(^{2+}\) ions in the development of the *P. vivax* parasite in the mosquito is needed to determine if Ca\(^{2+}\) interferes with eXflagellation and/or parasite adhesion to midgut cells.

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