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Tracking inorganic elements in GRMD blood dogs submitted to hASCs investigated by NAA technique

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Abstract Investigation in blood of specific elements concentration are an important step in the development of new treatments. Recently, studies with human adipose derived from stromal cells injected systemic (medication that affects the entire body) into a golden retriever muscular dystrophy dogs shown to be able to express improvements in skeletal muscle. To check the alterations that this cell therapy may cause in the dogs, elements concentration in blood of treated dogs were investigated using NAA. These results were compared with the control and affected (untreated) dogs groups showing an improvement in Ca and Fe blood levels in treated dogs.

Keywords Blood · NAA · Inorganic elements · Duchenne muscular dystrophy · hASCs treatment · Golden retriever

Introduction

Progressive muscular dystrophies (PMDs) are characterized by presenting an irreversible progressive degeneration of skeletal muscle. More than thirty different forms of muscular dystrophies have been identified, varying the complexity and all affect the muscles. However, the affected muscles may be different according to the type of PMDs [1]. The Duchenne muscular dystrophy (DMD) is the most prevalent and aggressive [2–4]. Currently, no effective treatment is available and the DMD research progress depends on animal models, faithful to human pathology. To verify the effectiveness and safety of new therapies preclinical testing need to be performed [5]. The DMD can be diagnosed by muscle biopsy (usually in the lower limbs). The muscle is evaluated and extremely low levels of dystrophin protein can be found. The dystrophin is responsible for the skeletal muscle repair. The reduction or absence of dystrophin on the muscle cells causes the rupture of the surface of the sarcolemma (muscle cell membrane). Through this opening there will be a greater influx of Ca in the muscle fiber [6]. Larger quantities of Ca increase oxidative stress, leading to lacerations and necrosis of the muscle fiber, both skeletal and cardiac muscles, and also in some cerebral neurons [7, 8]. In the last years, the DMD dystrophy has been investigated in Research Centers from Brasil using mice genetically modified, namely Dmd^{mdx}/J (these mutants do not express dystrophin and the resultant myopathology is much less severe compared to the human DMD disease course), SJL/J (it is the murine model of Limb-Girdle Muscular Dystrophy 2B and carries a deletion in the dysferlin gene) and A/J (this strain characteristic is a late onset from 4 to 5 months of progressive muscular dystrophy as a result of a homozygous retrotransposon insertion in the dysferlin gene) [9]. The elemental composition of blood in these dystrophic strains were analyzed and a comparison with human blood estimation revealed significant changes mainly in Ca and Mg blood levels [10–13]. Since mice don't show an acute muscle weakness the use of these animal models become limited. It's a good genetic and biochemical model but not as a human DMD model for a

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clinical investigation [5]. The animal model that has a similar phenotype to the DMD carrier patients are the Golden Retriever dogs [Golden retriever muscular dystrophy (GRMD)]. In this model, the changes of muscle degeneration and fibrosis predominates leading to a progressive loss of muscle structure and function resembling to a human pathogenesis [5]. Recently, the possibility of a new treatment for DMD with cellular therapy began to be investigated in the Human Genome Research Center (Sao Paulo city, Brazil). Vieira et al. [14] showed that cells derived from human adipose stromal cells (hASCs) injected in the SJL/J mice, were able to reach and engraft the host muscle, improving the functional performance of the animals. Currently, this treatment is being investigated in an experiment using the animal model GRMD [14]. In order to show in more detail the progress of the hASCs treatment on GRMD dogs, inorganic elements like Ca and Mg (which revealed change in blood of some dystrophic mice strains [10–13]) as well others elements of clinical relevance in blood, were investigated by NAA. These data help to evaluate the safety related to the inorganic elements toxicity (mainly in blood) that this cellular therapy may cause in this animal model before to perform tests in patients with muscular dystrophy.

Experimental

To perform this investigation, biological samples of 2–10 years old GRMD dogs, Control ($n = 5$) and Affected ($n = 7$) that were bred at Biosciences Institute/USP (São Paulo, Brasil), was collected. Two of the seven affected dogs were submitted to hASCs treatment. Serum samples have traditionally been used as the main source of clinical analysis. However, for this investigation we used whole blood samples [15]. This alternative procedure has been adopted in our clinical investigations with advantages over conventional procedures [16]. This procedure consists of collecting blood from a cephalic vein and subsequent deposit of a small amount of blood on a filter paper (100 μL, in duplicate) for short irradiation time (minutes). Using this procedure Br, Ca, Cl, Mg, Na and S were investigated. For Fe determination, 500 μL were stored in plastics cylinders and immediately frozen, lyophilized and stored at room temperature until 4hs of irradiation. Each biological sample and standard (certified standard solutions) were irradiated in the IEA-R1 nuclear reactor at IPEN (IEA-R1, 4.5 MW, pool type). The whole blood collections were held for all animals before the treatment started and after the transplantation stages (6 months period). The measurements of the gamma induced activity of the sample and standard were carried out using a 60 % efficiency high-purity Germanium detector (GEM-60195, ORTEC Model) and an amplifier (ORTEC- 671) coupled to a MCA (ORTEC-919E) connected to a PC. The concentration of each element in each blood sample was obtained by using in-house software [17]. The IAEA-A13 Blood Animal was used for analytical quality control and the *Z*-score values indicated that the results were satisfactory for a confidence interval of 95 %.

Results and discussion

In Table 1 the whole blood element concentrations of GRMD dog control and affected (but not submitted to hASCs treatment) are presented as the mean value (MV)

Table 1 The Br, Ca, Cl, Fe, K, Mg, Na and S concentrations for control group (CG) and affected group (AG)

Elements	CG ($n = 5$) [range]	AG ($n = 7$)
Br (mg L^{-1})	[2.3–4.7]	
MV ± 1 SD	3.5 ± 0.6	3.3 ± 1.3
Min	2.6	1.5
Max	4.3	4.6
Ca (g L^{-1})	[0.013–0.145]	
MV ± 1 SD	0.079 ± 0.033	0.124 ± 0.035
Min	0.049	0.066
Max	0.132	0.169
Cl (g L^{-1})	[1.78–3.74]	
MV ± 1 SD	2.76 ± 0.49	2.71 ± 0.83
Min	1.92	1.41
Max	3.19	3.33
Fe (g L^{-1})	[0.521–0.641]	
MV ± 1 SD	0.581 ± 0.030	0.584 ± 0.117
Min	0.555	0.375
Max	0.607	0.760
K (g L^{-1})	[0.081–0.173]	
MV ± 1 SD	0.127 ± 0.023	0.175 ± 0.055
Min	0.105	0.102
Max	0.152	0.267
Mg (g L^{-1})	[0.003–0.055]	
MV ± 1 SD	0.029 ± 0.013	0.019 ± 0.011
Min	0.017	0.002
Max	0.049	0.029
Na (g L^{-1})	[1.75–3.71]	
MV ± 1 SD	2.73 ± 0.49	2.85 ± 0.64
Min	2.00	1.64
Max	3.34	3.67
S (gL^{-1})	[0.05–1.29]	
MV ± 1 SD	0.67 ± 0.31	0.77 ± 0.28
Min	0.33	0.20
Max	0.97	1.07

n number of samples

from duplicate analyses, standard deviation (±1 SD), minimum (Min) and maximum (Max) values and the range for the control group (for a confidence interval of 95 % usually adopted for clinical practice). In Table 2, the whole blood element concentrations of the treated GRDM dogs (A_1 and A_2) are presented before and after hASCs treatment (after 6 months) as mean value from duplicate analyses and one standard deviation (68 %). In this table the data for untreated group were also included (blood collection was performed together with the treated group, i.e., before and after 6 months of starting treatment).

According to Table 1 (CG and AG not treated) the concentrations of Br, Cl, Na, Fe and S for affected group are in agreement with the normal range established by the control group. For Ca and Mg a significant variation in the blood ($p < 0.05$) can be noticed. Ca level showed an increase in 57 % of the affected group, probably due to disruption of the sarcolemma [5, 6] and Mg level, a decrease of 35 %. A decrease of Mg levels was also observed in the Dmd^{mdx}/J mice, however for Ca the behavior is reversed showing a decrease in the blood levels of the Dmd^{mdx}/J mice [12, 13].

Another aspect to consider is the increase in K of 38 % ($p < 0.05$), suggesting also a damage in the cardiac muscle on the affected GRMD [7, 8]. The same behavior was also observed in blood levels of Dmd^{mdx}/J mice strain [12]. A comparison between control and affected group dogs (CG and AG not treated) can be viewed in Fig. 1.

In Table 2 Cl, Na and S blood concentrations for treated group are keeping constante and in agreement with the

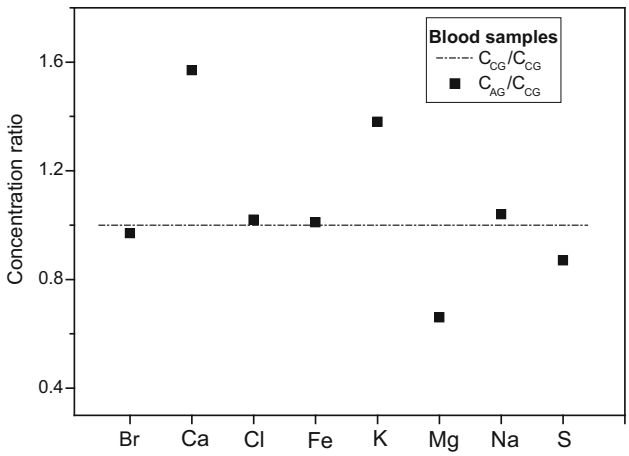

Fig. 1 Ratio between the mean of the affected group (C_{AG}) blood concentrations and the mean of the control group (C_{CG}) blood concentrations

normal range established by the control group. Yet, according to this table, Br blood levels after treatment were higher, near of the upper limit (5.3 mg L^{-1}) for a confidence interval of 99 %. This variation can be related to administration of different rations, which emphasizes the need for analysis in the diet of these animals. That will generate information for comparative analysis (between concentrations) and adequate nutritional monitoring of these animals. Figure 2 shows individual Br behavior emphasizing an increase, mainly for untreated animals (one of the GRMD dogs died before treatment started, which show a missing point on this figure). These results suggest

Table 2 The Br, Ca, Cl, Fe, K, Mg, Na and S concentrations for affected GRMD dogs before and after hASCs treatment

Elements (range)	Whole blood samples (A_n)	Before	After[a]
Br (mg L^{-1})	A_1	3.3 ± 0.4	5.9 ± 0.6
	A_2	3.9 ± 0.3	5.2 ± 0.6
Ca (g L^{-1})	A_1	0.169 ± 0.023	0.081 ± 0.019
	A_2	0.110 ± 0.015	0.071 ± 0.018
Cl (g L^{-1})	A_1	3.26 ± 0.18	3.35 ± 0.17
	A_2	3.33 ± 0.18	3.26 ± 0.15
Fe (g L^{-1})	A_1	0.375 ± 0.027	0.441 ± 0.028
	A_2	0.571 ± 0.040	0.463 ± 0.032
K (g L^{-1})	A_1	0.102 ± 0.014	0.109 ± 0.017
	A_2	0.216 ± 0.068	0.214 ± 0.017
Mg (g L^{-1})	A_1	0.0265 ± 0.0043	0.0490 ± 0.0034
	A_2	0.0272 ± 0.0037	0.0470 ± 0.0033
Na (g L^{-1})	A_1	2.64 ± 0.14	3.21 ± 0.14
	A_2	3.24 ± 0.18	3.05 ± 0.14
S (gL^{-1})	A_1	0.82 ± 0.25	0.75 ± 0.22
	A_2	0.77 ± 0.22	0.82 ± 0.24

A_n blood samples of GRMD affected dogs ($n = 1, 2$)

[a] After 6 months of cellular transplantation procedure

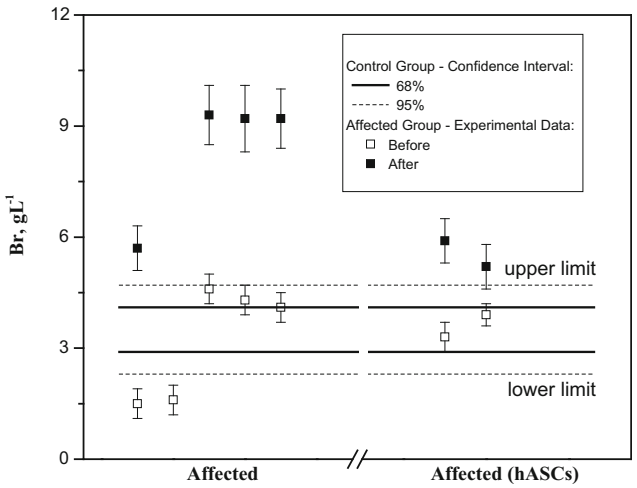

Fig. 2 Br concentrations for affected groups (untreated and hASCs treated)

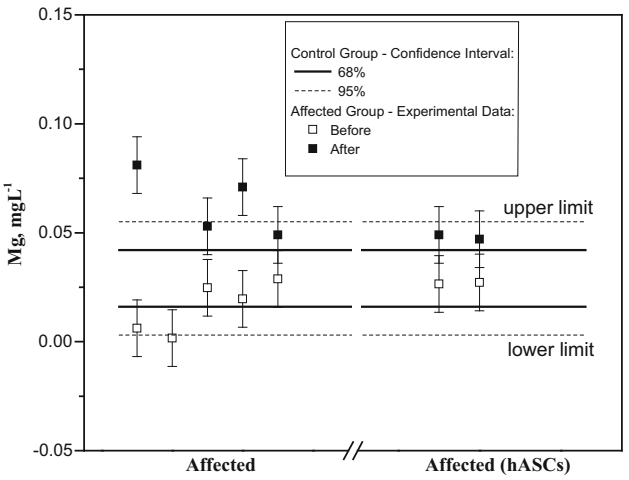

Fig. 4 Mg concentrations for affected groups (untreated and hASCs treated)

that an unbalanced diet can interfere with blood levels of patients with DMD. The study by Santos [18] also emphasizes that, the nutritional management of patients with mild muscular dystrophy, is critical to maintaining quality of life. In Figs. 3, 4 and 5 are shown the concentration values for Ca, Mg and Fe among affected groups (untreated and hASCs treated). According to the individual data for treated dogs, Ca and Fe showed an improvement while for Mg is maintained constant. However, in untreated animals there is an increase in Mg levels (Fig. 4) and a decrease in Fe levels (Fig. 5). Alterations in Mg levels can be related to the sarcolemma rupture. The decrease of Fe levels might be related to the replacement of muscle cells by adipose ones and the reduction of red blood cells (anemia). In dystrophic muscle, reduction or absence of

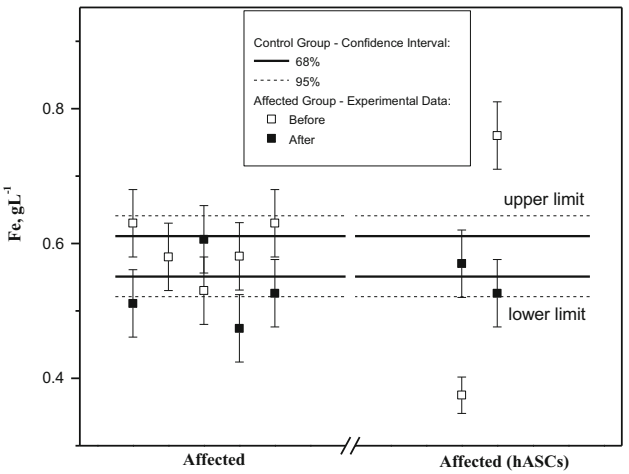

Fig. 5 Fe concentrations for affected groups (untreated and hASCs treated)

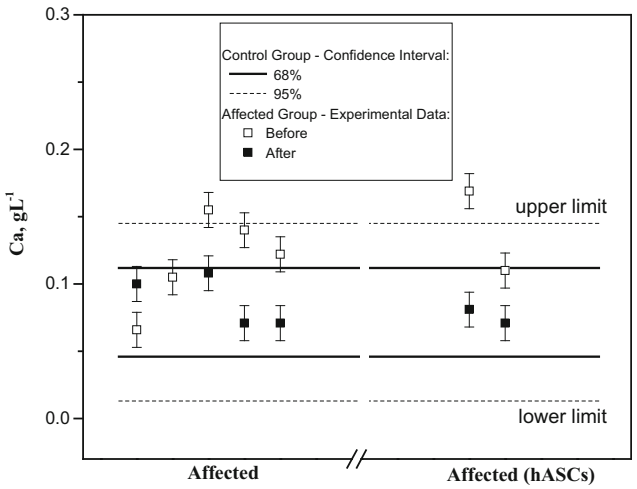

Fig. 3 Ca concentrations for affected groups (untreated and hASCs treated)

dystrophin protein leads the rupture of the surface of the sarcolemma (muscle cell membrane). As a result, through this opening, there is a great influx of Ca and Mg ions in the muscle fibers by altering the mechanism of membrane permeability control in these cells [4, 8]. Both Ca and Mg lead to muscle necrosis that can affect skeletal and cardiac muscle and also some cerebral neurons. When muscle necrosis occurs, it results in cell death that will be replaced by adipose cells [7, 8]. Related to the K levels, there is a decrease in untreated animals (Fig. 6) while for the treated animals these levels are maintained constant. Specifically, for one of the treated animals ($A_2 = 0.214 \pm 0.017$ g L^{-1}, see Table 2) the K concentration is keeping above the control group, even considering a confidence interval of 99 % (0.196 g L^{-1}), suggesting a probable damage to the cardiac muscle [7, 8].

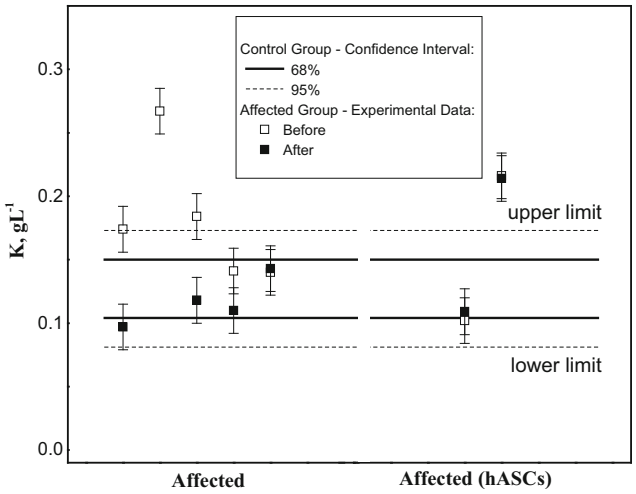

Fig. 6 K concentrations for affected groups (untreated and hASCs treated)

Conclusion

The blood analyses for the treated group showed no changes that might adversely affect the continuity of the GRMD treatment. Besides, Ca and mainly Fe blood levels are showing a significant improvement in the treated dogs while Mg and K are maintained constant. Only Br blood levels after treatment were higher, which can be related to the different diet of these animals. We intend to repeat this research during and after the treatment (every 6 months, period set for the cell treatment) over the next 2 years for best interpretation of the behavior of these elements.

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