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Determination of inorganic elements in blood of mice immunized with Bothrops Snake venom using XRF and NAA

L F F Lopes da Silva[1], C B Zamboni[1], V Bahovschi[1], S Metairon[1], M F Suzuki[1], O A Sant´Anna[2], M A Rizzutto[3]

[1]Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN-SP)
Av. Professor Lineu Prestes 2242 - 05508-000 São Paulo, SP- Brasil.
[2] Instituto Butantan
Av. Dr.Vital Brasil 1500 - 05503-900 São Paulo, SP – Brasil.
[3] Instituto de Física - Universidade de São Paulo, IFUSP, SP
Rua: do Matão Travessa R 187 - 05508-090, São Paulo, SP, Brasil.

E-mail: lais_de_fatima@ig.com.br

Abstract. In this work, mice genetically modified [H_{III} line] were immunized against different *Bothrops* snake venoms to produce anti-*Bothrops* serum (antivenom). The Neutron Activation Analysis (NAA) and Energy Dispersive X-Ray Fluorescence (EDXRF) techniques were used to evaluate Ca and Fe concentrations in blood of these immunized mice in order to establish a potential correlation between both phenotypes: antibody response and blood constituents after *Bothrops* venom administration. The results were compared with the control group (mice not immunized) and with human being estimative. These data are important for clinical screening of patients submitted to immunological therapy as well as the understanding of the envenoming mechanisms.

Introduction

In the last years we have performed investigations for checking the behaviour of ions and metal in blood of animal models as well as in antivenoms (such as, *anti-Bothrops, anti- Diphtheria, anti-Rabies and anti-Tetanus*) using Neutron Activation Analysis (NAA) technique [1-5]. The need to perform this kind of investigation in blood and also in antivenom is due to the fact that small changes (mainly excess) can lead to intoxication process to the victims of poisonous animals, mainly snakes. The main advantage to use NAA technique is the viability to analyze simultaneous elements in whole blood (for example: Br, Ca, Cl, K, Mg, Na, S) using small quantities (0.1 mL), an important condition when this biological material is scarce [1]. However, for Fe determination the amount of blood (0.1 mL) requires long neutron irradiation time (many hours) to obtain adequate uncertainty. This motivated us to check the viability of using a Portable X-Ray Fluorescence Spectrometry (PXRFS) for Fe determination in blood. Recently this portable spectrometer was used for Ca and Fe determination in our clinical research with success [6]. Now, we intend to quantify these inorganic elements in blood samples of mice immunized with a mix of different *Bothrops (B.)* species snake venoms (*B. jararaca, B. jararacussu, B. alternatus*, *B. moogeni, B. taeniata* and *B. neuwiedi)* using PXRFS and to compare these results with NAA procedure.

Experimental Procedure

For this study, biological samples of 20 genetically modified mice (H_{III}) were used. The blood samples were classified as a control group (CG) and immunized groups (IG) according to the mix of venoms. Several types of *Bothrops* venom *(B.)* obtained in the North of Brazil are used in the immunization procedure. The following classification was adopted:

CG: control group; mice genetically modified (H_{III}), but not immunized.

IG-1: *B.atrox* from the Brazilian Amazon in Rio Negro;

IG-2: *B.atrox* from Maranhão;

IG-3: *B. jararaca, B. jararacussu, B. alternatus, B. moogeni and B. neuwiedi;*

IG-4: *B. jararaca, B. atrox* from Rio Negro, *B. alternatus, B. moogeni and B. neuwiedi;*

IG-5: *B. taeniata* and Viperideae from Amazon;

The blood from retro–orbital venous plexus was collected from each adult male mice. A small amount of blood, 0.1 mL (±0.5%), was transferred to the filter paper and storage without refrigeration until to be used. The same procedure was used for certified standard solution preparation of Ca and Fe (Inorganic Ventures). More details about these steps are present in reference [1].

The NAA measurements were performed in the IEA-R1 nuclear reactor (4.0-4.5MW, pool type, at IPEN). Each blood sample and standards solutions of Ca and Fe, were irradiated in a thermal neutron flux and gamma counted using HPGe detector (GEM-60195, FWHM=1.92 keV for 1.33 MeV of ^{60}Co) connected to MCA (ORTEC-919E). To determine Ca, the irradiated time was 300s and the counted time was 600s. To determine Fe, the irradiated time was 8hs and the counted time was 12hs. All the quantitative analyses were performed using *ATIVAÇÃO* software and the quantitative values were determined using the certified concentration values and uncertainties of Ca (10.05 ± 0.44 μg/mL) and Fe (10.03 ± 0.54 μg/mL).

The XRF measurements were performed using a Portable X-ray Spectrometer (Amptek®) with Ag X-ray tube. The characteristic fluorescent X-rays emitted from the samples (K and L lines) were measured with a Si Drift detector (25 mm^2 x 500μm / 0.5 mil) with Be window (1.5"). The same blood sample investigated by NAA was analysed by XRF technique. All the blood samples as well as the certified standards solutions of Ca and Fe (Inorganic Ventures) were irradiated for 300s using 30 kV and 5 μA excitation. The spectra analysis was performed using WINAXIL software program and the quantitative values were determined using the same certified concentration values and uncertainties of Ca and Fe.

Results and Discussion

In figures 1 and 2 are presented the gamma ray spectrum of whole blood samples obtained by NAA using HPGe detector and in figure 3 using the PXRFS system. The element concentrations (mean value) and uncertainly propagation (combined uncertainty including contribution from all possible sources) using NAA and XRF techniques are shown in table 1. The Ca and Fe range for the control group and human being were included in this table for comparison [6, 7]. To illustrate, Ca and Fe concentrations data from XRF analyses are shown in figure 4 and 5, respectively. In these figures the confidence interval (considering ±1 and ± 2SD) for the control group (mice not immunized) was included for comparison.

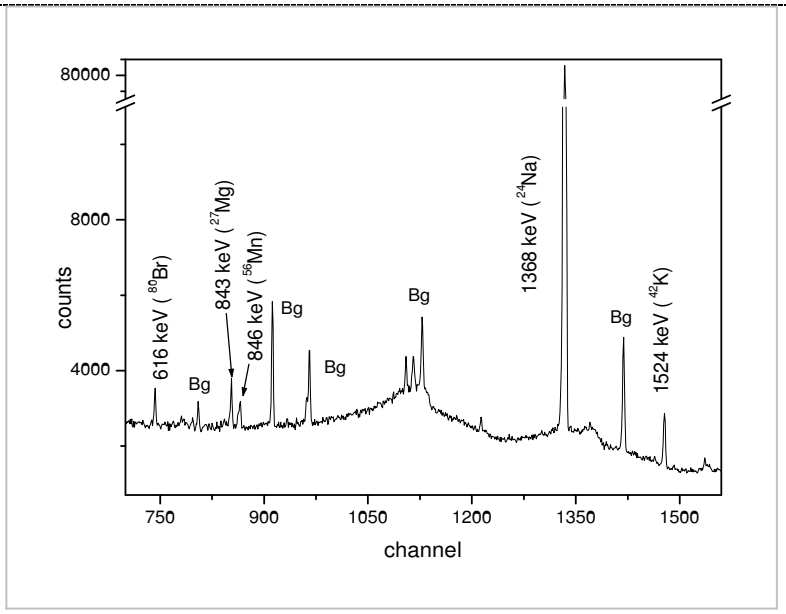

Figure 1. Partial gamma ray spectrum of whole blood obtained by NAA using HPGe detector

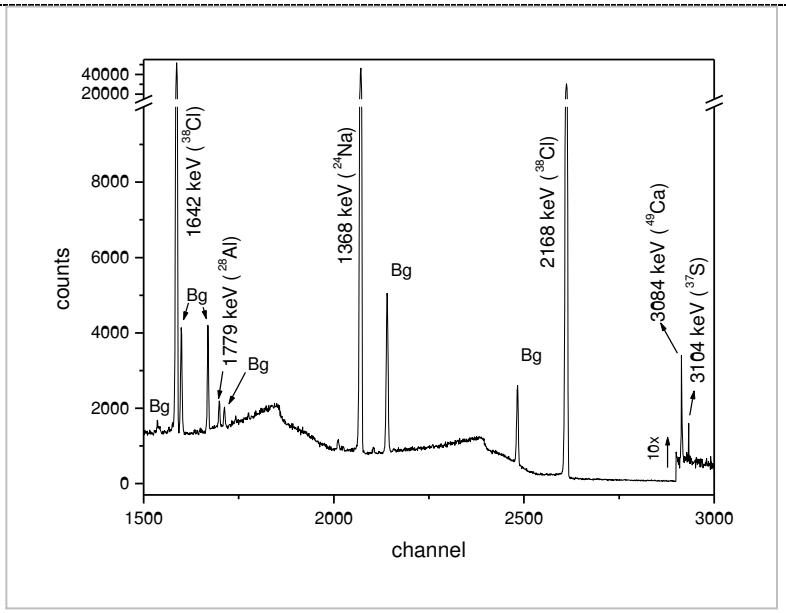

Figure 2. Partial gamma ray spectrum of whole blood obtained by NAA using HPGe detector

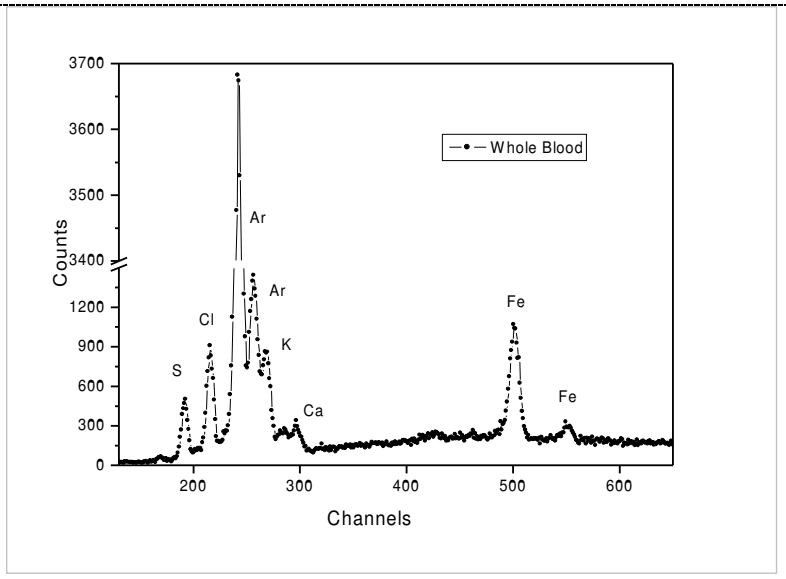

Figure 3. Spectrum of whole blood obtained by ED-XRF using PXRFS

Table 1. Element content (mean value ± uncertainty) of whole blood mice samples. The confidence interval (range) for the control group and human being were included for comparison

Groups (n)	Ca NAA	Ca XRF	Fe NAA	Fe XRF
		mgL^{-1}		
IG-1 (2)	240 ± 14	222 ± 17	390 ± 23	371 ± 26
IG-2 (3)	188 ± 11	191 ± 15	377 ± 23	362 ± 25
IG-3 (3)	201 ± 12	179 ± 14	483 ± 29	417 ± 29
IG-4 (3)	103 ± 6	118 ± 9	360 ± 24	371 ± 26
IG-5 (3)	177 ± 11	182 ± 14	354 ± 21	336 ± 23
CG [range]* (6)	[73 -355]	[48 - 356]	[304 - 477]	[268 - 376]
Human [range]*	[98 - 482][a]	nd	[277 - 513][b]	nd

n: number of samples
*confidence interval of 95%
[a] [7]
[b] [8]
nd: not determined

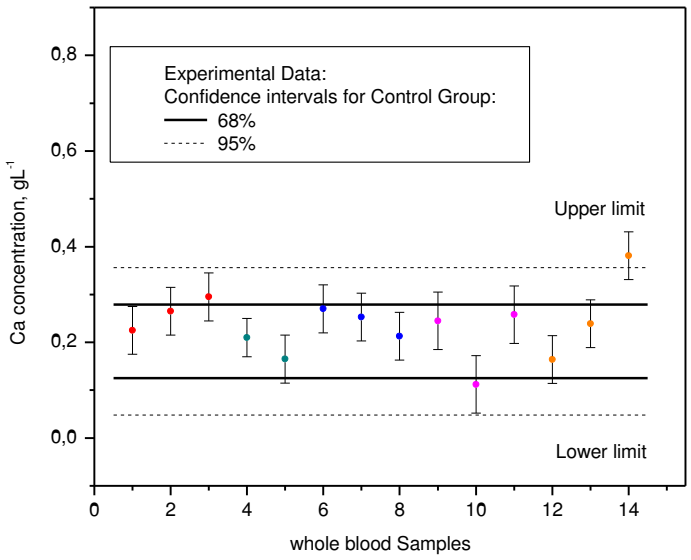

Figure 4. Ca concentration value and uncertainty in whole blood samples using PXRFS

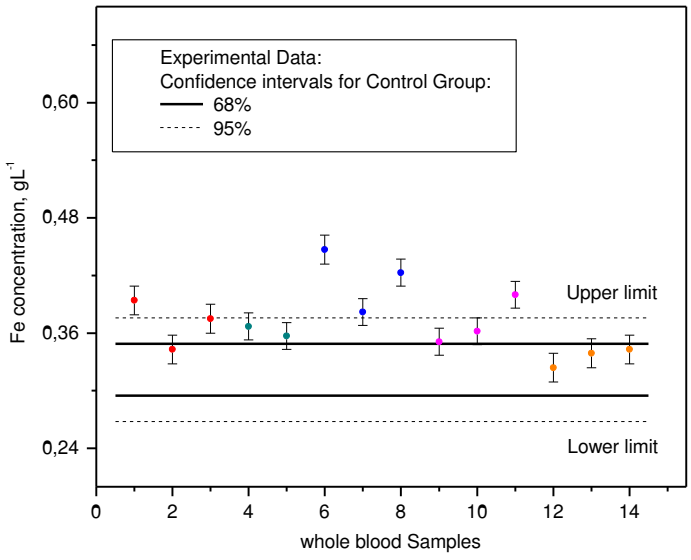

Figure 5. Fe concentration value and uncertainty in whole blood samples using PXRFS

According to table 1 the Ca data in immunized mice groups are in agreement with control group and human being estimative, while for Fe the data are close or above the upper level (considering the confidence interval of 95%). Related to the techniques (XRF and NAA) both shown to be appropriate for Ca and Fe blood analysis.

Conclusion

These data are important for clinical screening of patients were submitted to immunological therapy as well as the understanding of the envenoming mechanisms. Furthermore, considering that, in underdeveloped countries or isolated areas, when snakebites are a public health problem, as occurs in Brazil. The presented data can be also useful as markers for clinical diagnosis in snake envenoming episodes.

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