

Effects of bleeding in horses immunized with snake venoms for antivenom production

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Abstract: Hematological and clinical alterations were investigated in horses injected with snake venom and subjected to repetitive bleeding, followed by plasmapheresis, for the production of polyvalent (Crotalinae) antivenom. These horses were compared to a control group that had not been immunized with venom and was subjected to the same bleeding protocol. Significant differences were observed between the two groups before the onset of the bleeding program, as immunized horses had lower hemoglobin concentration and hematocrit and higher total serum protein concentration than the non-immunized group, probably as a consequence of venom inoculations. When subjected to the bleeding program, similar alterations were observed in the two groups. There was a slight drop in hemoglobin concentration and hematocrit, as well as a reduction in serum protein concentration. In addition, there was a slight reduction in serum sodium and potassium concentrations. No clinical alterations were observed in these animals neither as a consequence of bleeding nor after plasmapheresis. A conspicuous drop in the neutralizing ability of serum was observed along the bleeding program. Administration of Ringer's lactate solution at the last day of bleeding increased sodium and potassium concentrations to values similar to those before the initiation of the bleeding program. Our results indicate that no major clinical alterations, and only minor laboratory changes, develop in horses as a consequence of this bleeding program. Plasmapheresis and administration of Ringer's lactate solution are recommended in antivenom-producing laboratories, in order to minimize deleterious effects.

Key words: Snake venom, anti venom, bleeding, hematological parameters, neutralization.

Parenteral administration of heterologous antivenoms constitutes the mainstay in the treatment of snakebite envenomations (Warrell 1993, Gutiérrez 1995). Antivenoms are produced by fractionation of plasma from horses or sheep immunized with venoms (Russell 1988, Raw *et al.* 1991). Hyperimmune plasma is fractionated and refined by a variety of methods that yield either whole IgG molecules, F(ab')₂ or Fab fragments (Sullivan 1987, Smith *et al.* 1992, Rojas *et al.* 1994).

Polivalent and anticoral antivenoms are produced at Instituto Clodomiro Picado, Costa

Rica, for the treatment of crotaline and coral snake envenomations, respectively (Bolaños and Cerdas 1980). After an initial immunization protocol, in which increasing doses of venoms are injected subcutaneously (Bolaños and Cerdas 1980, Angulo *et al.* 1997), horses are subjected to a bleeding schedule for three consecutive days. This study was undertaken in order to evaluate the clinical and laboratory alterations in these animals as a consequence of these extensive bleeding.

MATERIALS AND METHODS

Horses and immunization: Six adult horses (ages between three and four years), weighing 450-550 Kg, were immunized with a mixture of equal parts of the venoms of the snakes *Bothrops asper*, *Crotalus durissus durissus* and *Lachesis muta stenophrys*, according to an immunization protocol previously described (Angulo *et al.* 1997). Venoms were obtained from adult specimens collected in Costa Rica and kept at the Serpentarium of Instituto Clodomiro Picado. Venoms were lyophilized and stored at -20°C. For preparing the immunization mixture, venoms were dissolved in sterile phosphate-buffered saline solution, pH 7.2 (PBS), sterilized through 0.22 µm filters and mixed with the corresponding adjuvants (Freund's complete, Freund's incomplete or sodium alginate) before injection. A group of two horses of similar characteristics that were not immunized with venoms was included as control. Throughout the study horses were fed with a combination of king grass, hay and a mixture of powder food reinforced with proteins, vitamins and minerals

Bleeding protocol: Ten days after the last venom injection, in the immunization schedule, clinical examination (auscultation of cardiorespiratory and digestive systems, conjunctival and gingival mucosal examination, capillary filling test and overall clinical evaluation), and hematological laboratory tests (hematocrit and hemoglobin concentration) were performed in these horses, in order to assess their general condition and to determine if bleedings could be performed. Then, animals were bled from the jugular vein according to the following scheme: the first day, 8 liters of blood were collected in sterile bottles containing anticoagulant ACD (0.093 M citric acid, 0.197 M sodium citrate, 0.6 M glucose). Erythrocytes were separated by sedimentation and resuspended in one volume of 0.85% NaCl. On the second day, 8 liters of

blood were collected again and, immediately afterwards, the resuspended erythrocytes were infused in the jugular vein. After separation of plasma and erythrocytes, the latter were resuspended in a volume of 0.85% NaCl. On the third day, 4 liters of blood were collected and the resuspended erythrocytes from day two were infused immediately after bleeding. Finally, on the fourth day, horses were divided in two groups: both received erythrocytes resuspended in 0.85% NaCl, but one of the groups also received an intravenous infusion of 10 l of Ringer's lactate solution (Na⁺ 130 mEq/l, K⁺ 4 mEq/l, Ca⁺⁺ 4 mEq/l, Cl⁻ 111 mEq/l and lactate 27 mEq/l). A group of two horses that had not been immunized with venoms (control group) was subjected to the same bleeding protocol, with the exception that, on the fourth day, these animals received erythrocytes resuspended in 0.85% NaCl only.

Laboratory tests: Blood samples were collected at the following stages during the bleeding program: (1) before each bleeding, (2) after each bleeding, (3) after each infusion of resuspended erythrocytes, and (4) seven days after the onset of bleeding. The following laboratory parameters were determined in these samples: hematocrit and hemoglobin concentration (Sáenz *et al.* 1987), serum protein concentration (Schosinsky *et al.* 1983), and serum sodium and potassium concentrations, performed by flame photometry. A general clinical evaluation was also carried out during the four days. In addition, changes in the neutralizing ability of horse sera against indirect hemolytic activity of *B. asper* venom were determined (Gutiérrez *et al.* 1988). Neutralization was expressed as $1/ED_{50} \times 10^5$, where ED_{50} corresponds to the ratio µl serum/mg venom in which the indirect hemolytic activity is reduced 50% when compared to the effect induced by venom alone. (Angulo *et al.* 1997).

Statistical analyses: The significance of the differences between the means of two

experimental groups was determined by the Student's *t* test.

RESULTS

Significant differences were observed between the immunized group and the control group of horses before the onset of the bleeding program. Clinically, the former had mild local lesions at the sites of venom injections (Angulo *et al.* 1997), whereas control horses were in an optimal clinical condition. Hemoglobin and hematocrit basal values of control horses were 15.9 ± 1.1 g/dl and 40 ± 3 %, respectively, whereas these parameters were significantly reduced ($p < 0.05$) in venom-injected horses (11.4 ± 0.5 g/dl hemoglobin and 30 ± 1 % hematocrit). In contrast, serum protein concentration was higher in horses immunized with venoms (8.8 ± 0.2 g/dl vs 7.6 ± 0.1 g/dl, $p < 0.05$).

When subjected to the bleeding protocol, the two experimental groups showed similar alterations. Hemoglobin and hematocrit values had a slight drop, when compared to basal values, being statistically significant ($p < 0.05$) at days 2, 3 and 4 in the immunized group (Figs 1 and 2). A reduction in these hematological parameters was also observed in control horses, although it was not significant ($p > 0.05$) (Figs 1 and 2). Seven days after the initiation of the bleeding program, hemoglobin concentration and hematocrit had returned to values very similar to basal values of day 1 (Figs 1 and 2).

A significant drop in serum protein concentration was observed in both groups of horses during the bleeding program (Fig 3). In venom-injected group, this drop was still observed seven days after the onset of bleeding. Serum sodium concentration was also reduced in both control and immunized horses in the last two days (Fig 4), whereas serum potassium concentration had a significant drop only in the control group of horses (Fig 5). Clinical examination showed no evidences of cardiovascular alterations, as judged by the normal appearance of conjunctival and gingival mucosae, an

adequate capillary and jugular vein filling and the absence of hyperventilation. Moreover, no reactions were observed after plasmapheresis during days 2, 3 and 4.

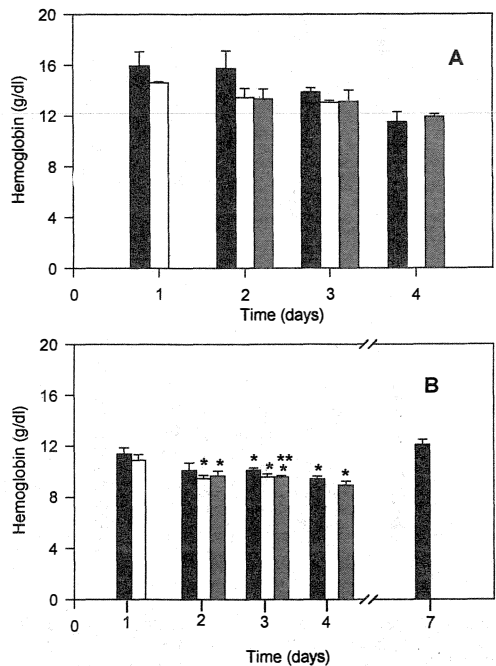


Fig.1. Changes in hemoglobin in control horses (A) and in horses immunized with venoms for production of polyvalent antivenom (B). Values correspond to samples before each bleeding (■), after each bleeding (□) and after each infusion of resuspended erythrocytes (▨). Results are expressed as mean \pm SEM. A (n=2) and B (n=5). * $p < 0.05$ when compared to the basal values of horses before the initiation of the bleeding program. ** $p < 0.05$ when compared to the values before the bleeding of the same day.

There was a conspicuous drop in the neutralizing ability of serum during the bleeding program, as there was a decrease in the neutralization of indirect hemolytic activity of *B. asper* venom (Fig 6). The largest drop was observed at day 4. Table 1 compares the results obtained in the group of immunized horses regarding the effects of administering Ringer's lactate solution. A significant increment in sodium and potassium serum concentrations was observed in horses receiving Ringer's lactate. These values were similar to those of basal samples collected on day 1. In contrast, no significant increments in

the concentration of these electrolytes were detected in the group that did not receive Ringer's lactate.

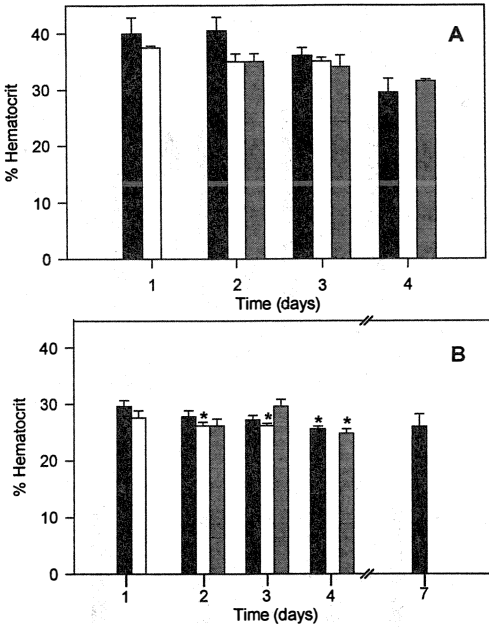


Fig 2. Changes in hematocrit in control horses (A) and in horses immunized with venoms for production of polyvalent antivenom (B). Values correspond to samples before each bleeding (■), after each bleeding (□) and after each infusion of resuspended erythrocytes (◻). Results are expressed as mean \pm SEM. A (n=2) and B (n=5). * $p < 0.05$ when compared to the basal values of horses before the initiation of the bleeding program.

DISCUSSION

Plasma from horses or sheep immunized with venoms constitutes the basic material for antivenom production. Laboratories vary in the bleeding protocols used (Raw et al 1991, Jadhav and Kapre 1991), but the common goal is to obtain relatively large volumes of blood while minimizing cardiovascular and hematological alterations in these animals. At Instituto Clodomiro Picado a bleeding protocol was established in which horses are bled during three consecutive days, with the introduction of plasmapheresis to reduce untoward effects. Assuming that total blood volume represents 8% of body weight

(Ganong 1983), blood loss due to the first day bleeding (8 liters) corresponds to approximately 20% of total blood volume for a 500 Kg horse.

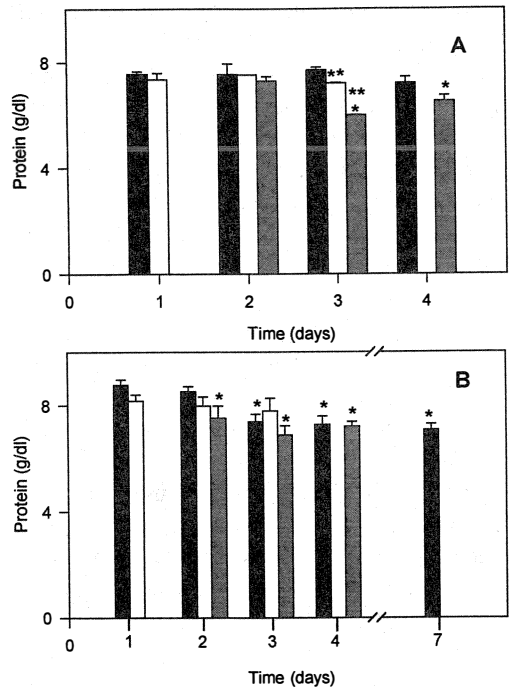


Fig 3. Changes in serum protein concentration in control horses (A) and in horses immunized with venoms for production of polyvalent antivenom (B). Values correspond to samples before each bleeding (■), after each bleeding (□) and after each infusion of resuspended erythrocytes (◻). Results are expressed as mean \pm SEM. A (n=2) and B (n=5). * $p < 0.05$ when compared to the basal values of horses before the initiation of the bleeding program. ** $p < 0.05$ when compared to the values before the bleeding of the same day.

Significant differences were observed, before the onset of bleeding, between a group of horses previously immunized with snake venoms and a control group of non-immunized animals in hematocrit, hemoglobin and serum protein concentrations. It has been described that hemoglobin concentration and hematocrit drop during immunization with venoms (Angulo *et al.* 1997), although the causes of these alterations have not been elucidated. In addition, a significant increase in serum protein concentration has been observed in horses immunized with venoms

(Estrada *et al.* 1992, Angulo *et al.* 1997), probably as a consequence of increased antibody synthesis. Thus, the observed differences before the initiation of bleeding are clearly due to the effects of immunization with venoms in one of the groups.

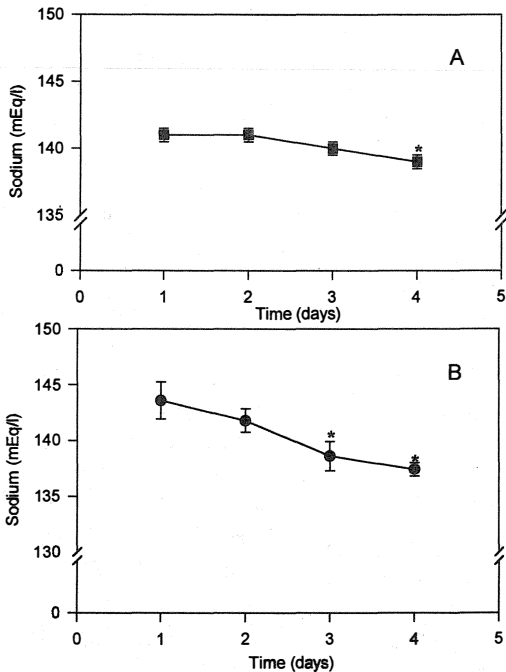


Fig 4. Changes in serum sodium concentration in control horses (A) and in horses immunized with venoms for production of polyvalent antivenom (B). Values correspond to samples before each bleeding. Results are expressed as mean \pm SEM. A (n=2) and B (n=5).

No clinical alterations such as cardiovascular or respiratory distress were detected in horses throughout the bleeding program, suggesting that major systemic disturbances do not occur in these conditions. Moreover, only small reductions in hemoglobin concentration and hematocrit were detected as a consequence of bleeding. It is likely that plasmapheresis was helpful in avoiding more drastic reductions in these hematological parameters. Moreover, despite the descriptions of various post-plasmapheresis reactions in antivenom-producing centers (Jadhav 1991), no clinical reactions were observed after plasmapheresis

in the seven horses used in this study. It is noteworthy that hemoglobin concentration and hematocrit values seven days after the onset of bleeding were not significantly different from basal values of the first day, evidencing a recovery of these hematological values. The changes described in laboratory parameters were clearly due to the acute blood loss associated with bleeding and not to the fact that animals had been injected with venom, since an overall similar pattern of alterations was observed in immunized horses as well as in the control group.

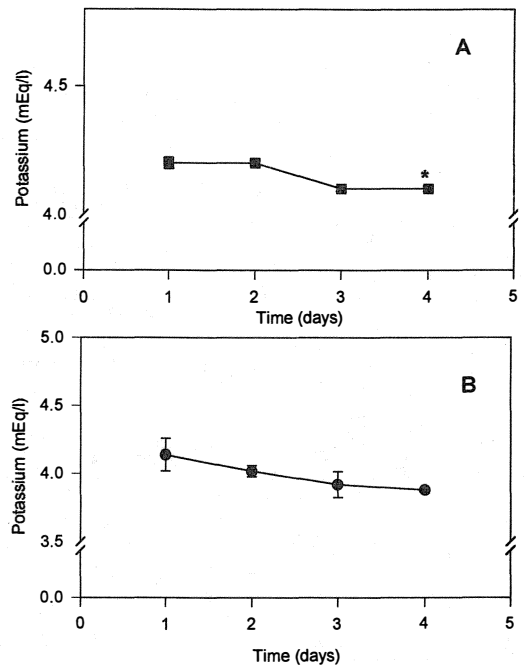


Fig 5. Changes in serum potassium concentration in control horses (A) and in horses immunized with venoms for production of polyvalent antivenom (B). Values correspond to samples before each bleeding. Results are expressed as mean \pm SEM. A (n=2) and B (n=5).

TABLE 1

Effects of administration of Ringer's lactate solution on different blood parameters in the horses on the fourth day of the bleeding protocol after immunization with venoms for production of polyvalent antivenom. Values correspond to samples before and after the infusion of resuspended erythrocytes and the Ringer's lactate solution. Results are expressed as mean \pm SEM. (*) $p < 0.05$ when compared to the values before the infusion of resuspended erythrocytes.

Parameter	Without lactate (n=2)	With lactate (n=3)
Hematocrit (%)		
Before	25.5 \pm 0.25	25.6 \pm 0.41
After	24.5 \pm 0.75	24.0 \pm 0.72
Hemoglobin (g/dL)		
Before	9.15 \pm 0.22	9.63 \pm 0.01
After	8.75 \pm 0.27	8.78 \pm 0.28 *
Protein (g/dL)		
Before	7.30 \pm 0.1	7.23 \pm 0.27
After	7.45 \pm 0.12	7.01 \pm 0.25
Sodium (mEq/L)		
Before	137.5 \pm 0.75	137.3 \pm 0.31
After	137.0 \pm 0.25	140.3 \pm 0.41 *
Potassium (mEq/L)		
Before	3.9 \pm 0.0	3.9 \pm 0.016
After	3.9 \pm 0.1	4.1 \pm 0.027 *

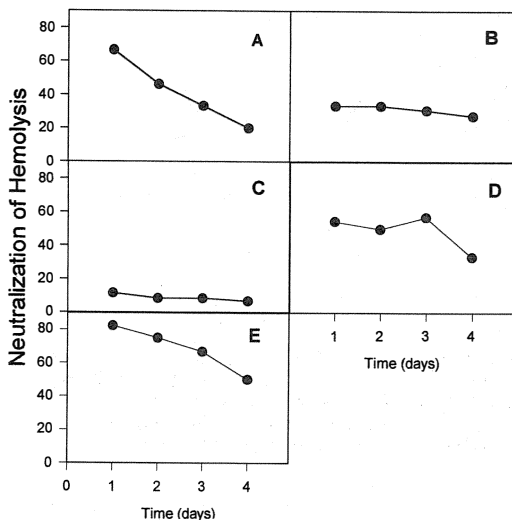


Fig 6. Changes in antibody titers during the bleeding program in the serum of horses immunized with venoms for production of polyvalent antivenom. The immune response was evaluated by the neutralization of indirect hemolytic activity of *B. asper* venom. Neutralization of hemolysis was expressed as $1/ED_{50} \times 10^5$ (see materials and methods for definition of ED_{50}). Samples were obtained every day before each bleeding.

Plasma protein concentration decreased during the bleeding program. This was expected since reinfused erythrocytes were resuspended in 0.9% NaCl solution. A parallel drop was observed in the neutralizing efficacy against indirect hemolytic activity of *B. asper* venom. This *in vitro* assay has a very good correlation with the neutralization of lethality (Gutiérrez *et al.* 1988) and is routinely used in our laboratory to monitor the development of immune response against *B. asper* venom (Estrada *et al.* 1991, Angulo *et al.* 1997). The neutralizing titer decreased so much by the fourth day that bleeding was not performed, in order to avoid unnecessary pathophysiological alterations in the animals.

Sodium and potassium serum concentrations also decreased at the last stages of the bleeding program. Interestingly, when Ringer's lactate was administered at the fourth day, sodium and potassium concentrations did not differ significantly from basal values of the first day. In conclusion, our results indicate that no major clinical alterations and only mild laboratory changes are observed in horses during the bleeding program followed at Instituto Clodomiro Picado for antivenom production.

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RESUMEN

Se investigaron las alteraciones hematológicas y clínicas en caballos inoculados con veneno de serpiente y sometidos a sangrías repetitivas, seguidas por plasmáferesis, para la producción de antiveneno polivalente (Crotalinae). Ese grupo de animales fue

comparado con un grupo control, el cual no había sido inmunizado con veneno y fue sujeto al mismo protocolo de sangría. Se observaron diferencias significativas entre los dos grupos antes de iniciar el programa de sangría, en donde los caballos inmunizados con veneno presentaban menor concentración de hemoglobina y hematocrito y mayor concentración de proteínas séricas totales, probablemente como consecuencia de las inoculaciones de veneno. Cuando ambos grupos se sometieron al programa de sangría, las alteraciones observadas fueron similares en los dos grupos, presentándose una leve disminución en la concentración de hemoglobina y hematocrito, así como una reducción en la concentración de proteínas séricas. Además se presentó una disminución en la concentración sérica de sodio y potasio. No se observaron alteraciones cardiovasculares ni respiratorias en esos animales como consecuencia de las sangrías, así como después de la plasmáferesis. A lo largo del programa de sangría se presentó una disminución evidente en la capacidad neutralizante del suero. La administración de solución de lactato de Ringer el último día de sangría incrementó las concentraciones de sodio y potasio a valores similares a los presentados antes del inicio del programa de sangría. Los resultados indican que no hay mayores alteraciones clínicas, y solamente pequeños cambios en los análisis de laboratorio, en los caballos sometidos al programa de sangría para la producción de antiveneno polivalente.

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