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NEUTRON ACTIVATION ANALYSIS OF Cl, K AND Na CONTENT IN WHOLE BLOOD OF HORSES USED IN HYPERIMMUNE SERA PRODUCTION

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ABSTRACT

Using Neutron Activation Analysis technique Cl, K and Na concentration were obtained in whole blood of equines used for antivenom production at Butantan Institute (São Paulo, Brasil). These data were compared with the human whole blood estimation. No significant difference was observed suggesting that this model animal is adequate sera production.

1. INTRODUCTION

Venomous snakes of the genus *Bothrops* are responsible by 80% of the snakebites in Brazil[1]. Butantan Institute (São Paulo, Brazil) is a centenarian institution that produces several types of antivenom including anti-*Bothrops* serum. Horses are significant in the development of this immunological therapy because they are used for snake antivenom production.

Since 1957 different methods of equine immunization were applied for optimizing antivenom production[2]. Recently, the use of crioulo horses and modification into immunogenic mixture (dose of venom mixture and Marcol Montanide adjuvant) for immunization with *Bothrops* venom (pool of several snake venom that has high prevalence in Brazil), has resulted in application of small venom quantities in the horses. Thus, the time necessary for antivenom production was reduced in about 80% and its production was duplicated.

In synthesis this new procedure involves three important steps: the immunization of the equines (injection of immunogenic mixture); the blood collection (about 5 to 7 liters/horse) following of its purification for serum production and the re-injection of the remainder cells blood in to the horses.

Considering that Na is the major component in the *Bothrops* venom[3] and also majority in blood (it is the main electrolyte in extra cellular space), the knowledge of its range in blood is an important data to be checked in the remainder cells blood for their re- injection, or not, in these animals.

The evaluation of K and Cl is also relevant because these elements are strongly correlated with Na in blood: while potassium is associated with Sodium by Na/K-ATPase the Chlorine ions, also majority in blood, are present in blood mainly in the form of NaCl. However, the conventional analysis for these elements is performed using serum[4], so we intend to use NAA technique as an alternative procedure for studying the behavior these elements in whole blood. This technique has been applied in our clinical researches with success for blood investigations in several model animals, due the simplicity in the sample preparation and agility in the analyses[5-7].

In this investigation the aim is to determine the concentrations of Na, Cl and K in whole blood of control group (not immunized horses) and also in immunizated groups (classified in function of the immunization period) that have been used during the last years in sera production at Butantan Institute for using these data as an indicator of quality control of cells blood. Furthermore, we intend to investigated the similarities between the control group and the human been whole blood for evaluation of this experimental model (crioulo race) for anti-*Bothrops* sera production at Butantan Institute.

2. EXPERIMENTAL PROCEDURE

For this study 20 equines from crioulo race not submitted to the hyperimmunization process yet (CG: Control Group) with 12-36 moths -old, (13) females and (7) males animals, from São Joaquim Farm at Butantan Institute (São Paulo city, Brasil) were investigated. Other three groups arranged in function of immunization period were also analyzed: just immunized (GI) composed by 17 equines from CG ; submitted to the immunization during 4 to 5 years (GI) and submitted to the immunization for 10 years (GIII), both composed by 20 equines (female).

For sample preparation about 2 ml of whole blood was taken from jugular vein in vacuum plastic tube, without anticoagulants, using needles of 25 x 8mm. Aliquots of 100μL (prepared in duplicate) were then transferred to the Whatman filter paper (~2.2 cm^2) and dried for few minutes using an infrared lamp. Standard solutions obtained from high purity metals and salts were prepared following the same procedure.

Sample and standard were irradiated in the pneumatic irradiation facility in the nuclear reactor at IPEN/SP (IEA-R1, 2-4MW, pool type) for 4 minutes in a thermal flux of ~$5 \cdot 10^{12}$ $n \cdot cm^{-2} \cdot s^{-1}$, allowed to decay time of 300s and gamma counted for 900s. HPGe detector (ORTEC) connected to an ADCAM multichannel analyzer and to a PC computer was used to measure the induced gamma-ray activity. The concentration of each element was obtained using in- house software[8].

3. EXPERIMENTAL PROCEDURE

The precision and accuracy of the results were checked by analyzing the NIST 8414 Bovine Muscle Powder standard reference material. The results are presented in Table 1.

Table 1. Element concentrations obtained in the analysis of NIST 8414 Bovine Muscle Powder standard reference material.

Element	Mean ± SD	RSD, %	Er, %	Z- score
Cl, %	0.192 ± 0.02 0.188 ± 0.01*	10.4	2.1	0.4
K,%	1.48 ± 0.10 1.517 ± 0.037*	6.7	-2.4	-1
Na,%	0.211 ± 0.013 0.210 ± 0.008*	6.2	0.5	0.1

* Certified values

RSD: Relative Standard Deviation

Er: Relative error

The concentration of the Cl, K and Na in blood samples of equines are shown in Table 2, 3 and 4 for Cl, K and Na, respectively. The *t*- test was used to evaluate the different concentrations between the control and immunized groups. Although male and female blood samples have been analyzed no significant difference was observed then, they were not shown in these tables.

Table 2. Concentration of Cl in whole blood samples of equines

Groups					
Range gL^{-1}	Mean ± 1SD	Median	Mode	Min Value	Max Value
CG					
1.89-2.93	2.41 ± 0.26	2.31	2.27	1.94	2.99
	3.02 ± 0.48*				
GI					
2.23–2.87	2.55 ± 0.16	2.48	-	2.41	2.81
[a] GII					
1.46–2.34	1.90 ± 0.22	1.90	1.88	1.46	2.29
[a] GIII					
1.78–2.34	2.06 ± 0.14	2.02	2.02	1.82	2.32

SD: standard deviation

* whole blood human estimation[9]

Min: minimum

Max: maximum

[a] *t- test*: no statistical differences (p>0.05) for GII and GIII

Table 3. Concentration of K in whole blood samples of equines

Groups					
Range gL^{-1}	Mean ± 1SD	Median	Mode	Min Value	Max Value
CG					
1.15–2.11	1.63 ± 0.24	1.62	2.13	1.10	2.13
	1.61 ± 0.28 *				
[a] GI					
1.00–2.20	1.60 ± 0.30	1.64	1.64	1.10	1.91
GII					
1.35–2.47	1.91 ± 0.28	2.06	2.08	1.30	2.23
GIII					
1.54–2.18	1.86 ± 0.16	1.88	1.86	1.54	2.16

SD: standard deviation

* whole blood human estimation[9]

Min: minimum

Max: maximum

[a] *t- test* : no statistical differences (p>0.05) for GI

Table 4. Concentration of Na in whole blood samples of equines

Groups	Range gL^{-1}	Mean ±1SD	Median	Mode	Min Value	Max Value
CG	1.48–2.44	1.96 ± 0.24 1.77 ± 0.29 *	1.98	1.78	1.56	2.41
[a] GI	1.63–2.79	2.21 ± 0.29	2.27	-	1.73	2.46
[a] GII	1.51–2.39	1.95 ± 0.22	1.91	2.05	1.62	2.51
[a] GIII	1.66–2.42	2.04 ± 0.19	2.02	2.23	1.75	2.42

SD: standard deviation

* whole blood human estimation[9]

Min: minimum

Max: maximum

[a] t- test *: significant differences for all groups*

According to these tables, Cl values present the most significant difference when compared to the other groups. Particularly for GII and GIII groups the alterations can be associated to the fact that the whole blood samples have been collected of horses from several races submitted to immunization for years while the for the GI the samples were collected at same day after the first immunization procedure. Another aspect that can be emphasized from these results is related to the clinical status of the animals: although some variations in the concentration values have been observed in the immunizeted groups when compared with the control one, no damage in their clinical chemistry performance can be associated considering a confidence interval of 95% usually adopted as reference for clinical practice[4]. Relate to the data analyses involving the comparison with human blood estimation[9] there is agreement for K (table 3)

and Na (table 4), considering a confidence interval of 68% for the CG but for the Cl concentration values the similarity can only be observed considering ±2SD (Standard Deviation).

4. CONCLUSIONS

The concentration for Cl, K and Na were determined in whole blood of equines used for snake antivenom production (Butantan Institute, Brazil) using NAA. The results for the control group (horse not immunized) could be used to interpret, in the future, the behavior of these elements in whole blood as well as in the cells blood (for re-injection), contributing for the immunization process validation nowadays in test at Butantan Institute. The results from the control group also indicated that the occurrence of the elements evaluated were similarly distributed when a comparison is performed with human being whole estimation, suggesting the choice of this race is an adequate model animal for anti-*Bothrops* sera production.

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