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Influence of blood storage on the haematological values in captive green iguanas (*Iguana iguana*)

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Abstract: The aim of this study was to evaluate the influence of long-term blood storage in citrate-phosphate-dextrose-adenine (CPDA-1) on selected haematological values in the green iguana. Seven adult captive-bred green iguanas (*Iguana iguana*) were enrolled in this study. Samples of CPDA-1-blood mixture (ratio 1:7) were kept at 4 °C for 28 days. Haematological examinations were performed immediately after the blood was collected (D0) and repeated at one-week intervals (D7, D14, D21 and D28). The total erythrocyte and leukocyte counts at D21 and D28 differed significantly from the values at D0 ($P < 0.05$). Significant differences ($P < 0.05$) in the haemoglobin concentrations (in comparison to D0) were detected at D14, D21 and D28. Starting at D14, marked erythrocyte degradation was present in the majority of the samples and various degrees of leukocyte degradation were present in all the samples. The mean haemolytic scores at D0, D7, D14, D21 and D28 were 0.14 ± 0.38, 0.71 ± 0.95, 1.43 ± 0.98, 2.14 ± 1.35 and 2.43 ± 1.62, respectively. The results indicate that it is possible to keep the whole blood of green iguanas in CPDA-1 at 4 °C for seven days, as statistically significant changes in the haematological values begin to appear on the 14th day of blood storage.

Keywords: citrate-phosphate-dextrose-adenine; erythrocytes; haematology; leukocytes; reptiles

Reptile patients are presented to veterinary clinics with chronic diseases as well as acute life-threatening traumatic injuries. In chronic diseases, physical examinations are very often unremarkable – revealing general symptoms such as apathy, dyspnoea, sunken eyes, exercise intolerance and/or pale oral mucosa (Gibbons and Darbo-McLeffan 2009), while the laboratory findings include severe hypoproteinaemia, low haematocrit and anaemia that may require intensive care, including blood transfusions (McCracken et al. 1994; Wack and Anderson 2004; Mehalick et al. 2007; Wu and Chie 2014; Louis et al. 2020). In acute cases, such as bone fractures, prolapse or severe soft tissues lesions, haemorrhages secondary to traumatic injuries are present. A homologous blood transfusion may prevent life-threatening blood loss (Gibbons and Darbo-McLeffan 2009) and should be considered in all cases where the reptilian haematocrit is 15% or less (Morrisey 2010). In wildlife hospitals, rescue or rehabilitation centres, healthy donor reptiles may be available (Louis et al. 2020). However, in private clinical practice, the stressful challenge of searching for a suitable blood donor could be overcome by storing preserved blood for emergency purposes. The effect of short-term blood storage has been studied in sea turtles (Philips et al. 2017), tortoises (Petersen 2016), and snakes (Harr et al. 2005). The effects of long-term blood storage have been evaluated and published

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for alligators (Emerson et al. 2014). The aim of this study was to evaluate the influence of long-term blood storage on selected haematological values in captive green iguanas (*Iguana iguana*).

MATERIAL AND METHODS

Animals

Seven adult, clinically healthy, captive-bred green iguanas (*Iguana iguana*) – six males and one spayed female – with a mean body weight of 1.61 ± 0.55 kg were submitted for this study. The lizards were handled in accordance with national and European legislation (EU Council Directive 86/609/EEC for the protection of animals). The iguanas were housed in a separate room and kept individually in terraria with a 12 h/12 h light/dark regime, a 12 h/day ultraviolet light regime (UVA/B, Repti-Glo 5.0; Hagen, Holm, Germany) with a temperature range of 24–35 °C and a humidity range of 60–80%. The health condition of the iguanas was checked daily by visual adspection, and by physical examination every 2 months. The iguanas were fed an herbivorous diet with the addition of calcium powder (Aquamin; Dr. Rhaco s.r.o., Prague, Czech Republic). Prior to the blood collection, all the iguanas were fasted for 48 hours.

Study design and procedure

Each lizard was manually restrained and the venepuncture site was disinfected with a chlorhexidine spray (SkinMed; Cymedica spol. s.r.o., Hořovice, Czech Republic). Three millilitres (3 ml) of blood were collected from the ventral tail vein (*v. coccygea ventralis*) into sterile 5 ml syringes (B. Braun, Melsungen, Germany) with 21Gx1 1/2'' needles (Chirana T. Injecta a.s., Stará Turá, Slovak Republic). The syringes were pre-filled with 0.4 ml of CPDA-1 (citrate-phosphate-dextrose-adenine) (Terumo CPDA-1 Blood Bag; Terumo Penpol Private Ltd., Thiruvananthapuram, India). The ratio of CPDA-1 to blood was 1 : 7. The first haematological examination was performed immediately after the blood withdrawal (day 0, D0). The blood samples with CPDA-1 were stored under refrigeration at 4 °C. The syringes with the blood and CPDA-1 were gently homogenised before the samples (0.3 ml) were taken for the haematological examinations, which were repeated at one-week intervals (day 7, D7; day 14, D14; day 21, D21; and day 28, D28).

Haematology

The evaluation of the haematocrit (PCV, packed cell volume) was performed by the micro haematocrit capillary centrifugation method (Campbell 2012). Blood samples were centrifuged for 5 min (14 500 *g*). The haemoglobin concentration was determined by the cyanmethemoglobin method. The blood mixture (20 µl) and Drabkin's reagent (5 000 µl; made in the Department of Pharmacy, St. Anne's Hospital Brno, Czech Republic) was kept at room temperature for 20 min and afterwards centrifuged (10 min, 2 000 *g*) to ensure that the nuclei of the erythrocytes had been separated. Spectrophotometry was then performed at 540 nm (Rodkey et al. 1979). For the total erythrocyte (RBC) and leukocyte counts (WBC), the haemocytometer technique was used. Twenty-five microlitres (25 µl) of blood were diluted with 4 975 µl of Natt-Herick's solution (Natt and Herrick 1952). The mixture was kept at room temperature for 10 min before counting the cells in a Bürker chamber (Meopta, Přerov, Czech Republic). The erythrocytes were counted in 20 rectangles (1/100 mm^2) and the leukocytes were counted in 100 large squares (1/25 mm^2). Blood smears were prepared with the use of the bevel-edge slide technique and stained according to the May-Grünwald-Giemsa method. All the differential counts of the leukocytes were assessed by the same investigator (MK) with the use of an optical microscope (Nikon Eclipse 50i, objective: CFI Plan Apo NCG 100X Oil; Nikon, Tokyo, Japan) and an immersion oil (Sigma-Aldrich, St. Louis, Germany). Magnification 1 000 × was used for the assessment of the erythrocytes and leukocytes (heterophils, eosinophils, basophils, lymphocytes, monocytes and azurophils). The differential counts and morphological assessments of the white blood cells were performed by examining 100 leukocytes. The morphological changes of the erythrocytes were assessed in ten different fields of view per each evaluated sample. The erythrocytes were assessed with an emphasis on cell degradation (membrane disintegration, vacuolisation, RBC swelling), the presence of immature cell forms, and hypochromasia. The leukocytes were also evaluated with an emphasis on cell deg-

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radation (membrane disintegration, karyorrhexis) as well as toxic granulation/degranulation, vacuolisation or the presence of phagocytosed particles.

The degree of haemolysis in the blood samples was determined with the use of a haemolytic score (0 – blood sample without haemolysis, 4 – blood sample with marked haemolysis) by the same investigator (MK). Despite the fact that the blood collection, handling and storage of the blood samples was undertaken under strict sterile conditions, samples from D28 were sent to the laboratory (Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) for standard bacteriological control.

Statistical analysis

All the statistical tests were performed using the PC-based software – UNISTAT for Excel (v6.5; Unistat Ltd., United Kingdom). Descriptive statistics (mean ± SD, median, minimum and maximum) were used for the data analysis. Based on an assessment of the population normality (Shapiro-Wilk test), a non-parametric repeated measures analysis of variance ANOVA (Friedman test) was used to determine the possible differences in the results of the haematological profiles received at D0, D7, D14, D21 and D28. A significance level (α) of 0.05 was used in this study.

RESULTS

The total erythrocyte counts at D21 and D28 were significantly lower ($P < 0.05$) than the total erythrocyte counts at D0 (Table 1). The right tail probability in Friedman's test for the RBC count was 0.019 1. The total leukocyte counts at D28 were significantly lower than the results obtained at D0 ($P < 0.05$). The right tail probability in Friedman's test for the WBC count was 0.029 2. A significant decrease ($P < 0.05$) in the haemoglobin concentration (in comparison to the values received at D0) was detected at D14, D21 and D28. The values of the PCV at D0 did not significantly differ from values at D7, D14, D21 or D28. There were no statistically significant differences found in the differential counts of leukocytes, other than lower eosinophil values (10^9/l)

Table 1. The influence of blood storage in CPDA-1 at 4 °C on haematological profile in seven green iguanas (*Iguana iguana*)*

Blood profile values (units)	Days of haematological examination				
	day 0	day 7	day 14	day 21	day 28
Haematocrit (l/l)	0.27 ± 0.14	0.28 ± 0.04[a]	0.26 ± 0.07	0.21 ± 0.09 [b]	0.21 ± 0.11
Haemoglobin (g/l)	87.19 ± 10.22[a]	85.75 ± 11.64[a]	76.94 ± 15.31[bc]	68.87 ± 14.48[d]	74.64 ± 8.86[d]
Erythrocytes (10^{12}/l)	0.98 ± 0.14[a]	0.94 ± 0.14[a]	0.87 ± 0.33[c]	0.76 ± 0.23[b]	0.72 ± 0.17[bd]
Leukocytes (10^9/l)	6.64 ± 2.19[a]	6.14 ± 1.73[a]	5.71 ± 1.89	6.36 ± 2.23[a]	4.86 ± 1.52[b]
Heterophils (%)	52.29 ± 13.73	54.57 ± 12.57	55.43 ± 12.74	52.29 ± 14.09	51.57 ± 14.85
Heterophils (10^9/l)	3.56 ± 1.59[a]	3.44 ± 1.33[a]	3.19 ± 1.42	3.56 ± 1.55[a]	2.66 ± 1.13[b]
Lymphocytes (%)	26.57 ± 8.10	25.43 ± 6.63[b]	27.00 ± 5.94	27.57 ± 5.71[a]	26.86 ± 6.64
Lymphocytes (10^9/l)	1.81 ± 1.05	1.58 ± 0.62	1.52 ± 0.77	1.72 ± 0.71	1.27 ± 0.44
Monocytes (%)	0.14 ± 0.38	0.57 ± 0.53	0.29 ± 0.49	0.14 ± 0.38	0.14 ± 0.38
Monocytes (10^9/l)	0.01 ± 0.02	0.03 ± 0.03	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.01
Eosinophils (%)	0.86 ± 0.69	0.86 ± 0.90	0.57 ± 1.13	1.00 ± 1.15	0.57 ± 1.13
Eosinophils (10^9/l)	0.16 ± 0.31[a]	0.04 ± 0.04	0.03 ± 0.06[b]	0.12 ± 0.23	0.02 ± 0.03[b]
Basophils (%)	6.29 ± 7.18	5.43 ± 7.63	6.00 ± 8.76	7.43 ± 10.31	7.29 ± 10.14
Basophils (10^9/l)	0.34 ± 0.31	0.25 ± 0.29	0.24 ± 0.30[b]	0.36 ± 0.22[a]	0.23 ± 0.19[b]
Azurophils (%)	13.71 ± 4.86	13.14 ± 4.10	10.71 ± 2.36	11.57 ± 2.51	13.57 ± 4.58
Azurophils (10^9/l)	0.76 ± 0.42	0.81 ± 0.39	0.57 ± 0.11	0.74 ± 0.30	0.68 ± 0.34
Haemolytic score	0.14 ± 0.38[d]	0.71 ± 0.9[5bc]	1.43 ± 0.98[a]	2.14 ± 1.35[a]	2.43 ± 1.62[a]

*Mean ± SEM; [a–b, c–d]Significant differences ($P < 0.05$) are indicated by pairs of different alphabetical superscripts

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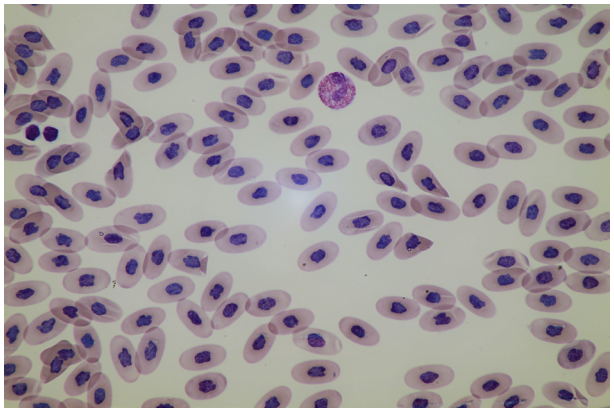

Figure 1. Physiological appearance of the erythrocytes, heterophils and thrombocytes in the blood smear of a green iguana (*Iguana iguana*)
D0 – individual no. 3; 1 000 × magnification, May-Grünwald-Giemsa staining

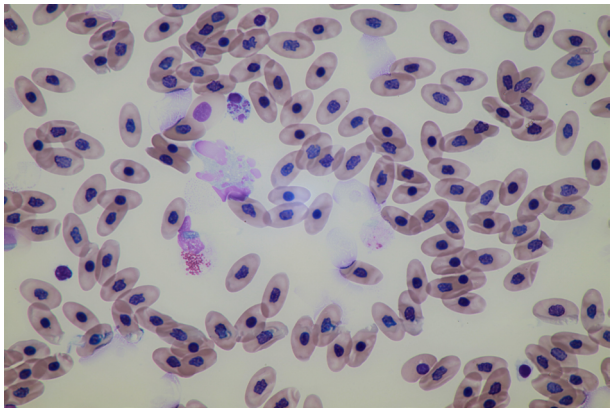

Figure 3. Degradation of the leukocytes (membrane disintegration, karyorrhexis) in the blood smear of a green iguana (*Iguana iguana*)
D21 – individual no. 5; 1 000 × magnification, May-Grünwald-Giemsa staining

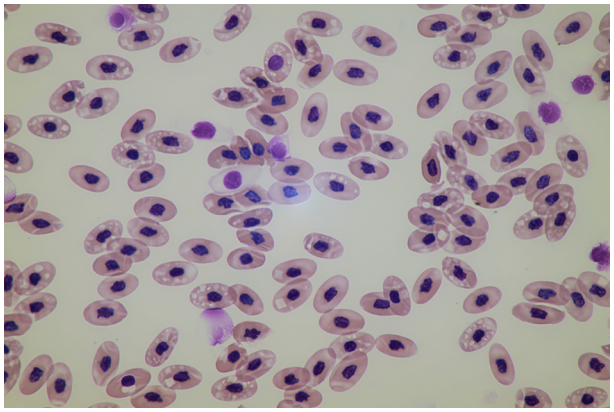

Figure 2. Degradation of the erythrocytes (cytoplasm vacuolisation and membrane disintegration) in the blood smear of a green iguana (*Iguana iguana*)
D14 – individual no. 1; 1 000 × magnification, May-Grünwald-Giemsa staining

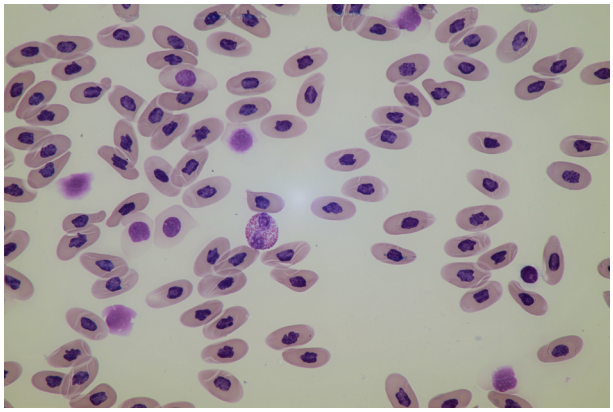

Figure 4. Heterophil with marked toxic granulation (round granules) in the blood smear of a green iguana (*Iguana iguana*)
D21 – individual no. 3; 1 000 × magnification, May-Grünwald-Giemsa staining

at D14 and D28 in comparison with D0. All the blood samples from D28 proved to be free of bacterial contamination.

The haematological evaluation performed at D0 did not reveal any abnormalities in the morphological characteristics of the blood cells (Figure 1). At D0, slight haemolysis was present in one blood sample and the mean haemolytic score was 0.14 ± 0.38. At D7, slight haemolysis was present in three blood samples and the mean haemolytic score was 0.71 ± 0.95. Erythrocyte degradation and leukocyte karyorrhexis (in 5% of WBC) were present in one blood sample (Figures 2 and 3). At D14, the mean haemolytic score was 1.43 ± 0.98 with various degrees of haemolysis in six blood samples. Erythrocyte degradation was present in five samples. Various degrees of leukocyte karyorrhexis (in 2–8% of WBC) was present in all the samples. The presence of vacuoles in azurophils was observed in two blood samples. At D21, the mean haemolytic score was 2.14 ± 1.35 with various degrees of haemolysis in six blood samples. Erythrocyte degradation was present in five samples. A slight degradation in the leukocyte morphology including karyorrhexis (in 5–30% of WBC) was present in all the samples. The presence of vacuoles in azurophils and toxic granulation of heterophils (Figure 4) was observed in three blood samples. At D28, the mean haemolytic score was 2.43 ± 1.62 with the presence of marked haemolysis in three

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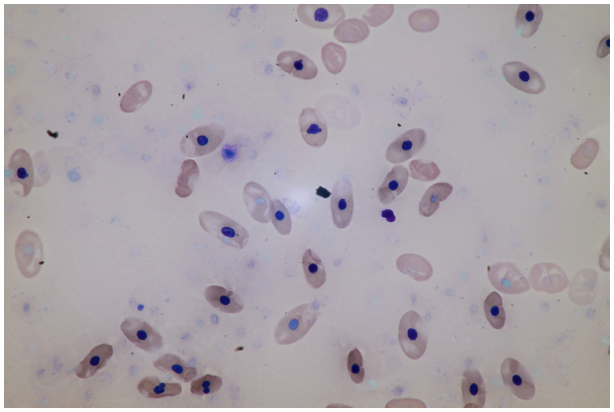

Figure 5. Extreme degradation of cells in the blood smear of a green iguana (*Iguana iguana*)
D28 – individual no. 2; 1 000 × magnification, May-Grünwald-Giemsa staining

samples. Erythrocyte degradation was present in six samples and marked leukocyte degradation including karyorrhexis (in 10–30% of WBC) was present in all the samples (Figure 5).

The mean haemolytic score at D0, D7, D14, D21 and D28 was 0.14 ± 0.38, 0.71 ± 0.95, 1.43 ± 0.98, 2.14 ± 1.35 and 2.43 ± 1.62, respectively.

DISCUSSION

Anaemia caused by acute blood loss is a common clinical presentation in reptiles (Wu and Chie 2014). While some authors mention that blood transfusions are uncommon in reptile practice as most severe cases of anaemia are chronic in nature (Divers and Camus 2020), a number of reports document that reptile patients with acute severe anaemia could benefit from single or multiple blood transfusions (McCracken et al. 1994; Wack and Anderson 2004; Mehalick et al. 2007; Wu and Chie 2014; Louis et al. 2020). Successful heterologous blood transfusions with or without cross-matching (a simple slide agglutination test between the recipient and the donors) have been performed in semi-aquatic terrapins (Wu and Chie 2014) and pythons (McCracken et al. 1994). Performing cross-matching is recommended before any repeated or heterologous transfusion in reptiles (McCracken et al. 1994). Transfusions should be intraspecific, because red cell isoantigens and serum agglutinins are determined, and physiologic determinants of blood types exist in reptiles (Bond 1940a; Bond 1940b; Hildeman 1962; Divers and Camus 2020).

In veterinary practice, heparin and dipotassium ethylenediaminetetraacetic acid (EDTA) are commonly used as anticoagulants for the haematological evaluation of fresh blood samples (Hanley et al. 2004). The use of heparinised blood for transfusion could be considered in situations when the reptile blood is stored short-term (up to 48 h) (Lichtenberger 2004; Harr et al. 2005). Long-term storage of heparinised blood is associated with significant amounts of clotting, cytoplasmic vacuolisation and membrane disintegration in the erythrocytes (Lichtenberger 2004; Harr et al. 2005; Petersen 2016).

There are several preservative solutions that prevent coagulation and maintain cell viability in blood stored for extended periods of time (Lichtenberg 2004). Citrate-phosphate-dextrose-adenine (CPDA-1), citrate-phosphate-dextrose (CPD), and acid-citrate-dextrose (ACD) are the most common anticoagulant-preservative solutions (APS) used in veterinary haematology (Lichtenberg 2004; Abrams-Ogg and Schneider 2010) and are preferred for long-term blood storage (Kumar 2017). CPDA-1 has been assumed to be the best anticoagulant preservative for long-term whole blood storage in exotic animal practice (Lichtenberg 2004). In feline practice, CPDA-1 has proven to be viable for blood storage up to 35 days (Spada et al. 2018). Studies have been undertaken to evaluate the practical use of anticoagulants for reptile blood samples. The purpose of one previous study was to evaluate the effects of lithium heparin, K3-EDTA, and CPDA-1 (with and without the addition of albumin) on the haematological values in blood samples of ten pythons. The blood with the anticoagulants was kept at 4 °C for 24 hours (Harr et al. 2005). The haemolysis was significantly increased in the citrated samples from pythons beginning at 12 hours. There were no significant differences in the haematological values in the samples from the pythons collected in heparin or EDTA at any point in time. No significant differences were found in the number of lysed cells or in the other haematological data in the samples with albumin (Harr et al. 2005). Based on the results of the study, the authors suggested that whole python blood anticoagulated with lithium heparin or EDTA should be evaluated within 24 h of collection and stored at 4 °C for the best results. Citrate should be avoided as it may result in increased cell lysis, which even the addition of albumin does not prevent (Harr et al. 2005).

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For the long-term storage of alligator erythrocytes, a CPDA-1 to blood ratio 1 : 9 was used (Emerson et al. 2014). The same CPDA-1 to blood ratio was previously used for short-term storage of sea turtle blood (Phillips et al. 2017) as well as for multiple heterologous blood transfusions in pythons and homologous blood transfusions in lizards (McCracken et al. 1994). A CPDA-1 to blood ratio of 1 : 9 was recommended for blood transfusions in exotic patients by Morrisey (2010). The present study uses a CPDA-1 to blood ratio of 1 : 7 in green iguanas. A 1 : 7 ratio of CPDA-1 to blood was used in a successful homologous blood transfusion in box turtles (Mehalick et al. 2007) and has been reported by other authors (Lichtenberger 2004; Abrams-Ogg and Schneider 2010). The evaluations of different anticoagulants (including CPDA-1) for short-term as well as long-term reptile blood storage were performed in alligators and sea turtles (Emerson et al. 2014; Phillips et al. 2017). Packed alligator erythrocytes can be stored in CPDA-1 at 4 °C for at least 35 days, at which point the cells appeared morphologically stable (Emerson et al. 2014). To evaluate the effect of CPDA-1 on the cell integrity, the PCV, total counts of erythrocytes and leukocytes, venous blood gas, glucose, electrolytes, and haemoglobin concentrations were assessed in sea turtles (Phillips et al. 2017). In the packed erythrocytes of alligators, the PCV decreased significantly from D7 to D35 in both anticoagulants (CPDA-1 and ACD). In the present study on the long-term storage of the whole blood of green iguanas, the PCV values at D0 did not significantly differ from values at D7, D14, D21 and D28. The difference may be related to various degrees of haemolysis in the blood of green iguanas when compared with the packed erythrocytes of alligators.

The results of this study indicate that it is possible to keep samples of green iguana blood in CPDA-1 at 4 °C for seven days, since statistically significant changes in the haematological values begin to appear on the 14th day of blood storage.

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Conflict of interest

The authors declare no conflict of interest.

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