J Radioanal Nucl Chem (2012) 291:373–378
DOI 10.1007/s10967-011-1275-8

Elemental analysis of biological tissues of animal models in muscular dystrophies investigation

Sabrina Metairon · Cibele B. Zamboni · Miriam F. Suzuki · Carlos R. Bueno Jr. · Osvaldo A. Sant'Anna

Received: 3 June 2011 / Published online: 19 June 2011

Abstract Element concentrations in biological tissues of Dmd^{mdx}/J and C57BL/6 J mice strains were determined using the neutron activation analysis technique. Samples of whole blood, bones and organs (heart and muscle) of these strains were irradiated in the IEA–R1 nuclear reactor at IPEN-CNEN/SP (Brazil). To perform this investigation biological samples of two-month-old adult females ($n = 10$) and males ($n = 9$) for Dmd^{mdx}/J (dystrophic mice), and males ($n = 12$) for C57BL/6 J (control group), originally obtained from the Jackson Laboratory (Maine, USA) and further inbred at IPEN–CNEN/SP (São Paulo, Brazil), were used. A significant change was observed in the analysis of the heart of dystrophic mice suggesting that this dysfunction affects severely the heart muscle. These data may, in the future, contribute to the healthcare area, in veterinary medicine and in the pharmaceutical industry allowing the evaluation of the best procedures in diagnosis, treatment and investigations of neuromuscular diseases (muscular dystrophy) of patients through the use of animal models.

S. Metairon · C. B. Zamboni · M. F. Suzuki (✉)
Instituto de Pesquisas Energéticas e Nucleares - IPEN/CNEN-SP, Av. Prof. Lineu Prestes, 2242 - Cidade Universitária, São Paulo, SP 05508-000, Brazil
e-mail: mfsuzuki@ipen.br

C. R. Bueno Jr.
Centro de Estudos do Genoma Humano, Instituto de Biociências, Rua do Matão, Travessa 13, 106 - Cidade Universitária, São Paulo, SP 05508-090, Brazil

O. A. Sant'Anna
Instituto Butantan, Av. Vital Brasil 1500 - Butantã, São Paulo, SP 05503-900, Brazil

Keywords Instrumental neutron activation analysis · Whole blood · Muscular dystrophy · Biochemistry analyses · Reference values

Introduction

"I thought humanity to be inflicted with enough evils already. I do not congratulate you, sir, upon the new gift you have made it." [1]. This dramatic announcement was made by Guillaume Duchenne in 1868, who wrote for the first time about the disease. He was referring to the historical description of a particularly progressive and destructive neuromuscular disease, which affects mainly boys. After the Duchenne observation, this condition would be known and commonly named as Duchenne muscular dystrophy (DMD).

Progressive muscular dystrophy is a dysfunction that affects approximately 1 in every 3600–6000 newborn boys in the world [2, 3]. In general, only males are affected and approximately 10% of female carriers can show some disease manifestations, which might affect the cardiac function. Previously considered rare, currently at least thirty different types have been identified. The worldwide incidence of genetic disorders among newborns in United States and in Europe is 3%. In Brazil, according to the latest census conducted by the IBGE (Instituto Brasileiro de Geografia e Estatística), this percentage means more than 5 million people will be affected, and in São Paulo State, the number will be about 1 million [4]. According to the IBGE, the estimated number of births per year of people with some type of muscular dystrophy in São Paulo State is 250, with 170 concentrated in metropolitan areas. As mentioned previously, the probability of a newborn boy being affected by DMD is 1/3500. Therefore, it is

estimated that each year 113 boys will be born suffering from DMD in São Paulo (Brazil) only [4].

All the forms of muscular dystrophy are genetic; they cannot be prevented or reverted. It is a disease of hereditary character and its main characteristic is the degeneration of the membrane that involves the muscular cell, causing its death. DMD is caused by a mutation of the dystrophin gene. The absence of dystrophin (a protein present in muscles) permits an excess of calcium to penetrate the sarcolemma (cell membrane) [5]. This protein is altered causing a critical muscular dysfunction in several body functions [6–8]. Under the microscope the DMD muscle generally shows an advanced stage of dystrophy, with muscle fibers abnormally bigger and surrounded by adipose tissues replacing healthier muscle. While there is no cure for DMD, substantial strategic research activities along with a search for new therapies are currently being tested in clinical trials and in animal models [9], with the goals of improving quality of life and extending life expectancy.

Particularly in Brazil, the Dmd^{mdx}/J strain mouse has been widely used as an animal model in progressive muscular dystrophy investigations. However there is no biochemical characterization for whole blood, bone and organs for this strain. Consequently, the determination of element concentrations in whole blood and biological tissues may help to evaluate the efficacy of the new treatments, as well as compare the advantages of different types of treatment schedules before performing tests in patients with muscular dystrophy. Moreover, a profound knowledge of all pathological characteristics of the animal models is crucial for its reliable use.

Nowadays, the DMD dystrophy is been investigated at IPEN, Instituto de Biociências and Instituto Butantã (Research Centers, from Brazil), using mice genetically modified such as: A/J, Dmd^{mdx}/J and SJL/J. Recently the elemental composition of blood in A/J, Dmd^{mdx}/J and SJL/J mouse lines [10–14] were analyzed and a comparison with human blood revealed physiologic differences among them.

In this study some element concentrations were determined in whole blood, bones and muscles of Dmd^{mdx}/J male and female, and C57BL/6 J (control group) male mice, using the instrumental neutron activation analysis (INAA) technique. These elements were selected due to the clinical relevance for evaluation of electrolyte disorders and nutritional relevance.

Experimental procedure

INAA was applied to conduct this investigation. The animal models, Dmd^{mdx}/J male ($n = 9$), Dmd^{mdx}/J female ($n = 10$) and C57BL/6 J male ($n = 12$), were obtained from Jackson Laboratory (Maine, USA) and later inbred at IPEN – CNEN/SP (São Paulo, Brazil).

Thirty-one mice (4 month-old) were sacrificed and the biological materials were collected. The first step was to collect the whole blood samples: 100 μL (duplicate) were withdrawn from an opening of the mice belly and transferred to $\sim$2.5 cm^2 pieces of Whatman filter paper using a calibrated micro pipette. Each sample was dried for few minutes using an infrared lamp. Standards were prepared from stock solutions of know concentration following the same procedure. The bones (tibiae) and organs (heart and muscle) were removed, weighed and immediately frozen until they were used.

For irradiation each biological sample was weighed and sealed into a polyethylene capsule and irradiated in the IEA-R1 nuclear reactor at IPEN-CNEN/SP (3.5 MW, pool type), in an irradiation position where the ratio between thermal and epithermal neutron flux is >14. The thermal neutron flux utilized ranged from 8.45 10^{11} to 1.19 10^{12} cm^{-2} s^{-1}. For elements determined in blood, a 240 s irradiation followed by 60 s decay and a 900 s count were performed. For organs and bones the experimental conditions are presented in Table 1. The organs and bone samples were transferred to a clean container before counting. Each sample (heart, bone, muscle) was analyzed two times at least. IAEA-A13 Animal Blood, Bovine Liver Powder (SRM 1557b) and Bone Powder (NIST 1486) were used as standards and for analytical quality control.

The measurements of the neutron induced activity of the samples were carried out using an ORTEC Model GEM-60195 detector and an ORTEC 671 amplifier, in pile up rejection mode, coupled to a MCA ORTEC 919E connected to a PC. The background radiation was reduced by employing the iron shield described by Medeiros et al. [15]. The source-detector distance in this experimental apparatus is 12.5 cm. The concentration of each element in each biological sample was obtained by using in-house software [16].

Results and discussion

In Table 2 the certified values and our results for IAEA-A13 Animal Blood, Bovine Liver Powder (NIST 1557b) and Bone Powder (NIST 1486) are presented. Cl and Na impurities were identified in very low concentrations in the filter paper (0.009–0.014 g kg^{-1} for Na and 0.060–0.073 g kg^{-1} for Cl). The Z score values obtained were $|Z\ \text{score}| < 2$, indicating that our results are satisfactory and are within the range of certified data at the 95% confidence level.

In Tables 3, 4, 5 and 6 the element concentrations in whole blood, tibia, muscle and heart of Dmd^{mdx}/J strain are

Table 1 Experimental conditions

Biological material (g) $N = 8$	MV (± 0.1%)	Mass (min)	Mass (max)	Element/Ti: Td: Tc
Tibia	0.0644^{CG}	0.0571	0.0734	Ca/30 s: 60 s: 120 s
	0.0749^{m}	0.0601^{m}	0.0930^{m}	Cl, Mg, Na/30 s: 60 s: 900 s
	0.0585^{f}	0.0464^{f}	0.0762^{f}	
Muscle	0.2118^{CG}	0.1552	0.2546	Ca/240 s: 60 s: 120 s
	0.3592^{m}	0.3321^{m}	0.4309^{m}	Br,Cl, K, Mg, Na/240 s: 60 s:
	0.2572^{f}	0.2014^{f}	0.2923^{f}	900 s
Heart	0.1004^{CG}	0.0893	0.1135	Br,Ca, Cl, K, Mg, Na, S/
	0.1393^{m}	0.1217^{m}	0.1691^{m}	240 s:60 s:900 s
	0.0989^{f}	0.0780^{f}	0.1201^{f}	

N number of samples, *MV* mean value (arithmetic mean wet mass in g), *mass (min)* smaller mass (wet mass in g), *mass (max)* largest mass (wet mass in g), *Ti* irradiation time, *Td* decay time, *Tc* counting time, *CG* control group C57BL/6 J (males), *m* males (Dystrophic–Dmd^{mdx}/J), *f* females (Dystrophic–Dmd^{mdx}/J)

Table 2 Element concentrations obtained in the analysis of NAA compared to the certified values

Element	This work mean ± 1σ	Certified values	RSD, %	Er, %	|Z score|
Br (g kg^{-1})	0.0211 ± 0.0025	0.0220 ± 0.0024[a]	11.85	−4.09	0.37
Ca (mg kg^{-1})	269 ± 11	265.8 ± 2.4[b]	4.09	1.20	1.33
	0.123 ± 0.014	0.116 ± 0.004[c]	11.38	6.03	1.75
	263 ± 44	286 ± 54[a]	16.73	−8.04	0.43
Cl (g kg^{-1})	2.76 ± 0.19	2.78 ± 0.06[c]	6.88	−0.72	0.33
Mg (mg kg^{-1})	4.51 ± 0.31	4.66 ± 0.17[b]	6.87	−3.22	0.88
	0.610 ± 0.054	0.601 ± 0.028[c]	8.85	1.50	0.32
	102 ± 33	99 ± 28[a]	32.35	3.97	0.11
Na (g kg^{-1})	2.5 ± 0.15	2.42 ± 0.06[c]	6.00	3.31	1.33
	13.1 ± 0.31	12.6 ± 1.01[a]	2.37	9.23	0.50
S (g kg^{-1})	7.92 ± 0.47	7.85 ± 0.06[c]	5.93	0.89	1.17
	7.1 ± 0.64	6.5 ± 0.52[a]	9.01	3.03	1.15
K (g kg^{-1})	2.03 ± 0.43	2.50 ± 0.35[a]	21.18	−18.80	1.34

RSD relative standard deviation, *Er* relative error

[a] IAEA A-13 whole blood animal

[b] NIST 1486 bone powder

[c] NIST 1557b bovine liver powder

presented as: the mean value, standard deviation (σ), minimum and maximum values, range for a confidence interval of 95% (adopted for clinical practice) and detection limit (DL). The control group (C57BL/6 J strain) data for males were also included for comparison.

The blood data for Br, Cl, K, Mg, Na and S for dystrophic groups (males and females) are near the lower limit, when compared to the control group (Table 3). However, for Cl, even considering the range of 3σ for the control group (2.51–4.31 gL^{-1}), there is no agreement between the dystrophic strains (1.64–2.36 gL^{-1}, for males and 1.57–2.17 gL^{-1}, for females). As for the females, values for Na (0.84–1.40 gL^{-1}) and S (0.44–1.48 gL^{-1}) are significantly lower, being close to the inferred limit of 3σ observed in the control group (1.43 gL^{-1} for Na and 1.50 gL^{-1} for S). With regard to Ca, which is significantly spread, especially in females, the results are in agreement with 2σ. The elements behavior in blood suggests that this biological material is a good indicator for monitoring and evaluation of DMD progression.

According to Br, Ca, Cl and Na whole blood concentrations, the results (Table 3) are in agreement when compared with the data from Ref. [12], but for K concentration there is no agreement, and this difference is related to the muscular degeneration stage [17].

Table 3 The Br, Ca, Cl, K, Mg, Na and S concentrations (gL^{-1}) in whole blood of Dmd^{mdx}/J and C57BL/6 J male mice ($N = 20$)

Elements (gL^{-1})	Mean ± 1σ	Minimum value	Maximum value	Range 95%	DL
Br	0.0053 ± 0.0007^{CG}	0.0042	0.0062	0.0039–0.0067	0.0009
	0.0019 ± 0.0008^{m}	0.0006	0.0021	0.0003–0.0035	
	$0.0020 \pm 0.0008^{m,a}$	0.0008	0.0022	0.0002–0.0034	
	0.0018 ± 0.0008^{f}				
Ca	0.327 ± 0.098^{CG}	0.190	0.460	0.131–0.523	0.014
	0.284 ± 0.067^{m}	0.241	0.382	0.150–0.418	
	$0.260 \pm 0.12^{m,a}$	0.212	0.458	0.105–0.593	
	0.349 ± 0.122^{f}				
Cl	3.41 ± 0.30^{CG}	2.90	3.65	2.81–4.01	0.01
	2.00 ± 0.18^{m}	1.85	2.06	1.64–2.36	
	$2.86 \pm 0.80^{m,a}$	1.77	2.11	1.57–2.17	
	1.87 ± 0.15^{f}				
K	2.71 ± 0.49^{CG}	2.63	3.43	1.19–3.15	0.45
	1.20 ± 0.11^{m}	1.13	1.37	0.98–1.42	
	$2.97 \pm 0.10^{m,a}$	1.08	1.25	1.02–1.30	
	1.16 ± 0.07^{f}				
Mg	0.027 ± 0.005^{CG}	0.023	0.032	0.017–0.037	0.001
	0.012 ± 0.005^{m}	0.008	0.020	0.002–0.022	
	0.014 ± 0.003^{f}	0.011	0.017	0.008–0.020	
Na	2.06 ± 0.21^{CG}	1.49	2.28	1.64–2.48	0.01
	1.22 ± 0.27^{m}	0.97	1.61	0.68–1.76	
	$1.54 \pm 0.41^{m,a}$	0.94	1.28	0.84–1.40	
	1.12 ± 0.14^{f}				
S	2.40 ± 0.30^{CG}	1.87	2.50	1.80–3.0	0.53
	1.11 ± 0.26^{m}	1.06	1.62	0.59–1.63	
	0.96 ± 0.26^{f}	0.71	1.32	0.44–1.48	

N number of samples, *DL* detection limit, *CG* control group C57BL/6 J (males), *m* males (Dystrophic - Dmd^{mdx}/J), *f* females (Dystrophic–Dmd^{mdx}/J), *a* from ref [12]

Table 4 The Ca, Cl, Mg and Na concentrations (g kg^{-1}) in bone of Dmd^{mdx}/J and C57BL/6 J male mice

Elements, g kg^{-1} $N = 8$	Mean	1σ	Minimum value	Maximum value	Range, 95%	DL
Ca	144.9^{CG}	3.7	89.0	117.5	137.5–152.3	0.46
	122.4^{m}	11.4	107.8	131.7	99.6–145.2	
	121.8^{f}	15.4	108.4	145.3	91.0–152.6	
Cl	1.13^{CG}	0.11	1.03	1.28	0.91–1.35	0.36
	1.13^{m}	0.15	0.97	1.32	0.83–1.43	
	1.10^{f}	0.10	1.01	1.24	0.90–1.30	
Mg	2.10^{CG}	0.30	1.79	2.44	1.50–2.69	0.28
	2.48^{m}	0.14	1.33	2.69	2.20–2.76	
	2.10^{f}	0.39	1.60	2.65	1.32–2.88	
Na	3.50^{CG}	0.61	2.96	4.16	2.28–4.72	0.05
	3.79^{m}	0.60	2.92	4.24	2.59–4.99	
	3.66^{f}	0.58	2.63	4.20	2.50–4.82	

N number of samples, *DL* detection limit, *CG* control group C57BL/6 J (males), *m* males (Dystrophic–Dmd^{mdx}/J, f: females (Dystrophic–Dmd^{mdx}/J))

Table 5 The Br, Ca, Cl, K, Mg, Na and S concentrations (g kg^{-1}) in muscle of Dmd^{mdx}/J and C57BL/6 J male mice ($N = 8$)

Elements (g kg^{-1})	Mean	1σ	Minimum value	Maximum value	Range 95%	DL
Br	0.00030^{CG}	0.00010	0.00021	0.00043	0.00010–0.00050	0.00010
	0.00047^{m}	0.00003	0.00044	0.00050	0.00041–0.00053	
	0.00042^{f}	0.00011	0.00031	0.00055	0.00020–0.00064	
Ca	0.069^{CG}	0.012	0.055	0.083	0.045–0.093	0.007
	0.075^{m}	0.022	0.046	0.094	0.031–0.119	
	0.069^{f}	0.023	0.053	0.095	0.023–0.115	
Cl	0.55^{CG}	0.06	0.47	0.60	0.43–0.67	0.003
	0.58^{m}	0.07	0.49	0.62	0.44–0.72	
	0.65^{f}	0.07	0.57	0.73	0.51–0.79	
K	5.15^{CG}	0.59	4.57	5.96	3.97–6.33	0.13
	5.05^{m}	0.72	4.19	5.94	3.61–6.49	
	4.63^{f}	0.56	4.11	5.28	3.51–5.75	
Mg	0.266^{CG}	0.018	0.247	0.285	0.230–0.302	0.002
	0.223^{m}	0.019	0.201	0.247	0.185–0.261	
	0.223^{f}	0.014	0.209	0.38	0.195–0.251	
Na	0.60^{CG}	0.06	0.53	0.67	0.48–0.72	0.004
	0.61^{m}	0.08	0.50	0.69	0.45–0.77	
	0.64^{f}	0.06	0.56	0.71	0.52–0.76	
S	2.34^{CG}	0.21	2.20	2.64	1.92–2.76	0.36
	2.22^{m}	0.54	1.55	2.86	1.14–3.30	
	2.03^{f}	0.38	1.57	2.43	1.27–2.79	

N number of samples, *DL* detection limit, *CG* control group C57BL/6 J (males), *m* males (Dystrophic–Dmd^{mdx}/J), *f* females (Dystrophic–Dmd^{mdx}/J)

Table 6 The Br, Ca, Cl, K, Na, Mg and S concentrations (g kg^{-1}) in heart of Dmd^{mdx}/J and C57BL/6 J male mice

Elements, (g kg^{-1}) $N = 8$	Mean	1σ	Minimum value	Maximum value	Range, 95%	DL
Br	0.00124^{CG}	0.00022	0.00094	0.00145	0.00080–0.00169	0.00035
	0.00138^{m}	0.00021	0.00117	0.00167	0.00096–0.00180	
	0.00103^{f}	0.00027	0.00065	0.00127	0.00049–0.00157	
Ca	0.035^{CG}	0.016	0.019	0.050	0.003–0.067	0.003
	0.027^{m}	0.004	0.023	0.031	0.019–0.035	
	0.016^{f}	0.005	0.010	0.025	0.006–0.026	
Cl	1.61^{CG}	0.48	1.21	2.28	0.65–2.57	0.009
	1.81^{m}	0.16	1.74	2.06	1.49–2.13	
	1.19^{f}	0.34	0.82	1.63	0.51–1.87	
K	2.64^{CG}	0.53	2.03	3.25	1.58–3.70	0.15
	2.37^{m}	0.47	2.37	2.66	1.43–3.31	
	2.78^{f}	0.48	2.16	3.31	1.82–3.74	
Mg	0.162^{CG}	0.024	0.132	0.185	0.114–0.210	0.007
	0.153^{m}	0.019	0.139	0.179	0.115–0.191	
	0.152^{f}	0.016	0.134	0.172	0.120–0.184	
Na	1.21^{CG}	0.38	0.92	1.75	0.45–1.97	0.007
	1.04^{m}	0.10	0.91	1.12	0.84–1.24	
	1.03^{f}	0.24	0.76	1.36	0.55–1.51	
S	3.03^{CG}	0.52	2.35	3.59	1.99–4.07	0.51
	2.23^{m}	0.48	1.55	2.68	1.27–3.19	
	2.01^{f}	0.63	1.14	3.15	0.75–3.27	

N number of samples, *DL* detection limit, *CG* control group C57BL/6 J (males), *m* males (Dystrophic–Dmd^{mdx}/J), *f* females (Dystrophic–Dmd^{mdx}/J)

Related to the bone results (Table 4), the concentrations for the majority of the elements in Dmd^{mdx}/J subjects fall within the range of 2σ from the control group (137.5–152.3 g kg^{-1}), only for Ca the results (122.4 ± 5.0 g $kg^{-1}$for male and 121.8 ± 4.9 g kg^{-1} for female) are near the lower limit.

For muscle results (Table 5) the concentration values for Br, Cl, Ca, K, Na and S are in agreement with the range of the control group at 2σ, but for Mg the lower limits (0.185 g kg^{-1} for male and 0.195 g kg^{-1} for female) are below this range.

Regarding the analysis performed in the heart (Table 6), the concentrations obtained for all elements in male and female were substantially altered; this may be due to the impact on the heart muscle resulting from DMD that was already manifested [4].

Conclusion

The determination of elements relevant for biochemical analysis in whole blood, bones and organs of Dmd^{mdx}/J and C57BL/6 J mice strain were evaluated by INAA. This research reports the first results for DMD mice organs which may help researchers of MD evaluate and describe the stage of disease progression. This would potentially help, in the diagnosis of DMD and possibly assist in the evaluation of carriers of DMD. Furthermore, these results may also, in the future, with more statistical analysis, help to evaluate and compare the advantages of different types of treatments using an animal model prior to testing in humans. This could lead to an improved quality of life and survival for both humans and companion animals such as Golden Retrievers suffering from DMD.

Moreover, a profound knowledge of all pathological characteristics of the animal model is crucial for its reliable use.

Acknowledgments The authors would like to thanks, Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN/SP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Modern Trends in Activation Analysis–13 (MTAA-13) for the financial support.

References

1. Ivory P (2011). http://www.mdausa.org/ Accessed 26 May 2011
2. Matsuo M (1995) Southeast Asian J Trop Med Public Health 26:166–171
3. Bushby K (2010). http://www.treat-nmd.eu/home.php. Accessed 10 Aug 2010
4. Associação Brasileira de Distrofia Muscular (ABDIM) (2010). http://www.abdim.org.br/ Accessed 22 Nov 2010
5. Han R, Campbell KP (2007) Curr Opin Cell Biol 19:409
6. Blake DJ, Weir A, Newey SE, Davies KE (2002) Physiol Rev 82:291
7. Meola G, Sansone V (2000) Neurol Sci 21:S953
8. Berchtold MW, Brinkmeier H, Muntener M (2000) Physiol Rev 80:1215
9. Muntoni F, Wells D (2007) Curr Opin Neurol 20:590
10. Zamboni CB, Suzuki MF, Metairon S, Carvalho MFD, Sant'Anna OA (2009) J Radioanal Nucl Chem 281:97
11. Zamboni CB, Zahn GS, Sant'Anna OA (2007) VI Latin American Symposium on Nuclear Physics and Applications. In: Kreiner A J et al. (eds) AIP conference proceedings, vol 884. Springer, New York, p 507
12. Zamboni CB, Suzuki MF, Sant'Anna OA (2008) J Radioanal Nucl Chem 278:585
13. Aguiar R, Zamboni CB, Genezini FA (2009) XXXI workshop on nuclear physics in Brasil. In: Guimarães V et al. (eds) AIP Conference Proceedings, vol 1139. American Institute of Physics, Melville, p 204
14. Zamboni CB, Metairon S, Suzuki MF, Furtado MF, Sant'Anna OA, Tambourgi DV (2009) J Radioanal Nucl Chem 282:37
15. Medeiros JAG, Zamboni CB, Lapolli AL, Kenchian G, Da Cruz MTF (2001) Appl Radiat Isot 54:245
16. Medeiros JAG, Zamboni CB, Zanh GS, Oliveira LC, Dalaqua Jr L (2005) Proceedings of 39th Congresso Brasileiro de Patologia Clínica, 19 oct. São Paulo, CD ROM
17. The Jackson Laboratory (JAX) (2011). http://www.jax.org/ Accessed 21 May 2011