Bothrops jararaca envenomation: Pathogenesis of hemostatic disturbances and intravascular hemolysis

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Abstract

To attain fully functional biological activity, vitamin-K dependent coagulation factors (VKDCF) are γ -carboxylated prior to secretion from liver. Warfarin impairs the γ -carboxylation, and consequently their physiological function. *Bothrops jararaca* snake venom (BjV) contains several activators of blood coagulation, especially procoagulant enzymes (prothrombin and factor X activators) and thrombin-like enzymes. In order to clarify the relative contribution of prothrombin and factor X activators to the hemostatic disturbances occurring during experimental *B. jararaca* envenomation, warfarin was used to deplete VKDCF, prior to BjV administration. Male Wistar rats were pretreated with saline (Sal) or warfarin (War) and inoculated subsequently with BjV or saline, thus forming four groups: Sal + Sal (negative control), Sal + BjV (positive control), War + Sal (warfarinization control), and War + BjV. Three hours after inoculation, prothrombin and factor X levels fell 40% and 50%, respectively; levels of both factors decreased more than 97% in the War + Sal and War + BjV groups. Platelet counts dropped 93% and 76% in Sal + BjV and War + BjV, respectively, and plasma fibrinogen levels decreased 86% exclusively in Sal + BjV. After 6 and 24 h, platelet counts and fibrinogen levels increased progressively. A dramatic augmentation in plasma hemoglobin levels and the presence of schizocytes and microcytes in the Sal + BjV group indicated the development of intravascular hemolysis, which was prevented by warfarin pretreatment. Our findings show that intravascular thrombin generation has the foremost role in the pathogenesis of coagulopathy and intravascular hemolysis, but not in the development of thrombocytopenia, in *B. jararaca* envenomation in rats; in addition, fibrinogenases (metalloproteinases) may contribute to coagulopathy more than thrombin-like enzymes.

Keywords: Vitamin K, factor II, kidney, platelets, hemolysis, metalloproteinases

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Introduction

Bothrops jararaca snake envenomation induces hemostatic disturbances, characterized mostly by ecchymosis, petechiae, purpura, epistaxis, and gingival bleeding.^{1–4} Blood coagulation factors are targets for snake venom toxins, once minute changes in blood fluidity promote rapid death of the prey and facilitate their capture and subjugation. Snake venoms contain various classes of toxins (e.g. metalloproteinases [SVMP], serine proteinases [SVSP], C-type lectin-like proteins, and phospholipases A₂), which activate or inhibit blood coagulation.⁵ Particularly, venoms from *Bothrops* snakes (family Viperidae), which account for around 20,000 snakebites in Brazil annually,⁶ contain several toxins that interfere in blood coagulation: (a) procoagulant enzymes that directly activate prothrombin^{7–15} or factor X^{8,16,17} zymogens, eventually generating meizothrombin or α -thrombin (prothrombin and factor X activators,

respectively); (b) thrombin-like enzymes, i.e. toxins that cleave fibrinopeptides from fibrinogen, converting it into fibrin;¹⁸ (c) fibrinogenolytic enzymes, which hydrolyze fibrinogen A α and B β chains, but do not convert fibrinogen into fibrin;^{19–22} (d) protein C activators, which activate protein C;²³ and (e) C-type lectin-like proteins that inhibit the biological activities of thrombin^{24,25} or factors IX/X.²⁶ Despite this plethora of toxins that disturb blood coagulation, it is an open question which of these toxins is the most important for promoting the hemostatic disturbances characteristic of human envenomation. However, SVSP have been recently reported to be minimally implicated in the genesis of thrombocytopenia and coagulopathy induced by the injection of *B. jararaca* venom (BjV) in rats.²⁷

Vitamin K is an essential cofactor for γ -glutamyl carboxylase, an enzyme involved in the metabolic synthesis (γ -carboxylation) of the amino acid γ -carboxyglutamic acid

(Gla) from precursor glutamate residues found in certain proteins. Blood coagulation factors II, VII, IX, X, and proteins C, S, and Z are essential to maintain hemostasis, and to attain fully functional biological activity, some N-terminal Glu residues must be γ -carboxylated prior to secretion from hepatocytes. These factors are known as vitamin-K dependent coagulation factors (VKDCF), since the lack of γ -carboxylation in these Glu residues, which are conditioned to the presence of vitamin K, does not render biological activity to these proteins. In the presence of Ca^{2+} ions, Gla residues provide the binding of VKDCF to negatively charged phospholipid surfaces, supplied by activated cells.^{28,29}

Warfarin, a coumarin derivative, is a vitamin K antagonist widely used clinically as an oral anticoagulant for the treatment of thromboembolism. Warfarin exerts a powerful depressant effect on the formation of VKDCF, as it limits the γ -carboxylation process. In humans³⁰ and bovines^{31,32} treated with coumarin anticoagulants, des- γ -carboxylated prothrombin is synthesized and defectively secreted in plasma. On the other hand, warfarin treatment in rats induces accumulation of des- γ -carboxylated prothrombin in the liver microsomes, and only 5–10% of this form is secreted into blood circulation.^{33–36} Likewise, factor X is accumulated in the rat liver during warfarin treatment.³⁷ The coagulant activity of factors VII, IX, and X also decreased in rats and humans treated with warfarin.^{38,39} Interestingly, although des- γ -carboxylated prothrombin does not operate physiologically, it is normally activated by the prothrombin activating enzyme from *Echis carinatus* venom.^{31,33–35,39,40}

The finding that warfarin impairs γ -carboxylation of VKDCF and limits the release of prothrombin and factor X into circulation of rats^{33–37} prompted us to investigate the contribution of snake venom prothrombin and factor X activators, and indirectly of thrombin-like enzymes and fibrinogenases, to the pathophysiology of hemostatic disturbances occurring during *B. jararaca* envenomation in warfarinized rats. Thus, we evaluated hemostatic and hematological parameters in experimentally envenomed rats that had been depleted of VKDCF by previous treatment with warfarin. We verified that (a) defibrinogenation was significantly dependent on the presence of VKDCF, i.e. on thrombin generation, and that (b) microangiopathic intravascular hemolysis was observed in rats and also blocked by warfarin pretreatment.

Materials and methods

Materials

A pool of lyophilized venom from specimens of adult *B. jararaca* snakes was obtained from the Laboratory of Herpetology, Institute Butantan. The venom was stored at -20°C , weighed, and dissolved in sterile saline at the moment of use. *Oxyuranus scutellatus* and *Daboia russellii* venoms and warfarin were purchased from Sigma-Aldrich® (USA), and the commercial *Bothrops* antivenin (lot 0611203; each mL neutralizes 5 mg of *Bothrops* venom) from Institute Butantan. Prothrombin time (PT) was determined using the DiaPlastin®, DiaMed (Brazil). Factor X deficient plasma was from Instrumentation Laboratory (Lexington, USA), and halothane (Tanohalo®) from

Cristália (São Paulo, Brazil). VKDCF-depleted plasma was prepared by adsorbing a pool of citrated rat plasma with 0.1 mol/L BaCl_2 .⁴¹

Animals

Male Wistar rats, weighing 250–300 g, were obtained from Institute Butantan Animal House. Rats had free access to food and water, and all procedures using animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996), and were approved by the Institutional Animal Care and Use Committee in Institute Butantan (CEUAIB) (protocol number 570/09). Rats were anesthetized with halothane.

In vivo depletion of VKDCF

To deplete VKDCF, whose half-life range from 8 to 60 h in humans,⁴² rats were pretreated with warfarin (5 mg/kg³⁶) once daily for three days. Briefly, warfarin (2 mg/mL) was dissolved in 154 mmol/L NaCl, and the pH of the solution was adjusted to around 10 in order to dissolve it. Rats were then injected intravenously (i.v.) with 0.5 mL of this solution during three days. Control rats received saline instead, under the same conditions.

In vitro experiments

Warfarinized and control rats were anesthetized, and blood samples were taken by puncture of abdominal aorta. Blood samples were anticoagulated with 3.8% trisodium citrate (9 volumes of blood: 1 volume anticoagulant), centrifuged at 3800g for 15 min at 4°C , and stored at -80°C until use. Plasma pools from control ($n=10$) and warfarinized ($n=10$) animals were made. In addition, an aliquot of the control plasma pool was used to deplete VKDCF using BaCl_2 adsorption.⁴¹ To evaluate the coagulant activity of BjV on those different plasma pools, the minimum coagulant dose (MCD)—i.e. the least amount of venom that clotted a solution of plasma or fibrinogen in 60 s at 37°C ⁴³—was determined. Estimates and the associated uncertainty, expressed as a 95% confidence interval,⁴⁴ of MCD were calculated by linear regression analysis in Stata™, using logarithmic transformation of data.

In vivo experiments

To evaluate the *in vivo* effect of VKDCF depletion on the severity of hemostatic disturbances induced by BjV, four experimental groups were designed (Figure 1). Group War+BjV: rats were pretreated with warfarin, as mentioned above, and inoculated with BjV at the dose of 1.6 mg/kg, subcutaneously (s.c.); group Sal+BjV: rats were administered with 154 mmol/L NaCl i.v. instead of warfarin, and injected with BjV; group War+Sal: in this group, a control of the warfarin treatment, rats were warfarinized and injected with saline; group Sal+Sal, animals were pretreated and inoculated with saline solution, under the same conditions reported earlier. At 3, 6, or 24 h after venom or saline injection, rats were anesthetized, and blood samples were obtained by puncture of abdominal aorta and immediately dispensed in plastic bottles containing

Figure 1 Schematic representation of timing of pretreatment (saline/warfarin), treatments (saline, *B. jararaca* venom) and collection of blood samples. (A color version of this figure is available in the online journal.)

anticoagulants. Anesthetized rats were exsanguinated and immediately euthanized following blood collection.

Blood sampling

Red blood cell (RBC) counts, hematocrit, and hemoglobin concentration were determined in an automated cell counter Advia 60[®] (Hematology System, Bayer Healthcare, USA) using blood samples anticoagulated with 2.7 mmol/L K₂-EDTA. RBC morphology was evaluated manually in blood smears stained with Romanowsky panchromatic stain. Platelet counts were determined manually using a buffered hypotonic solution⁴⁵ in a Neubauer hemocytometer. Blood samples were anticoagulated with 3.8% trisodium citrate, as mentioned earlier, to obtain citrated plasma. To neutralize the *in vitro* action of BjV, *Bothrops* antivenin was added to all blood samples (10 μ L/mL of blood), including the controls.

Assays in plasma

Citrated plasma samples were used to assay plasma fibrinogen⁴⁶ and plasma hemoglobin.⁴⁷ Prothrombin activity on plasma was assayed by the one-stage method, using *O. scutellatus* snake venom and a pool of normal citrated rat plasma ($n = 10$) adsorbed with 0.1 mol/L BaCl₂.^{48,49} Factor X activity on plasma was assayed similarly, except that *D. russellii* snake venom and factor X deficient plasma were used.⁵⁰ Clotting times were determined on a fibrometer (Fibrosystem. Becton Dickinson[®], USA).

Data handling and statistical analyses

Statistical analyses for *in vivo* experiments were performed using the softwares SigmaStat[®] 3.5 and STATA[®] 10. Statistical differences were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Whenever necessary, data transformation was carried out in STATA to provide homocedasticity and normal distribution. Differences with $P < 0.05$ were considered statistically significant. Data were expressed as mean $\pm$ standard error of mean (SEM).

Results

Warfarinization reduces the *in vitro* coagulant activity of BjV

Prior to testing the coagulating activity of BjV *in vivo* and *in vitro*, we evaluated the efficiency of warfarinization to deplete VKDCF. Three-day warfarinization in rats prolonged PT, as expected, from 22.2 ± 0.4 s in controls pretreated with saline to 94.7 ± 0.6 s in rats pretreated with warfarin. Accordingly, prothrombin and factor X activities fell drastically, respectively, from $106 \pm 7\%$ and $152 \pm 15\%$ in controls to $0.27 \pm 0.01\%$ and $0.24 \pm 0.01\%$ in warfarinized animals. Fibrinogen levels increased around 60% in warfarinized animals (347 mg/dL) in comparison to controls (219 mg/dL), but they were still within the normal reference range for the species. In fact, warfarin has been shown to raise the plasma levels of interleukin-6 and acute phase proteins, including fibrinogen, implying that it induces a mild inflammatory status in rats.⁵¹

The coagulating activity of BjV was firstly evaluated in plasma pools from animals pretreated with saline and warfarin, as well as in a rat plasma pool that has been adsorbed with BaCl₂ to remove VKDCF; adsorption with barium was used to test if warfarin *per se* interfered in BjV coagulating activity. The efficiency of BaCl₂ adsorption was tested by assaying PT, which was markedly prolonged (greater than 200 s) in the BaCl₂-treated plasma pool. Evaluating MCD curves in these plasma pools (Figure 2) demonstrated that VKDCF depletion, by either warfarin or BaCl₂, substantially decreased (approximately 4 times) the coagulant activity of BjV. Altogether, these results indicate that warfarinization was effective to deplete VKDCF from rats, and that the *in vitro* coagulating activity of BjV was markedly dependent on the presence of prothrombin and factor X in plasma.

Warfarin treatment reduces fibrinogen consumption in envenomed rats

In regard to the Sal + Sal group, similarly to what is shown above, warfarin treatment alone (War + Sal) increased mean fibrinogen levels by 70–103% ($P < 0.001$). However, remarkable fibrinogen consumption developed at 3 and 6 h after venom administration, and fibrinogen levels tended to return to normal levels at 24 h (Figure 3a). At 3, 6, and

Figure 2 MCD curves of BjV on rat plasmas. The coagulating activity of BjV was assayed on plasma pools from saline- (black circles) and warfarin-treated (red squares) rats, as well as in normal plasma pools previously depleted of VKDCF by BaCl_2 precipitation (blue triangle). The curves are representative of two different experiments. (A color version of this figure is available in the online journal.)

Figure 3 Plasma levels of fibrinogen (a), prothrombin (b), and factor X (c) activities, and platelet counts (d) in rats pretreated or not with warfarin for three days, and injected s.c. with BjV (1.6 mg/kg) or 154 mmol/L NaCl. Blood samples were collected at 3, 6, and 24 h after venom or saline injection. Statistically significant difference between groups: † $P < 0.02$; * $P < 0.001$. Data are expressed as mean $\pm$ SEM ($n = 5-6$ animals/group). (A color version of this figure is available in the online journal.)

24 h, mean fibrinogen levels in Sal + BjV were around 14%, 18%, and 41%, respectively, of those found in Sal + Sal group ($P < 0.001$). On the other hand, fibrinogen levels in War + BjV were 41%, 57%, and 72% of those obtained for

War + Sal at 3, 6, and 24 h, respectively ($P < 0.001$). Warfarinization thereby protected animals from BjV-induced fibrinogen consumption, since those ratios between War + BjV/War + Sal groups were statistically

Figure 4 RBC counts (a) and plasma hemoglobin levels (b) in rats pretreated or not with warfarin for three days, and injected s.c. with BjV (1.6 mg/kg) or 154 mmol/L NaCl. Blood samples were collected at 3, 6, and 24 h after venom or saline injection. Statistically significant difference between groups: † $P < 0.03$; # $P = 0.007$; * $P < 0.001$. Data are expressed as mean $\pm$ SEM ($n = 5$ –6 animals/group). (A color version of this figure is available in the online journal.)

higher than the respective ratios for Sal + BjV/Sal + Sal groups at each time interval ($P < 0.002$). These findings show that despite the presence of thrombin-like enzymes and fibrinogenases in BjV, defibrinogenation was remarkably reduced when VKDCF had been depleted, reinforcing the *in vitro* results presented above showing that thrombin-like enzymes have a secondary role in defibrinogenation.

Envenomation-induced consumption of prothrombin and factor X

As depicted in Figure 3b and c, the Sal + Sal group had prothrombin and factor X activities within the normal range over time. In the Sal + BjV group, prothrombin activity was reduced 40% at 3 h ($P < 0.001$), and rapidly recovered at 6 and 24 h, close to values noticed in the Sal + Sal group. On the other hand, factor X activity showed a statistically significant drop (around 50%) in the Sal + BjV group at 3 h ($P < 0.001$); however, the fall in factor X levels was more accentuated at 6 h (around 80%), and by 24 h factor X levels had already reached control levels. Warfarin alone was effective in reducing the activities of prothrombin and factor X, which achieved mean values lower than 0.3% over time. As expected in War + BjV, prothrombin and factor X activities were practically maintained below 1% over time. Thus, BjV-induced moderate consumption of prothrombin and factor X, which, nonetheless, was effective to promote fibrinogen consumption.

Warfarin mildly protects rats from BjV-induced thrombocytopenia

Sal + BjV showed a marked fall in platelet counts at 3 and 6 h, around 93% and 78%, respectively, in comparison with those from Sal + Sal (Figure 3d). By 24 h, platelet counts of Sal + BjV recovered to the values found in Sal + Sal, but the difference between both groups was still statistically significant ($P < 0.001$). In War + Sal, a mild decrease in platelet count was observed, which was statistically different from

results obtained in Sal + Sal at 6 h ($P = 0.017$) and 24 h ($P = 0.010$). Such falls in platelet counts were possibly related to the warfarinization protocol used to deplete VKDCF, which promoted occult bleeding in rats over time (see Figure 4a). BjV exerted an intense fall in platelet counts in warfarinized animals at 3 and 6 h, of around 76% and 73%, respectively, compared to those of War + Sal, and by 24 h platelet counts of the War + Sal and War + BjV groups were of the same magnitude (Figure 3d). These findings indicate that thrombin engendered by procoagulant toxins (prothrombin and factor X activating enzymes) does not seem to be the main mechanism that accounts for platelet consumption in *B. jararaca* envenomation.

BjV induces mild hemolysis and RBC fragmentation

RBC counts were not statistically different among groups at 3 h (Figure 4a). However, a progressive drop was noticed in RBC counts of warfarinized groups (War + Sal and War + BjV) in regard to non-warfarinized animals (Sal + Sal and Sal + BjV) at 6 and 24 h ($P < 0.038$) (Figure 4a). This drop was followed by a similar and parallel drop in hematocrit and hemoglobin levels (data not shown), and they were likely due to the high dose of warfarin used herein to deplete VKDCF, which resulted in occult bleeding and anemia over time. However, RBC counts among War + Sal and War + BjV, and Sal + Sal and Sal + BjV were not statistically different over time. On the other hand, we observed that the color of the plasma and urine samples from Sal + BjV was reddish, suggesting hemoglobinemia. Accordingly, levels of plasma hemoglobin were remarkably elevated in the Sal + BjV group (Figure 4b), particularly at 3 and 6 h, returning to normal levels at 24 h; this assay also showed that warfarin protected rats from developing hemolysis, since War + BjV only experienced a mild increase in hemoglobinemia at 3 h. Besides hemoglobinemia, we noticed a higher frequency of pathological RBC, such as schizocytes and microcytes,

Figure 5 Representative pictures of red blood cell morphology of experimental groups. Rats were pretreated with warfarin or saline for three days, and then injected s.c. with BjV (1.6 mg/kg, s.c.) or saline. Blood samples were collected at 3, 6, and 24 h after BjV or saline injection. Note the presence of schizocytes (black arrowheads) in Sal + BjV group at all time intervals analyzed, but not in Sal + Sal, War + BjV, and War + Sal groups at 3 h. Increased polychromasia was also observed in blood smears from Sal + BjV, particularly at 3 and 6 h. (A color version of this figure is available in the online journal.)

in blood smears from Sal + BjV at 3 h (Figure 5). Poikilocytosis was also observed less frequently at 6 and 24 h in blood smears from Sal + BjV group. Nonetheless, poikilocytosis was not noticeable in warfarinized animals, evidencing that warfarin protected animals from fragmentation of RBC. Taking into account those results, we demonstrated that mild hemolysis occurred following BjV administration, which seemed to be dependent on thrombin generation, since previous warfarinization could prevent it.

Discussion

BjV contains various toxins that interfere in hemostasis, and they exert deleterious effects in victims in different ways. Although fibrinogen consumption in *Bothrops* snakebites had been previously thought to be primarily due to thrombin-like enzymes,⁵² results shown here and elsewhere²⁷ have recently demonstrated that, at least in rats, SVMP are the main class of toxins involved in defibrinogenation.

Warfarinization considerably reduced fibrinogen consumption in rats, showing that defibrinogenation was mostly due to intravascular generation of α -thrombin or

meizothrombin. These findings corroborate the results obtained by *in vitro* MCD tests in plasma treated with BaCl_2 or from warfarinized rats, showing that depletion of VKDCF impairs the coagulant activity of BjV. Thus, both *in vitro* and *in vivo* tests demonstrate that the coagulant activity of BjV in rat plasma is primarily due to prothrombin and factor X activating enzymes, and not to thrombin-like enzymes, in agreement with a previous report.²⁷ However, since warfarinization could not completely abrogate fibrinogen consumption, other SVMP, such as fibrinogenases, seem to also play a role in the etiology of defibrinogenation.

If prothrombin and factor X-activating toxins are generating thrombin/meizothrombin and factor Xa, one may question why levels of prothrombin and factor X are so modestly reduced in Sal + BjV. The intermediate steps of the coagulation cascade are vitally controlled by inhibitors found in plasma (e.g. antithrombin and activated protein C), and particularly on endothelial cell surface (e.g. thrombomodulin, tissue factor pathway inhibitor, endothelial protein C receptor, and heparan sulfate), which rapidly promote the inactivation of these activated factors, so that only moderate consumption of prothrombin and factor X are

noticed. Limited amounts of factor Xa and thrombin formed in the fluid phase by snake procoagulant toxins are easily inhibited by antithrombin-glycosaminoglycan complexes in microvascular endothelium.^{53–55} These results evidence that studies of crude snake venoms or isolated hemostatically active-toxins, which are exclusively carried out *in vitro*, without taking into account the participation of rheological factors and microvascular endothelium, cannot explain all the effects that venoms can induce *in vivo*.

Herein, rats also manifested intravascular hemolysis, which lasted for at least 6 h after venom administration. Intravascular hemolysis has been shown to occur sporadically in *B. jararaca* envenomation.^{56–59} Interestingly, intravascular hemolysis was also notably diminished in the War + BjV group, indicating that thrombotic microangiopathy due to intravascular thrombin generation was taking place during envenomation. Microangiopathic hemolysis is characterized by the appearance of intravascular hemolysis and the presence of poikilocytosis in blood smears, which we did notice in the Sal + BjV group. Schizocytes and microcytes are formed by the passage of RBC through fibrin strands deposited in the vascular bed, which were formed and cross-linked by thrombin generated following *B. jararaca* envenomation. As a result of mechanical RBC fragmentation, plasma hemoglobin levels increase. In fact, microcytes and schizocytes were observed in animals from Sal + BjV group, and warfarinization abolished the presence of poikilocytosis in blood smears. The rise in plasma hemoglobin levels and the presence of microcytes and schizocytes in blood smears demonstrate that thrombin generation is clearly involved in microangiopathic hemolysis resulting from snakebites.^{56,60–62} Although BjV has been reported to induce indirect hemolytic activity *in vitro*, due to phospholipase A₂ activity,⁶³ conspicuous hemolysis has not been reported in *B. jararaca* envenomation.⁶⁴ Since intravascular hemolysis was remarkably reduced by warfarinization, phospholipases A₂ in BjV do not seem to be involved in the genesis of hemolysis. Intravascular hemolysis and fibrin deposition in glomerular capillaries have been implicated in the genesis of acute kidney injury, the most frequent cause of death in patients bitten by *B. jararaca*.^{59,60,65} Since warfarinization prevents both intravascular hemolysis and fibrinogen consumption, our results clearly suggest that acute kidney injury in *B. jararaca* envenomation is intrinsically associated with thrombin generation and fibrin formation and deposition in the vascular bed. On the other hand, our results also demonstrated that platelet consumption and defibrinogenation are independent events in *B. jararaca* envenomation,²⁷ inasmuch as platelet consumption was not prevented by warfarinization. This finding indicates that intravascular generation of α -thrombin and meizothrombin is not crucial for inducing thrombocytopenia.

In conclusion, using warfarinization as a strategy to study the role of snake venom procoagulant toxins (prothrombin and factor X activators) in *B. jararaca* envenomation in rats, we showed herein that intravascular thrombin generation elicited by these toxins has a crucial role in evoking fibrinogen consumption, thrombotic microangiopathy,

and intravascular hemolysis, but not in platelet consumption.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. MLS conceived and designed the experiments. LVS and KMY performed the experiments. LVS, KMY, and MLS analyzed the data. MLS supplied reagents, analysis tools, and materials. MLS, LVS, and KMY wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that there is no conflict of interest.

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