J Radioanal Nucl Chem (2009) 282:37–39
DOI 10.1007/s10967-009-0275-4

Quantitative evaluation of blood elements by neutron activation analysis in mice immunized with *Bothrops* snake venoms

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Received: 1 July 2009 / Published online: 29 July 2009

Abstract Mice genetically selected for high antibody responsiveness (H_{III}) were immunized against different *Bothrops* species snake venoms from distinct region of Brazil. The Neutron Activation Analysis technique was used to evaluate the whole blood concentrations of elements of clinical relevance [Ca, Cl, K, Mg and Na] in order to establish a potential correlation between *antibody response* and *blood constituents* after *Bothrops* venom administration for clinical screening of envenomed patients.

Keywords Neutron activation analysis · Bothrops · Snake venoms · Blood elements

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Introduction

Animal model is an important tool for many researches in life science. Particularly mice are significant in experiments for development of immunological therapy and in the advancement of the understanding of immune mechanisms intervening in envenoming. In Brazil, venomous snakes of the genus *Bothrops* are responsible for 80% of the snakebites [1] and still a critical public health problem. Butantan Institute is an institution that produces several types of therapeutic antivenom including anti-*Bothrops* serum obtained in horses immunized with a mixture of venoms from five species: *Bothrops jararaca* (*B. jararaca*), *Bothrops jararacussu* (*B. jararacussu*), *Bothrops alternatus* (*B. alternatus*), *Bothrops moogeni* (*B. moogeni*) and *Bothrops neuwiedi* (*B. neuwiedi*). The final product (the antivenom) contains antibodies used for the treatment of victims of poisonous animals. Constantly these antivenoms are modified aiming to increase its therapeutic actuation in the organism. But, the efficacy of this product must be tested first in animal model, in order to check the antibody response.

Using NAA we proposed to determine the concentrations of Ca, Cl, K, Mg and Na in whole blood of mice immunized with *Bothrops* venom and to perform a comparison with the corresponding control line (mice not immunizated) for checking the behavior of these ions and metals in blood. Although all elements in blood are important to be quantified these are majority, they are also present in *Bothrops* venom [2] as well as in the antivenom [3], then small variations could be related to intoxication or other adverse reaction, mainly if high concentration were determined in mice blood due the immunization procedure.

Experimental

Materials and methods

Mice genetically selected for high antibody responsiveness [H_{III} line] were immunized against different *Bothrops* genus snake venoms and the specific IgG antivenom production determined. Groups of mice were immunized with: *Bothrops atrox* (*B. atrox*) venoms from snakes of distinct Amazon Brazilian regions, i.e., Alto Rio Negro [Group G1] and from Maranhão [Group G2]; the venom mixture used for obtaining the therapeutic sera, namely, *B. jararaca, B. jararacussu, B. alternatus*, *B. moogeni* and *B. neuwiedi* [Group G3]; the venom mixture with *B. atrox* from Rio Negro instead of *B. jararacussu* venom [Group G4]; *Bothriopsis taeniata* (B. *taeniata*) from Amazonia [Group G5]. The control group (H_{III} line not immunized) was composed by six animals while the immunized G1, G2, G4 and G5 by three and G3 by animals.

The animal model used in this experiment is the mouse genetically modified denominated H_{III}. They present high (H) antibody responsiveness and were selected by Instituto Biológico, São Paulo, Brazil according to the study performed by Boumsell et al. [4].

To perform these measurements the blood was collected by the retro–orbital venous plexus (at least 300 μL) from twenty adult mice (3–4 month old and average mass 30 g), 3 months after inoculation (period for antibody production) of 5 μg of venom with a subcutaneous injection. Certified Standard Solutions from Spex Certiprep Chemical (USA) was used as multi-elemental standard. The NIST 8414 Bovine Muscle Powder was analyzed for analytical quality control.

Sample preparation

Each biological sample was prepared by pipetting whole blood (exactly 100 μL) onto Whatman 41 filter paper ($\sim$2.4 cm^2). To perform this procedure a calibrated pipette was used to draw up the correct amount of blood in it. After that, the sample was dried for few minutes using an infrared lamp. Although anticoagulants, such as Sodium and Potassium salts EDTA or Trisodium Citrate, are very suitable for routine hematological work, particularly to perform the nuclear analysis they cannot be used because, if they are added, they can interfere in the results mainly for Sodium concentration determination. All the samples were prepared in triplicate and the results are the mean value. As standards, convenient aliquots of standard solutions of Ca, Cl, K, Mg and Na were prepared in a similar way as the samples. Each pair (standard-sample) was then sealed into individual polyethylene bag and irradiated in the nuclear reactor (IEA-R1, 2–4 MW, pool type) at IPEN under a thermal neutron flux of 3.3×10^{12} n cm^{-2} s^{-1}. For Cl and Na determination 30 s irradiations were carried out and for Ca, Mg and K 4 min irradiations. After the irradiation, the activated materials were gamma-counted by adequate time: 2 min for Cl and Na and 15 min for Ca, Mg and K. Considering that the biological material is scarce the adopted procedure present the advantage of uses small quantities of blood when compared with the conventional methods for biochemistry analyzes which needs $\sim$5 mL of whole blood for serum-plasma separation (a significant quantity for a small size animal) and different reagent by element determination [5]. An HPGe Spectrometer of High Energy Resolution (FWHM = 0.88 for 122 keV of ^{57}Co and 1.87 keV of ^{60}Co) and an ORTEC 671 amplifier, in pile-up rejection mode, coupled to a MCA ORTEC Model 919E connected to a PC were used. The background radiation as well as the escape peaks was reduced by employing the iron shield described by Medeiros et al. [6]. The source-detector distance in this experimental apparatus is 13.4 cm. In this experimental condition the detector dead time is about 2–3%. The radionuclide used in whole blood analyses were ^{49}Ca, ^{38}Cl, ^{42}K, ^{56}Mg and ^{24}Na. The concentration of each element was obtained using in-house software by evaluation of the peak position, FWHM and net area with the associated uncertainty.

Results and discussion

The certified and determined values as well as the Z-score values are presented in Table 1. The concentration values for Ca, Cl, K, Mg and Na in whole blood mice immunized (H_{III}) are presented in Table 2. The mean value, the limits of detection and the normal range for the control group were also presented.

Considering that Na is the majority in blood ($\sim$g L^{-1}), in *Bothrops* snake venoms ($\sim$mg L^{-1}) and also in antivenom ($\sim$mg L^{-1}) an increase in its concentration could be observed. According to Table 2 the results revealed high concentration for Na for all groups: while G2 (1.84 ± 0.10 g L^{-1}) and G5 (1.85 ± 0.07 g L^{-1}) are near of the upper limit for a confidence interval of 68% (1.55–1.93 g L^{-1}), for G3 (2.06 ± 0.08 g L^{-1}) and G4 (2.07 ± 0.09 g L^{-1}) they are near of the upper limit for a confidence interval of 95% (1.36–2.12 g L^{-1}) and, for G1(2.17 ± 0.08 g L^{-1}) there is an agreement only for a confidence interval of 99% (1.17–2.31 g L^{-1}). Although 80% of the data are inside of confidence interval of 95%, usually adopted for checking the clinical status of the organism, these data suggest that Na must be severely monitored in blood in immunological therapies because small variations in the majorities elements in blood can be lethal for the organism.

Table 1 Element concentrations obtained in the analysis of NIST 8414 bovine muscle powder SRM

Element	Mean ± SD	Certified values mean ± SD	Z-score
Ca, mg kg^{-1}	154 ± 15	145 ± 20	0.5
Cl, %	0.196 ± 0.018	0.188 ± 0.015	0.5
K, %	1.589 ± 0.066	1.517 ± 0.037	1.9
Mg, mg kg^{-1}	1015 ± 64	960 ± 95	0.6
Na, %	0.218 ± 0.007	0.210 ± 0.008	1.1

Table 2 The concentration (mean value) for Ca, Cl, K, Mg and Na in whole blood mice immunized with distinct *Bothrops* snake venoms

Venom group (*n*)	Ca (mg L^{-1})	Cl (g L^{-1})	K (g L^{-1})	Mg (mg L^{-1})	Na (g L^{-1})
G1 (3)	246 ± 15	2.56 ± 0.09	2.00 ± 0.10	34 ± 3	2.17 ± 0.08
G2 (3)	179 ± 11	2.31 ± 0.09	1.94 ± 0.11	35 ± 3	1.84 ± 0.10
G3 (2)	206 ± 12	2.43 ± 0.11	1.93 ± 0.15	26 ± 3	2.06 ± 0.08
G4 (3)	93 ± 7	2.30 ± 0.09	1.80 ± 0.12	30 ± 3	2.07 ± 0.09
G5 (3)	178 ± 11	2.08 ± 0.11	1.78 ± 0.12	36 ± 4	1.85 ± 0.07
Control					
CG(6)	202 ± 10	2.62 ± 0.09	1.95 ± 0.12	36 ± 3	1.74 ± 0.08
Range (±1 SD)	125–279	2.40–2.83	1.89–2.01	26–46	1.55–1.93
3σ (ppm)	78	7	151	42	5

n number of animals, *SD* standard deviation

For Ca and Mg the concentration values in immunized mice agreed with those of the control group considering a confidence interval of 68%, except for Ca in immunized mice with mixture with *B. atrox* venoms [Group G4] however this lower value is in agreement with a confidence interval of 95% (48–356 mg L^{-1}) .

No serious alteration in the concentration of the Cl in blood samples of all groups was observed when compared with the control group: while for G1, G2, G3 and G4 the concentration values are in agreement with a confidence interval of 68% (2.41–2.83 g L^{-1}) for G5 the lower value (2.08 ± 0.11 g L^{-1}) has compatibility only for ± 3 Standard Deviation (1.99–3.25 g L^{-1}).

Mice immunized with mixture with *B. atrox* [Group G4] and *B. taeniata* [Group G5] there are an agreement for K values since a 99% of confidence interval were considered (1.77–2.13 g L^{-1}). The relevance of this ion in blood suggests that it must be monitored in snake envenoming episodes for administration of the anti-*Bothrops* serum.

Conclusion

The NAA technique is very helpful for the determination of several blood elements of small animals because only 100 μL are collected from a 30 g mouse. For the first time it was applied for measurement of Ca, Cl, K, Mg and Na blood concentrations in mice after snakebites. Considering that snakebites are a public health problem in Brazil, the presented data can be useful as markers for clinical diagnosis in snake envenoming episodes. This study suggests that Na and K in blood were influenced by the venom of *Bothrops* and provide information that could be employed for immunological therapies.

Acknowledgments The authors thank the financial support of FAPESP, CNPq - INCTTOX and CENP. O. A. Sant'Anna and D. V. Tambourgi are researchers of CNPq.

References

1. MINISTÉRIO DA SAÚDE (2001) Manual de diagnóstico e tratamento de acidentes por animais peçonhentos, 2nd edn. Fundação Nacional de Saúde, Brasília
2. LAporta-Ferreira IL, Santos SMA, Santoro ML, Saiki M, Vasconcelos MBA (1997) J. Natural Toxins 6:103
3. Baptista TS, Zamboni CB, Medeiros JAG, Marcelino JR, Higashi HG, Freitas MG (2008) Blood evaluation in equines using NAA: study of the similarities equines versus humans. In: XXXI Reunião de trabalho sobre Física Nuclear no Brazil, proceedings of RTFNB, p 22
4. Boumsell L, Bernard A (1980) J Immunol Methods 3(38):225
5. Fréjaville JP, Kamoun P (1981) Guide des examens de laboratoire. Flammarion Médecine-Sciences, Paris
6. Medeiros JAG, Zamboni CB, Lapolli AL, Kenchian G, da Cruz MTF (2001) Appl Radiat Isot 54:245