

Research Article

Preclinical Studies with Umbilical Cord Mesenchymal Stromal Cells in Different Animal Models for Muscular Dystrophy

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Umbilical cord mesenchymal stromal cells (MSC) have been widely investigated for cell-based therapy studies as an alternative source to bone marrow transplantation. Umbilical cord tissue is a rich source of MSCs with potential to derivate at least muscle, cartilage, fat, and bone cells *in vitro*. The possibility to replace the defective muscle cells using cell therapy is a promising approach for the treatment of progressive muscular dystrophies (PMDs), independently of the specific gene mutation. Therefore, preclinical studies in different models of muscular dystrophies are of utmost importance. The main objective of the present study is to evaluate if umbilical cord MSCs have the potential to reach and differentiate into muscle cells *in vivo* in two animal models of PMDs. In order to address this question we injected (1) human umbilical cord tissue (hUCT) MSCs into the caudal vein of *SJL* mice; (2) hUCT and canine umbilical cord vein (cUCV) MSCs intra-arterially in GRMD dogs. Our results here reported support the safety of the procedure and indicate that the injected cells could engraft in the host muscle in both animal models but could not differentiate into muscle cells. These observations may provide important information aiming future therapy for muscular dystrophies.

1. Introduction

Mesenchymal stromal cells (MSCs) have been extensively explored over the last years to understand their stem cell properties and clinical application. MSCs were first isolated from bone marrow (BM), but similar populations have been reported afterwards in other tissues, such as adipose tissue, dental pulp, placenta, umbilical cord, and fallopian tube [1–4]. They comprise a population of cells with ability to self-renew and differentiate into specific functional cell types including chondrocytes, osteocytes, adipocytes, and myocytes *in vitro*. However, examining the differentiation potential of MSCs *in vivo* still stands as one of the most important way to address their stemness capacity and direct their use for future cell-based therapies.

Among the genetic diseases of great medical relevance are the progressive muscular dystrophies (PMDs), a clinically and genetically heterogeneous group of disorders for which

there is no cure. They are caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function [5]. Duchenne muscular dystrophy (DMD), which affects 1 in 3500 male births, is the most common and severe form. It is caused by mutations in the dystrophin gene leading to the absence of the muscle dystrophin protein, an essential component of skeletal muscle [6]. The clinical course of DMD is severe and progressive. Affected individuals exhibit muscular weakness by the age of 5 years, lose their independent ambulation around 12 years, and, without special care, they succumb due to respiratory failure or cardiomyopathy in their late teens or early twenties [5].

The murine model for DMD, the mdx mouse, also lacks muscle dystrophin. However, in opposition to affected boys, they have an almost normal phenotype [7, 8]. On the other hand, the golden retriever muscular dystrophy dog (GRMD) has a frameshift point mutation within the splice acceptor

site in intron 6 of the dystrophin gene, which results in the complete absence of the muscular protein [9]. Although the disease course is variable, and neonatal death is frequent, with very rare exceptions [10], GRMD dogs are severely affected and represent the best animal model for human DMD.

Differently from DMD, the limb-girdle muscular dystrophies (LGMDs) constitute a subgroup of 22 different forms identified until now, most of them with autosomal recessive inheritance [11, 12]. They are characterized by the involvement of the pelvic and shoulder girdle musculature. Among the autosomal recessive forms, one of the most prevalent is caused by mutations in the dysferlin gene resulting in two phenotypes: miyoshi myopathy (MM) which affects distal muscles at onset, with preferential early involvement of the gastrocnemius and LGMD2B with a more pronounced limb-girdle involvement [13]. Dysferlin expression is reduced or absent in these patients [14, 15].

A 171-bp in-frame deletion in the murine dysferlin cDNA was identified in a mouse model, the *SJL* mice, with a corresponding reduction in dysferlin levels to 15% of normal. The spontaneous myopathy of the *SJL* mice begins at 4–6 weeks of age and is nearly complete by 8 months of age with a progressive inflammatory change in muscle [16]. The *SJL* mice deletion is in-frame and, therefore, does not cause a total absence of the protein.

Adult skeletal muscle has the potential to regenerate new muscle fibers by activating a population of mononucleated precursors, which otherwise remain in a quiescent and non-proliferative state [17]. However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to a depletion of satellite cells, and, consequently, the capability to restore the skeletal muscle is lost [18, 19]. The possibility to repair the defective muscle through cell therapy is a promising approach for the potential treatment of PMD, independently of specific mutations.

We have recently shown that human umbilical cord tissue (hUCT) is a rich source of MSC with ability to differentiate into skeletal muscle cells *in vitro* [3, 20]. We also described that canine MSCs could be isolated from umbilical cord vein (cUCV) and that they represent a good candidate for preclinical studies [21]. Human umbilical cord MSCs are obtained after full-term delivery of the newborn, from a sample that would be inevitably discarded. The process is noninvasive, painless, and without harm for the mother or the infant. These cells also lack the expression of the major histocompatibility complex (MHC) class I and II antigens which render them to be highly tolerated in transplantations [22, 23] and excellent candidates for cell replacement therapy in PMDs.

However, it is not known if umbilical cord MSCs show the same *in vitro* muscle differentiation capacity as *in vivo*. In order to address this question we injected umbilical cord MSCs in two different animal models of PMDs, *SJL* mice, and GRMD dogs, aiming to compare their ability to engraft into the host muscle and express muscular proteins. Although the injected cells could reach the musculature in both animal models, they were unable to differentiate into muscle cells. In GRMD dogs, it is very difficult to evaluate the therapeutic effect of any procedure due to their great clinical

variability. However, we observed that *SJL*-injected mice had a functional performance significantly better than the control noninjected animals. These results may have important implications for future therapeutic approaches.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the human research ethics committee (*Comitê de ética em pesquisas humanas*—CEP) and by the animal research ethics committee (*Comissão de ética no uso de animais em experimentação*—CEUA) of Institute of Bioscience and University Hospital of University of São Paulo. hUCT MSC were collected from donated umbilical cord (UC) units, after all mothers sign the written informed consent, in accordance with the ethical committee of Institute of Bioscience and University Hospital of University of São Paulo (CEP), permit number 040/2005. Animal care and experiments were performed in accordance with the animal research ethics committee (CEUA) of the Biosciences Institute, University of São Paulo, permit number 034/2005.

2.2. Animal Models. *SJL* mice were purchased from the Jackson Laboratory. The GRMD dog colony was established with a female GRMD carrier, Beth, donated by Dr. Joe Kornegay (University of North Carolina). All animals were housed and cared for in the University of Sao Paulo.

GRMD dogs were genotyped, at birth, from blood genomic DNA extracted with the kit GFX Genomic (GE Healthcare). For PCR reaction, the primers GF2 and GR2 and the temperature conditions were used as previously reported [24]. DMD diagnosis was confirmed by the digestion of PCR products with the enzyme Sau96I (New England Biolabs) and by elevated serum creatine kinase (CK) levels.

Dog leukocyte antigen (DLA)-identical littermate donor/recipient pairs were determined based on the identity for highly polymorphic MHC class I (C.2200) and MHC class II (C. 2202) microsatellite markers, formerly described [25].

2.3. Harvesting and Expansion of hUCT and cUCV MSC. Human umbilical cord (UC) units were collected and transferred to the laboratory under sterile conditions. hUCT and cUCV were isolated, characterized, and expanded as described elsewhere [3, 21]. Briefly, UC of full-term deliveries were filled with 0.1% collagenase (Sigma-Aldrich) in phosphate-buffered saline (PBS, Gibco) and incubated at 37°C for 20 minutes. Then, each UC was washed internally with proliferation medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco) supplemented with 10% of fetal bovine serum (FBS; Gibco). Detached cells were harvested after gentle massage of the umbilical cord and centrifuged at 300 g for 10 minutes. Cells were resuspended in proliferation medium, seeded in 25-cm² flasks, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours of incubation, nonadherent cells were removed, and culture medium was replaced every 3 days. Adherent cells were cultured until reaching 90% confluence and passaged using TrypLE (Invitrogen).

2.4. Cell Transplantation

2.4.1. SJL Mice. Two-month-old SJL mice were divided into two groups ($n = 7$): experimental (group A) and control (group B). Each animal from group A was injected in the tail vein with 1×10^6 of hUCT MSCs in 0.1 mL of Hank's Buffered Salt Solution (HBSS, Gibco). The animals were injected for 6 months, weekly in the first month and then monthly. The control group B were uninjected animals. All results were analyzed blindly. The code for each of the mice groups was disclosed only after the completion of all the studies. Two months after the last cell transplantation, the animals were euthanatized using a CO₂ chamber.

2.4.2. GRMD Dogs. Approximately 1×10^7 cUCV or hUCT cells were injected through the femoral artery of 3 GRMD dogs. Dogs were sedated, and the injections were performed using a 22 gauge intravenous catheter connected to the injection syringe containing the cells resuspended in a final volume of 10 mL of HBSS. Transplantation protocol started when dogs were 51-day old, and each dog received 7 consecutive injections with 30-day interval. All dogs were given standard supportive care and have been followed up during all experimentation. Completion time of the study was determined when the dogs died of natural DMD-related causes.

2.5. Muscle Biopsies. In the SJL study, muscle biopsies were collected after animals were euthanized. Samples were taken from distal and proximal muscles localized in the hind leg and foreleg of both experimental and control animals.

In GRMD dogs, biopsies were obtained from *biceps femoralis*. The first procedure (B144) was realized two days after the third injection, when dogs were 144-day old. The second procedure (B312) was done 3 days after the seventh injection, when dogs were 312 days old. During these procedures, the animals were under effect of anesthesia and sedation.

Each biopsy was divided into two pieces. The first piece was reserved for histological analysis and prepared by embedding in optimal cutting temperature compound and stocked in liquid nitrogen. The other fragment was used for molecular analysis and prepared by snap freezing in liquid nitrogen.

2.6. Engraftment Analysis. DNA samples were obtained from muscle biopsies using DNeasy Blood & Tissue Kit (Qiagen). The presence of human DNA in the host was evaluated as described in Pelz et al. [26].

To detect the presence of Y chromosome in the female-injected animals, we evaluated the amplification of the sex-determining region Y (SRY) gene by PCR using the primers and temperature conditions previously described [27]. PCR products were separated by electrophoresis on 6% polyacrylamide gels and stained with ethidium bromide. Nonsaturated digital images were obtained using ImageQuant imaging system (GE HealthCare).

2.7. Protein Analysis. Immunohistochemistry (IHC) and western blot (WB) were performed according to the methodologies previously described [28]. The following primary antibodies were used: antidystrophin NCL-DYS1 and NCL-DYS2 (Novocastra Laboratories); specific antihuman-dystrophin MANDYS106 2C6 and MANDYS108 4D8 (a kind gift from Dr. Glenn Morris and Dr. Nguyen thi Man, from the North East Wales Institute, Wrexham, UK); specific antihuman nuclei MAB1281 (Chemicon).

For IHC, samples were incubated with antimouse IgG-Cy3-conjugated secondary antibody (Chemicon), and, when necessary, slides were counterstained with 4', 6-diamidino-2phenylindole (DAPI; Sigma). Slides were examined in Axiovert 200 microscope (Carl Zeiss), and images were captured using Axiovision 3.1 software (Carl Zeiss).

For WB, horseradish peroxidase (HRP)-conjugated antimouse secondary antibody (Santa Cruz Biotechnology) was used to detect immunoreactive bands with enhanced chemiluminescence (ECL) plus kit (GE Healthcare).

2.8. Functional Assessment. In order to verify whether injected hUCT MSCs would improve motor ability in SJL-injected mice, we performed motor ability tests before and after 2 months of the last injection. Mice were examined, weighed, and submitted to the following tests: (a) the *inclined plane test* evaluated by measuring the maximal angle of a wood board on which the animal was placed until it slipped; (b) the *wire hanging test* to determine the ability of the mouse suspended on a horizontal thread by its forelegs, to reach it with its hind legs and the length of time they were able to stay hanging; (c) the *ambulation test* which was performed to determine the mean length of a step measured in hindfoot ink prints while mice freely run in a corridor (length: 50 cm; width: 8 cm; height of lateral walls: 20 cm) [29].

2.9. Statistical Analysis. Observations were quantified blindly. Numerical data are the mean \pm sd (standard deviation). The statistical analysis of the equivalence between the injected and uninjected mice was achieved by the one-tailed Student's *t*-test, at the significance level of $P = 0.05$, and the results were expressed by the percentage variation between their performance before and after hUCT MSC transplantation.

3. Results

3.1. DMD Typing, DLA Matching of Littermates and Transplantation Setup. For this study, we had 3 affected GRMD dogs available from same litter: 1 affected male, L3M6; two affected females, L3F1 and L3F2. However, only the dogs L3M6 and L3F1 had DLA-histocompatible pairs from the male littermates L3M7 and L3M5, respectively. The donor-recipient pairs with DLA identity were chosen where the recipients possessed the dystrophin mutation, and the donors were wild-type littermates. Thus we injected cUCV cells from DLA-compatible donors into the dogs L3M6 and L3F1. Since L3F2 did not have any DLA-compatible donor, we injected this dog with male hUCT MSCs. Both cell type

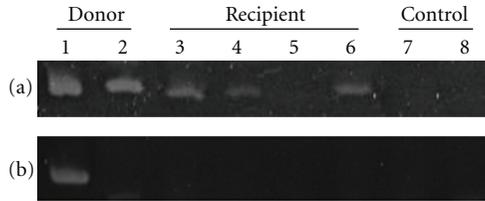


FIGURE 1: Engraftment of male human and canine umbilical cord MSCs into muscle of female GRMD dogs. Polymerase chain reaction analysis for (a) sex-determining region Y (SRY) sequence and (b) canine-specific dystrophin sequence. Muscles samples shown are the following: (1) male cUCV MSC from L3M5; (2) male hUCT MSC; (3, 4) B144 and B312 from affected female L3F1; (5, 6) B144 and B312 from affected female L3F2; (7) female canine control DNA; (8) female human control DNA.

injected were previously characterized by immunophenotyping and differentiation potential [3, 21].

3.2. Capacity of Umbilical Cord MSCs to Reach and Engraft at the Host Muscle of Transplanted Animals. In order to verify if human and canine umbilical cord MSCs were able to reach and colonize the host muscle, we analyzed the biopsies of transplanted female dogs, L3F1 and L3F2, which received male cUCV and hUCT cells respectively. By PCR analysis, we detected the presence of the Y-chromosome marker SRY in muscle biopsies of both affected females, indicating the presence of the injected cells in the musculature of these animals (Figure 1). In addition, scattered human cells were confirmed in the affected female L3F2 by immunohistochemistry (IHC) using specific antibody for human nuclei (Figure 2).

We also found similar results in the *SJL* mice model injected with hUCT MSCs into the caudal vein [30]. PCR analysis detected human DNA in the foreleg and hind leg muscles of all seven injected mice (data not shown).

3.3. Muscle Proteins in Transplanted Animal Models. To explore the myogenic differentiation followed by the engraftment of umbilical cord MSCs, we analyzed the expression of dystrophin in the host muscle of transplanted GRMD dogs. Through western blot (WB) analysis (Figure 3(a)), no dystrophin was found in the muscles of injected animals indicating that the engrafted cells were unable to produce muscular proteins. In addition, we did not observe the expression of human dystrophin, by IHC analysis (Figure 4) or RT-PCR analysis (data not shown), in the muscle biopsies of the affected female L3F2 that received hUCT MSCs.

Three months after we finalized the injections in GRMD animals, the dog L3M6 died of natural GRMD-related causes. Aiming to investigate if the injected cells into the femoral artery were able to spread all over his body, reach different muscular groups, and restore the dystrophin expression, we collected eight different muscle samples at his necropsy. However, no dystrophin expression was found by WB analysis in any analyzed tissue (Figure 3(b)).

Similarly from what we observed in GRMD dogs, hUCT MSCs were able to engraft in the host muscle of injected *SJL*

mice but were not able to differentiate into muscle cells and express human dystrophin (data not shown) [30].

3.4. Functional Assessment. Clinical assessment in GRMD dogs is very difficult due to the great variability in their clinical course [10]. From the 3 transplanted animals, one affected male dog (L3M6) and one affected female dog (L3F2) died 3 months after the last cell injection at 414 days of age and at 3 years and 5 months of age, respectively. However, the female affected dog L3F1 is alive at the age of 4 years.

In *SJL* mice, we performed three standardized motor ability tests and compared their performance before and after cell transplantation [30]. Our results showed a statistically significant difference between the two groups. While uninjected animals worsen significantly their performance, in the injected group, the disease remained stable ($35.14 \pm 9.55\%$ versus $13.47 \pm 10\%$; $P = 0.0014$, Student's *t*-test, $n = 7$).

4. Discussion

Repairing skeletal muscle damage is a challenge for cell-based therapies, given the unique architecture of the tissue, which comprises around 640 types of skeletal muscles that make up about 40 percent of the body's weight in a normal individual. Thus, the successful use of stem cell for clinical application in PMDs will depend on finding an easily obtainable source that could be expanded in quantities suitable to reach the entire musculature, engraft, and restore the defective protein. Although high levels of engraftment are very difficult to be achieved, it has been reported that levels of 20–30% are able to ameliorate dystrophic pathologic lesions [31, 32].

In the past decades, human umbilical cord has been used as an alternative source to bone marrow for cell-based therapies because of its hematopoietic and mesenchymal cell components. We recently showed that hUCT is a richer source of MSCs in comparison to human umbilical cord blood (hUCB) [3, 20]. In addition, we demonstrated that MSC from hUCT and hUCB have different gene expression profiles [33]. Since umbilical cord is easily obtained and a rich source of MSCs, we investigated their ability to originate muscle cells *in vivo* and restore the expression of defective muscular proteins in different animal models of PMDs.

Jazedje et al. [34] and Gang et al. [35] demonstrated that both hematopoietic and mesenchymal stromal cells, respectively, from umbilical cord blood were able to differentiate into skeletal muscle *in vitro*. In addition, Secco et al. [3] reported the myogenic potential, *in vitro*, of MSCs from human umbilical cord tissue. Although different cell populations from umbilical cord show apparently a similar ability to differentiate into muscle cells at least *in vitro*, preclinical studies are of utmost importance to verify if this also happens *in vivo*.

Kong et al. [36] injected human umbilical cord blood cells intravenously into *SJL* mice. These authors reported that a small number of cells engrafted in the recipient muscle and were capable of myogenic differentiation. More recently, Kang et al. [37] reported a boy that was cured of chronic granulomatous disease (CGD) after being transplanted with

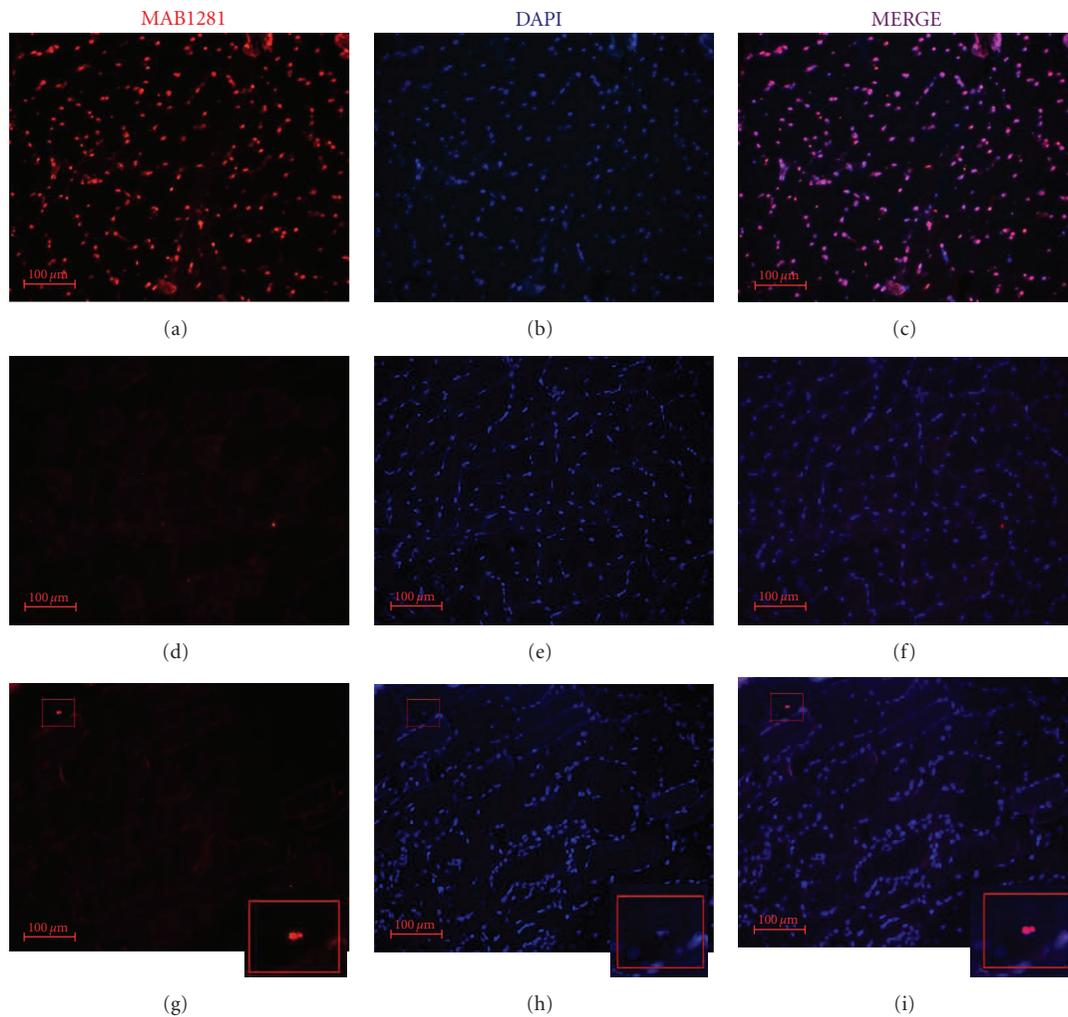


FIGURE 2: Presence of human nuclei at recipient dog muscle after umbilical cord MSCs transplantation. Scattered human cells into *biceps femoralis* (B312) of affected female L3F2 identified by the antihuman nuclei antibody MAB1281. Preparations were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). (a–c) human muscle; (d–e) nontransplanted canine muscle; (g–h) B312 from L3F2. Insets in (g–h) show details of human nucleus. Images were acquired with the same exposure time and magnification of 200x.

allogeneic umbilical cord blood cells. Unfortunately, two years later, he was diagnosed with DMD, and analysis of his muscle biopsy demonstrated no expression of donor dystrophin.

In the present study, we were interested to investigate the potential of MSCs from umbilical cord tissue for *in vivo* muscle regeneration. In our first trial, we did nine injections of one million cells into the caudal vein of *SJL* mice, the murine model of limb-girdle muscular dystrophy 2B. DNA analysis in transplanted animals showed that the hUCT MSCs were able to reach the host muscle through systemic delivery. However, we did not find human dystrophin through WB in the same muscle samples where the human DNA was present. In addition, the functional ability tests did not show any clinical improvement. These results were expected since the human umbilical cord MSCs were not able to originate human muscle proteins. However, surprisingly, the performance of noninjected animals was significantly

worse than the “treated” animals [30]. The results reported here were done with the same methodologies used in our previously report where we injected human adipose multipotent mesenchymal stromal cells (hASCs) [38]. Differently from hUCT MSCs, hASCs injected in *SJL* mice resulted in *in vivo* expression of human muscle proteins and functional amelioration. These results suggest that although MSCs from different sources show apparently similar properties *in vitro*, they may be more or less efficient to differentiate into specific cell lineages *in vivo* according to the niche where they come from.

In the second trial, we used the golden retriever muscular dystrophy (GRMD) dogs, the canine model of Duchenne Muscular Dystrophy, aiming to evaluate the ability of MSCs from umbilical cord to regenerate the dystrophic muscle in a large animal model using a protocol already described [39]. As reported by Sampaolesi et al. [39], intra-arterial delivery of wild-type dog mesoangioblasts resulted in an extensive

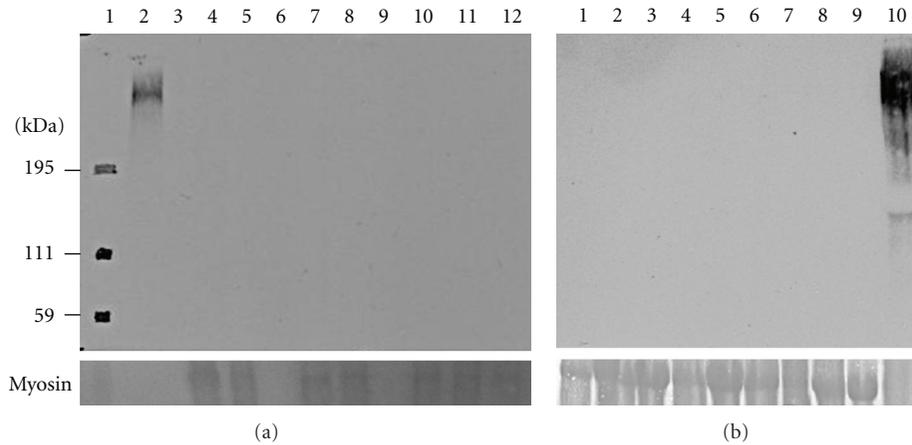


FIGURE 3: Dystrophin expression analysis. Western blot using antidystrophin rod-domain DYS1 antibody. Samples shown are the following: (a) (1) kaleidoscope protein standard; (2) wild-type canine muscle; (3, 6, 9) blank; (4, 5) B144 and B312 from affected male L3M6; (7, 8) B144 and B312 from affected female L3F1; (10, 11) B144 and (12) B312 from affected female L3F2. (b) (1) Biceps femoralis; (2) biceps brachialis; (3) triceps brachialis; (4) quadriceps femoralis; (5) tibialis cranialis; (6) diaphragm; (7) sartorius; (8) gastrocnemius; all from affected male L3M6 at necropsy. (9) GRMD muscle; (10) wild-type canine muscle. Myosin content in the Ponceau S prestained blot was used to assess the amount of loaded proteins.

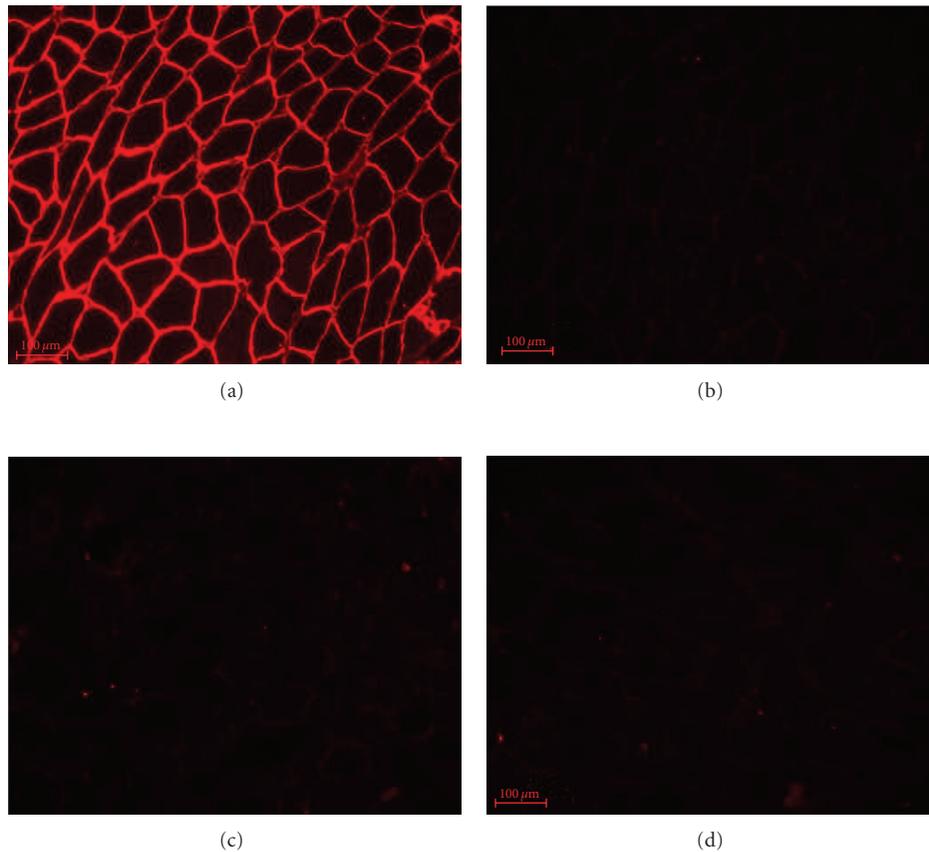


FIGURE 4: Human dystrophin expression analysis. Immunofluorescence using specific antihuman dystrophin antibody, Mandys106/2C6. (a) human normal muscle; (b) canine wild-type muscle; (c) B144 and (d) B312 from affected female L3F2. Images were acquired with the same exposure time and magnification of 200x.

recovery of dystrophin expression in transplanted animals. In the present study, we injected seven consecutive injections of one billion cells into the femoral artery of GRMD dogs. Using the Y chromosome as a track marker, we could show the successful engraftment of male cells into the *biceps femoralis* muscle of female affected dogs that received both canine and human umbilical cord MSCs. Furthermore, human cell engraftment into the canine muscle was also confirmed using the antihuman nuclei antibody. Similarly to what we observed in the mouse model, human and canine umbilical cord MSCs were able to reach the musculature in injected affected dogs, but no dystrophin expression were detected in those animals after transplantation.

Although no relevant number of GRMD dogs were evaluated in preclinical cell transplantation assays by us and others [39–41], due to the difficulty and high cost of such studies, its very important to test the safety and efficiency of different cell sources in a large animal model of PMD before starting any attempt of clinical trials. In addition, since the disease course in GRMD dogs is extremely variable, it is very difficult to analyze any amelioration or better performance due to any preclinical study [10]. Although one of the injected dogs did not survive long after the last injection and one affected female dog died at age of 3 years and 5 months, we still have in our kennel in Sao Paulo one injected female at age of 4 years that is being followed up.

In this study, we showed, in both animal models, that even without differentiating in muscle cells, systemic injections of umbilical cord MSCs are apparently safe and may possibly have a positive effect when interacting with the host muscle. Therapeutic effects of MSCs are believed to occur not only by direct differentiation into injured tissues but also by productions of paracrine factors that inhibit apoptosis, stimulate endogenous cell proliferation, and/or activate tissue resident stem cells in the site of injury. As reported by Prockop [42], MSCs secrete, in response to injury, large quantities of bioactive molecules, such as cytokines, antioxidants, proangiogenic, and trophic factors. Also, there are growing evidences that umbilical cord MSCs possess important immunomodulatory properties that may enable them to survive in an allogeneic or xenogeneic environment [43]. First, UCT MSC have low immunogenicity and suppress the proliferation of activated splenocytes and T cells. Second, UCT MSC do not express human leukocyte antigen (HLA)-DR and costimulatory molecules CD40, CD80, and CD86 that are required for T-cell activation. Third, UCT MSC synthesize HLA-G6, an immunosuppressive isoform of HLAs [44–46]. Finally, UCT MSC can be tolerated in animal models. These cells are not rejected when transplanted into SCID mice or even as xenografts in immune-competent rats [47–50]. As suggested by Chen et al., prostaglandin E₂ is the principal mediator of this potent immunomodulatory property of umbilical cord MSC [51].

In short, here we analyzed, for the first time, the ability of mesenchymal stem cells obtained from human and canine umbilical cord tissue to engraft into recipient dystrophic muscle after systemic delivery, express muscle proteins in the dystrophic host, and the safety of the procedure. Our results showed that, in both murine and canine models of PMD,

umbilical cord MSCs were able to reach the host musculature but were not able to complete full differentiation in skeletal muscle cells.

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