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Repeated Systemic Transplantation of Mesenchymal Stromal Cells: a therapeutic strategy for Machado-Joseph Disease?

Dissertação de mestrado em Investigação Biomédica, orientada pela Doutora Catarina Oliveira Miranda e pelo Professor Doutor Henrique Girão e apresentada à Faculdade de Medicina da Universidade de Coimbra

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ABBREVIATIONS

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AST – Aspartate aminotransferase

a.u. – Arbitrary unit

BDNF – Brain-derived neurotrophic factor

bFGF – Basic fibroblast growth factor

BM-MSCs – Bone-marrow mesenchymal stromal cells

CFU-F – Colony forming unit fibroblastic cells

CMEs – Células mesenquimatosas do estroma

CNTF – Ciliary neurotrophic factor

CSF – Cerebrospinal fluid

DMEM – Dulbecco's Modified Eagle Medium

DMJ – Doença de Machado-Joseph

EGF – Epidermal growth factor

EVs – Extracellular vesicles

FACS – Fluorescence activated cell sorting

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

GDNF – Glial cell line-derived neurotrophic factor

GFP – Green fluorescence protein

HA – Haemagglutinin

HBSS – Hank's Balanced Salt Solution

HGF – Hepatocyte growth factor

hMSCs – Human mesenchymal stromal cells

HU-MSCs – Human umbilical mesenchymal stromal cells

IGF-1 – Insulin-like growth factor

LV – Lentiviral

MJD – Machado-Joseph disease

ML – Molecular layer

MSA-C – Multiple system atrophy-cerebellar type

MSCs – Mesenchymal stromal cells

NGF – Nerve growth factor

NGS – Normal goat serum

NT-3 – Neurotrophin-3

PBS – Phosphate-buffered saline

PCP-4 - Purkinje cell protein 4

PFA – Paraformaldehyde

PolyQ – Polyglutamine

SCA – Spinocerebellar ataxia

SCAs – Spinocerebellar ataxias

SCA-1 – Spinocerebellar ataxia type 1

SCA-2 – Spinocerebellar ataxia type 2

SCA-3 – Spinocerebellar ataxia type 3

SNP – Single nucleotide polymorphism

UC-MSCs – Umbilical cord mesenchymal stromal cells

UPS – Ubiquitin-proteasome system

VEGF – Vascular endothelial growth factor

WT – Wild-type

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ABSTRACT

Machado-Joseph disease (MJD) is an autosomal dominant neurodegenerative disorder caused by the expansion of a CAG codon repeat in the *MJD1* gene. This genetic mutation translates into a polyglutamine tract conferring a toxic function to ataxin-3 protein, a deubiquitinating enzyme. The mutated protein tends to aggregate and form intranuclear inclusions, leading to neuronal dysfunction and degeneration of several tissues of the nervous system, being the cerebellum the main region affected. This results in a severe neuropathologic disorder characterized by ataxia, dyshartria, dysphagia, parkinsonism, among other symptoms. Currently, there is no cure for this disease. A possible approach to prevent the progression of MJD would be the use of mesenchymal stromal cells (MSCs) as a cellular therapy. MSCs are multipotent stem cells that can promote tissue preservation and regeneration through the secretion of several molecules such as growth factors, cytokines or exosomes. MSCs have been proven to exert therapeutic effects in several neurodegenerative disorders. Moreover, they are currently being tested in human clinical trials for hereditary ataxias, but there are no pre-clinical studies showing the benefits of using MSCs in MJD, and since each neurodegenerative disorder has its proper neuropathological features, MSCs mode of action may be different according to its clinical *scenario*. Previous work from our group has determined that a single injection of MSCs in a transgenic mouse model for MJD, via local injection in the cerebellum or in the lateral ventricle, has resulted in temporary alleviation of transgenic mice phenotype and neuropathology (unpublished data). Furthermore, some preliminary results from clinical trials have reported that some patients regressed to the previous stage only a few months after the treatment. Therefore, the aim of this study was to provide evidences that a continuous systemic treatment with MSCs would produce sustainable therapeutic effects in MJD. In the present study, we have used a transgenic mouse model of MJD to test a periodic treatment with bone marrow-derived MSCs. Four intravenous administrations of MSCs were performed, with a 2-week interval between each other, and both behavioral testing and neuropathology analysis were performed in MSCs-treated and non-treated MJD transgenic mice to evaluate their progressive status. Results obtained have shown that MSCs treatment improved motor impairments that were maintained throughout time. Accordingly, MSCs could alleviate the characteristic cerebellar atrophy in treated mice.

With the present work, we provided evidences that treatment with MSCs, if administered periodically, can become an excellent therapy for MJD patients, and probably patients with other spinocerebellar ataxias. Future studies more prolonged in time should be however performed to guarantee that MJD symptoms do not worsen in later stages of the disease.

Key words: Machado-Joseph disease, mesenchymal stromal cells, MJD transgenic mice, intravenous transplants, repeated injections.

RESUMO

A doença de Machado-Joseph (DMJ) é uma doença neurológica hereditária, transmitida à descendência de forma autossómica dominante. É causada por uma mutação no gene *MJD1*, que se traduz por uma expansão de repetições do códão CAG neste gene. Esta alteração genética leva ao aumento do número de poliglutaminas na proteína ataxina-3 que, por sua vez, adquire toxicidade. Quando a ataxina-3 se encontra mutada, ocorre a formação de agregados intranucleares e, consequentemente, diversos tecidos do sistema nervoso sofrem degenerescência. Estas alterações traduzem-se num fenótipo neuropatológico bastante severo, caracterizado por ataxia, disartria, disfagia, entre outros sintomas. Actualmente, não existe nenhum tratamento eficaz para esta patologia. Uma terapia celular baseada no uso de células mesenquimatosas do estroma (CMEs) surge como uma possível estratégia terapêutica para prevenir o avanço do processo de neurodegenerescência. As CMEs são progenitores estaminais multipotentes, que promovem a preservação e regeneração de tecidos lesados através da secreção de diversas moléculas, como factores de crescimento, citoquinas e exossomas. O seu potencial terapêutico foi demonstrado em várias doenças neurodegenerativas e, neste momento, existem ensaios clínicos a decorrer que estão a avaliar as CMEs como estratégia terapêutica em ataxias hereditárias. No entanto, não existem estudos pré-clínicos que avaliem os efeitos das CMEs em DMJ e, uma vez que cada doença neurológica apresenta alterações patológicas características, o mecanismo de acção das células poderá divergir de acordo com o quadro clínico de cada patologia. Foi anteriormente desenvolvido um trabalho no nosso grupo que revelou que uma única injecção de CMEs efectuada num modelo de ratinho transgénico de DMJ, quer através de injecção local no cerebelo, quer no ventrículo lateral, resultou em melhorias temporárias fenotípicas e neuropatológicas dos ratinhos transgénicos (resultados não publicados). Para além disso, alguns resultados preliminares de ensaios clínicos demonstraram que alguns pacientes regrediram para o estado inicial da doença apenas alguns meses após o tratamento. Assim, o objectivo deste estudo foi mostrar evidências de que um tratamento periódico com CMEs, através da via sistémica, poderia induzir efeitos terapêuticos que fossem mantidos ao longo do tempo em DMJ. Para isso, no presente estudo utilizámos um modelo transgénico de ratinho de DMJ para testar um tratamento repetido com CMEs derivadas da medula óssea nesta doença. O tratamento

consistiu em quatro injecções intravenosas de CMEs, espaçadas por intervalos de duas semanas entre si. Animais transgénicos tratados e não tratados foram sujeitos a testes comportamentais para análise da função motora ao longo do tempo, bem como à avaliação da sua condição neuropatológica. Os resultados obtidos demonstraram que o tratamento com CMEs induziu melhorias a nível motor, que foram mantidas ao longo do tempo. Além disso, a atrofia cerebelar característica dos ratinhos transgénicos de DMJ foi mitigada em animais tratados com CMEs. Este estudo mostrou evidências de que o tratamento com CMEs, quando administrado periodicamente e de forma continuada, pode ser uma boa estratégia terapêutica para pacientes com DMJ e, possivelmente, outras ataxias espinocerebelares. No futuro, deveriam ser realizados estudos com um tempo de duração superior, de modo a garantir que os sintomas observados em DMJ não são agravados em fases mais tardias da doença.

Palavras-chave: doença de Machado-Joseph, células mesenquimatosas do estroma, ratinhos transgénicos de DMJ, transplantes intravenosos, injecções repetidas.

CHAPTER I - INTRODUCTION

1.1. Machado-Joseph Disease

Machado-Joseph Disease (MJD) is a neurodegenerative disorder also known as spinocerebellar ataxia type 3 (SCA-3). This disease belongs to a heterogeneous group of autosomal dominant inherited spinocerebellar ataxias (SCAs), being MJD the most common worldwide (Bettencourt and Lima, 2011). These hereditary conditions are characterized by the expansion of a CAG (glutamine) trinucleotide repeats in the coding regions of causative genes, leading to a pathogenic polyglutamine (polyQ) tract in the translated proteins. Regarding MJD, the affected gene is *MJD1* and the mutated protein translated is denominated ataxin-3 (Kawaguchi et al., 1994). In a normal condition, ataxin-3 has 10-51 glutamines while the mutated protein carries 55-84 consecutive glutamines. The mutant protein form gains a toxic function resulting in a severe phenotype (de Almeida and Nóbrega, 2012).

Machado-Joseph Disease was first described between 1972 and 1977 in four Azorean descendant families living in the North America (Costa and Paulson, 2012). Due to discrepant symptoms presented by these families, four distinct diseases were defined at that time: "Machado disease" (Nakano et al., 1972), "nigro-spino-dentatal degeneration" (Woods and Schaumburg, 1972), "Joseph disease" (Rosenberg et al., 1976) and "Azorean disease of the nervous system" (Romanul et al., 1977). These four different denominations were maintained until 1978, when Coutinho and Andrade reported a study involving 15 families from Azores, revealing that these four disorders were a single disease, named "Machado-Joseph disease", which was described as a disorder with high degree of clinical variability (Coutinho and Andrade, 1978). MJD has been identified in several countries worldwide, including Germany, China, Brazil and Japan, among others with the general prevalence estimated to be 1-2 in 100,000. In a specific island of Azores named Flores, the prevalence of MJD is much higher, reaching 1/239 (Bettencourt and Lima, 2011).

1.1.1. Genetics and protein physiology

The causative gene of MJD, the *MJD1* gene, is located on the chromosome 14q32.1 (Takiyama et al., 1993). This gene encodes for the protein ataxin-3 (Fig.1), a deubiquitinating enzyme, which has been reported to play a role in the ubiquitin-mediated proteolysis (Burnett et al., 2003; Doss-Pepe et al., 2003). However, its function is not fully understood. The mutation occurs at the C-terminus of ataxin-3 protein and results in an expanded polyglutamine stretch in the translated protein (Kawaguchi et al., 1994).

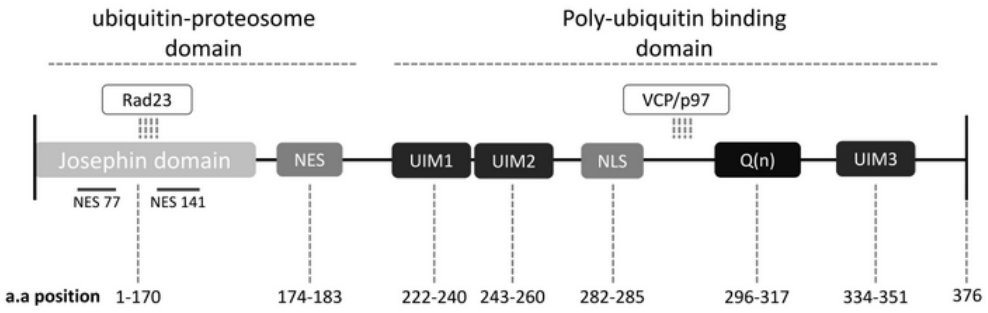


Figure 1: Representative image of the structure of ataxin-3 protein. Adapted from: (de Almeida and Nóbrega, 2012).

Ataxin-3 protein has a molecular weight of 42kDa (de Almeida and Nóbrega, 2012) and is expressed in several tissues across the organism, suggesting to be of significant biological importance (Matos et al., 2011). However, only neuronal cells seem to be affected by the presence of mutant ataxin-3. Inside the cell, wild-type (WT) ataxin-3 is localized both in the nucleus and in the cytoplasm (Paulson et al., 1997a; Trottier et al., 1998). When ataxin-3 is mutated, it aggregates in affected regions by neurodegeneration and forms intranuclear inclusions (Paulson et al., 1997b; Schmidt et al., 1998) . These intranuclear inclusions are eosinophilic spherical assemblies, with a variable size, ranging from 0.7 to 3.7 μm that also include other proteins like ubiquitin (Paulson et al., 1997a).

Besides the fact that the genetic basis of MJD is well known, the molecular mechanisms are not fully understood and present some controversy within the research community. Numerous events have been proposed to contribute to the pathogenic mechanisms in MJD. On one hand, the polyglutamine tract present in ataxin-3 protein is thought to induce neuronal cytotoxicity. It was shown that β-strand conformations acquired by polyglutamine monomers of ataxin-3 were cytotoxic in cultured cells (Nagai et al., 2007), and could assemble into oligomers (Bevivino and Loll, 2001), referred as the most toxic structures in other neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and also polyglutamine diseases (Kayed et al., 2004; Walsh et al., 2002). Moreover, proteolytic cleavage could produce smaller toxic fragments that can enter in the nucleus and lead to the formation of aggregates.

Another event that could contribute to cell death is the impairment of the ubiquitin-proteasome system (UPS) and autophagy. These protein degradation systems are extremely important to cell function and viability, and when damaged could lead to the accumulation of misfolded proteins inside the cells, resulting in cellular death. As ataxin-3 is a polyubiquitin-binding protein, a loss of function of the mutant protein could disrupt the UPS. When ataxin-3 is mutated, nuclear inclusions were shown to be ubiquitinated and to contain proteasome components, suggesting an impairment in UPS (Chai et al., 1999; Paulson et al., 1997b). Furthermore, in a lentiviral model of MJD, it was observed that essential proteins for autophagy are sequestered by mutant ataxin-3 inclusions as well as accumulate in the brain of MJD patients (Nascimento-Ferreira et al., 2011). This data supports the hypothesis that autophagy system might be disrupted in MJD and, therefore, contribute to pathogenesis of this disorder.

Additionally, other molecular mechanisms could lead to neurodegeneration in MJD. The transcription factor TATA-binding protein and transcription co-factor CREB-binding protein were reported to be present in mutant ataxin-3 nuclear inclusions, suggesting that transcription might be dysregulated in the disease (McCampbell et al., 2000; Uchihara et al., 2001). Mitochondrial function (Yu et al., 2009), axonal transport (Seidel et al., 2010) and intracellular Ca^{2+} homeostasis (Chen et al., 2008) are also proposed to be altered in this disorder, suggesting that several cellular mechanisms are impairment in MJD.

1.1.2. Clinical features and neuropathology

The clinical hallmark of MJD is the progressive ataxia defined as abnormal uncoordinated movements. However, affected individuals present a high range of clinical manifestations, which include progressive external ophthalmoplegia, pyramidal signs, dysphagia, dystonia, dysarthria, rigidity, visual and speech disorders, among other neurological complications (Wu et al., 2015).

The age of onset occurs during adulthood and varies according to the number of glutamines that mutated ataxin-3 carries. The longer the polyglutamine stretch in ataxin-3, the earlier the appearance of the symptoms (Maciel et al., 1995). Due to the high degree of clinical variability, four subtypes of the disorder have been described. Subtype 1 is characterized by an early onset and a rapid progression of the disorder with pyramidal (rigidity and spasticity) and extrapyramidal (bradykinesia and dystonia) signs, along with cerebellar ataxia. The subtype 2 of MJD is the most common among all with an intermediate age of onset (20-50 years) and the principal symptoms are progressive ophthalmoplegia and extrapyramidal signs. Subtype 3 presents a late onset of the disease (40-75 years) and manifests as motor neuropathy and muscle atrophy ataxia. Subtype 4 presents Parkinsonism symptoms and is regarded as a rare subtype of MJD (de Almeida and Nóbrega, 2012).

The pathology observed in MJD results from a progressive degenerative process that affects several brain regions (Fig.2), mainly occurring in the cerebellum, spinal cord, substantia nigra and striatum (Taroni and DiDonato, 2004). As already referred, the neuropathological hallmark of the disease is the presence of the mutant ataxin-3 inclusions in the nucleus of neurons (Paulson, 1999). Imaging studies demonstrated a decreased volume of the cerebellum as well as the enlargement of the fourth ventricle (Etchebehere et al., 2001; Murata et al., 1998). Moreover, a loss of Purkinje cells mainly in the vermis of the cerebellum is also observed in MJD patients (Muñoz et al., 2002).

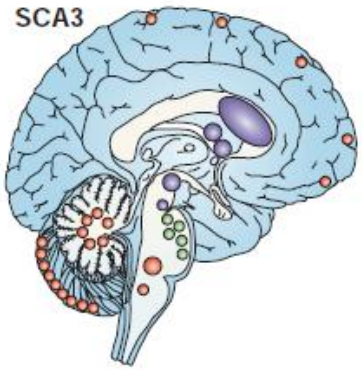


Figure 2: Principal sites of neuronal loss and organ dysfunction in MJD. Adapted from: (Taroni and DiDonato, 2004).

1.1.3. Treatment

Despite several attempts to develop a therapeutic approach for MJD have reached encouraging results, there is still no effective treatment to delay or stop the progression of the disease. There are numerous potential targets to treat MJD and, therefore, many therapeutic approaches can be used. RNA interference-based strategies were very efficacious in reducing the levels of expression of the mutant protein, although selective silencing is required, as the wild-type ataxin-3 protein is crucial to cell survival (Rodrigues et al., 2010). A study in a rat model of MJD (Alves et al., 2008) demonstrated that a lentiviral-mediated silencing approach of the human mutant ataxin-3 was efficient in alleviating the neurophatological phenotype and was selective for the mutant protein form, leading to preservation of the wild-type construct. However, this allele-specific silencing targeted a single nucleotide polymorphism (SNP) present in only in 70% of the patients with MJD, meaning that approximately 30% of patients would not benefit from this therapy. Another therapeutic strategy that could be used in MJD would be the stimulation of the UPS and autophagy mechanisms in order to reduce the accumulation of the misfolded proteins. Consequently, mutant ataxin-3 degradation would be promoted, delaying MJD progression. Accordingly, the administration of rapamycin ester, an activator of autophagy, could alleviate motor deficits in a transgenic mouse model of MJD (Menzies et al., 2010). This improvement was accompanied by a reduction in the number of aggregates and levels of mutant ataxin-3 soluble form in the cytoplasm, while the wild-type protein levels kept unchanged. However, rapamycin is an immunosuppressive drug and consequently, its use could result in low effectiveness of the immune system, among other adverse effects (Bamgbola, 2016). Moreover, the use of drugs or compounds with neuroprotective purposes could have a benefic effect in alleviating the phenotypic impairments observed in MJD. In 2014, the results of a phase II clinical trial using lithium carbonate in MJD patients were published (Saute et al., 2014). However, besides the fact that lithium was proven to exert neuroprotective effects in a Drosophila model of MJD (Jia et al., 2013), the same therapeutic results were not observed in patients subjected to the lithium administration. Therefore, further research is essential to develop an effective therapy to delay or reverse the progression of this fatal disorder.

Stem cells therapy is a relatively recent research field that has been shown to bear great potential to treat neurodegenerative disorders and could, in that sense, meet this purpose. From the group of therapies using adult stem cells, mesenchymal stromal cells have been in particular focus as they have been shown to be very promising for the treatment of several diseases that at the moment do not have any effective therapy available. This issue will be discussed in the following section.

1.2. Mesenchymal stromal cells: a promising therapy for neurological disorders

1.2.1. Ontology, biology and phenotype of MSCs

Mesenchymal stromal cells (MSCs) are adult multipotent progenitor cells that were first described by Friedenstein and colleagues (Friedenstein et al., 1970). When they were isolating bone-marrow of guinea-pig, they observed that a population of cells distinct from the hematopoietic fraction adhered to plastic. Due to their fusiform morphology and their capacity to form colonies they were determined as "colony forming unit fibroblastic cells" (CFU-F). Later on, these cells were designed "Multipotent mesenchymal stromal cells" (Horwitz et al., 2009).

MSCs represent 0,01-0,001% of the total nucleated cells from the bone-marrow and promote functional and structural support for hematopoietic cells (Sadan et al., 2009). Despite the fact these cells were originally isolated from the bone-marrow, later it was shown that they exist in almost all adult connective tissues, though in a very low percentage (da Silva Meirelles et al., 2008). Presently, MSCs are most frequently obtained from the bone-marrow, fat tissue and cord blood.

MCSs are multipotent stem cells meaning they can differentiate into cells belonging from mesoderm-derived tissues such as osteoblasts, adipocytes and chondroblasts (Pittenger, 1999) (Fig.3). Phenotypically, they are defined by the expression of a group of specific CD markers present in cell membrane. Human MSCs are positive for CD73, CD90, and CD105 surface markers and negative for the hematopoietic markers CD11b or CD14, CD19 or CD79α, CD34, CD45 and HLA class II (Dominici et al., 2006; Paul and Anisimov, 2013). The niche of MSCs *in vivo* is still unknown. However, they were suggested to reside in the perivascular space and are thought to be pericytes-alike (da Silva Meirelles et al., 2008).

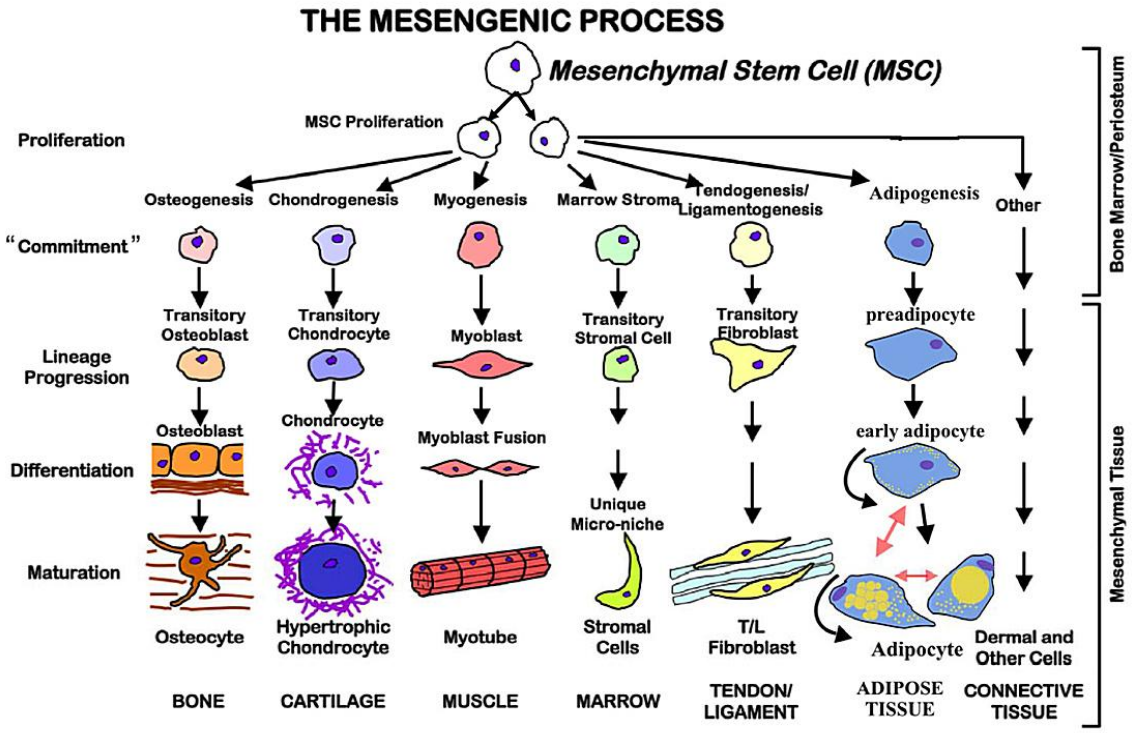


Figure 3: Mesenchymal stromal cells: lineage. Adapted from (Caplan and Dennis, 2000).

1.2.2. Advantages of MSCs for cell therapy

MSCs have been used to treat a wide range of disorders including various neurodegenerative diseases due to their advantageous features. They possess several properties that make them excellent targets for cellular therapies. MSCs can be easily harvest and expanded *ex vivo* in order to obtain sufficient number of cells for transplantation. Nowadays, there are already several companies that expand MSCs and whose goal is to develop autologous mesenchymal stem cell therapies for neurodegenerative diseases, including Multiple Sclerosis, Parkinson's disease, Huntington's disease and Amyotrophic Lateral Sclerosis. Moreover, MSCs were reported to present low immunogenicity as they do not express co-stimulatory molecules such as CD40, CD80 and CD86 making them negative for MHC II (Paul and Anisimov, 2013). However, other studies showed that allogeneic MSCs transplantation can induce alloimmunity and graft rejection (Deuse et al., 2011; Nauta et al., 2006). Further research, particularly in the assessment of immune responses in clinical trials using MSCs transplantation, should be done to address this important question

(Consentius et al., 2015). Furthermore, MSCs were described to have the ability to migrate to injured sites where they secrete paracrine factors, promoting cell survival and tissue regeneration and repair (Paul and Anisimov, 2013). Also, these cells were also reported to be non-tumorigenic (Choumerianou et al., 2008). Finally, as they are adult stem cells, their use for cell therapies is regarded as a safer cell source than other pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells. Because of these valuable properties, MSCs have been considered as a safe and effective approach for the treatment of many neurological diseases (Tanna and Sachan, 2014).

1.2.3. Mechanisms of action of MSCs

The exact mechanisms through which MSCs exert beneficial effects remain unidentified. Recently, the paracrine effect was considered the principal mechanism through which MSCs act (Paul and Anisimov, 2013). It has been shown that it would not be necessary direct contact between MSCs and injured tissues in order to promote release of the therapeutic factors. Therefore, the same benefic results may be achieved through the transplantation of the cells via a less invasive route such as the intravenous injection (Paul and Anisimov, 2013). The paracrine effect of MSCs can be mediated by four main groups of molecules: neurotrophic factors, cytokines, extracellular vesicles such as exosomes and extracellular matrix proteins. Neurotrophic factors are released by MSCs in response to environmental stimuli and will promote tissue repair/neuroprotection. The most common secreted neurotrophic factors are the following: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and ciliary neurotrophic factor (CNTF) (Chen et al., 2005). Extracellular matrix molecules and adhesion molecules, such as collagen, laminin and fibronectin, also contribute for the regenerative function of MSCs (Chen et al., 2007). These compounds may act by trophic support and molecular signaling events, inducing alterations in the stem cell niche, or contributing to tissue regeneration through self-biological activity (Aizman et al., 2009; Paul and Anisimov, 2013). Another paracrine

model proposed to explain MSCs effect is through the secretion of immunomodulatory molecules. It was shown that MSCs can modulate the immune system's response via anti-inflammatory actions. They have the ability to inactivate cells of the adaptive immune system, such as B-cells (Fan et al., 2016), T-cells (Di Nicola, 2002), or cells from the innate immune system, like natural killer cells (Aggarwal and Pittenger, 2005; Spaggiari et al., 2006). Through the prevention of the inflammatory response, which is a common mechanism in the pathology of neurodegenerative disorders, MSCs might promote cell survival and ameliorate the phenotype of neurologic diseases (Paul and Anisimov, 2013). The last mechanism through which MSCs might exert a benefic effect is the release of extracellular vesicles (EVs) which are plasma membrane-bound fragments. The EVs interact with recipient cells through contact at cell membrane, internalization into endocytic structures and fusion with plasmatic membranes, and then transfer molecules such as DNA, proteins/peptides, mRNA, microRNAs, and lipids to the damaged cells, with the mean to repair them. The exosomes are the most known EVs derived from MSCs (Abreu et al., 2016), and thus are the most commonly referred in the MSCs effect through EVs.

1.2.4. Pre-clinical studies with MSCs in SCAs

Recently, numerous pre-clinical studies have been performed in mouse models of spinocerebellar ataxias. In 2010, a group of investigators transplanted bone-marrow mesenchymal stromal cells (BM-MSCs) into the cerebellum of Lurcher mutant mice, a mouse model featured by the selective death of Purkinje cells in the cerebellum. Two months after MSCs transplantation, treated mice exhibited better results in motor function tests as well as a decrease death of Purkinje cells. Interestingly, MSCs were found near the Purkinje cell layer, expressing neurotrophic factors such as BDNF, NT-3 and GDNF. In conclusion, transplantation of MSCs was able to ameliorate pathophysiological features as well as motor deficits of a spinocerebellar ataxic mouse model (Jones et al., 2010). Similarly, other studies in mouse models of spinocerebellar ataxias achieved equally promising results. In 2011, two studies involving MSCs transplantation in mouse models of ataxia were performed. In the first study, it was used an ataxic model induced by cytosine β-D-arabinofuranoside which resembles the ataxic

phenotype observed in patients (Zhang et al., 2011). Treated mice received human umbilical mesenchymal stromal cells (HU-MSCs) via intravenous injection. Improvements of motor function and preservation of neuronal cells were observed in mice treated with HU-MSCs. Regarding the second study, a transgenic mouse model of spinocerebellar ataxia type 2 (SCA-2) received human mesenchymal stromal cells (hMSCs) intravenously or intracranially (Chang et al., 2011). Assessment of motor performance showed that several intravenous infusions of hMSCs alleviated motor impairments in SCA-2 transgenic mice while intracranial administration of hMSCs failed to produce benefic effects. Similarly, intravenous treatment was more effective in inducing Purkinje cells survival than intracranial transplantation. Furthermore, another study involving MSCs and spinocerebellar ataxias was done in order to confirm the potential of MSCs in treating spinocerebellar ataxia type 1 (SCA-1) (Matsuura et al., 2014). For this purpose, a single intrathecal injection of MSCs was administered into a transgenic mouse model of SCA-1 before any significant degeneration and loss of Purkinje cells had occurred. The results showed that a single intrathecal transplantation of MSCs was able to alleviate the neuropathological phenotype observed in SCA-1, and MSCs partially restored the Purkinje cells organization into a monolayer, when compared to non-treated mice, which displayed a multilayer arrangement. Also, MSCs ameliorated the motor performance in behavioral tests of treated mice for 15 weeks after the treatment. This group of studies showed the great potential of MSCs in treating SCAs, anticipating that probably this cellular therapy would also produce benefic effects in MJD.

1.2.5. Clinical trials using MSCs

The promising results obtained from pre-clinical studies in other neurodegenerative disorders led to the development of clinical trials using MSCs. In 2010, a phase I clinical trial showed the safety of the usage of mesenchymal cells in patients with amyotrophic lateral sclerosis (Mazzini et al., 2010). In this trial, autologous bone marrow MSCs were transplanted into the spinal cord of the patients. The clinical trial report described MSCs as a safe approach that promoted the preservation of brain functions through neuronal replacement. Similarly, some clinical

trials have been also performed in hereditary spinocerebellar ataxias to assess the safety and the efficacy of MSCs transplantation. Presently, the following clinical trials including patients with SCAs are running (www.clinicaltrials.gov: ClinicalTrials.gov Identifiers: NCT01489267, NCT01958177, NCT01360164, NCT01649687, NCT02540655). A few other clinical studies using MSCs in SCA patients were already completed and results were reported. One of the studies used umbilical cord mesenchymal stromal cells (UC-MSCs) to treat 14 SCA and 10 multiple system atrophy-cerebellar type patients (MSA-C) (Dongmei et al., 2011). In this study, $1x10^6$/kg UC-MSCs and 5mg dexamethasone were administered via intrathecal injection weekly during 4 weeks. Motor skills and quality of daily life were assessed during 6-15 months and compared with those scores obtained before transplantation. The results showed that after 1 month, symptoms were alleviated in all patients, except for one case in which no response was observed. The side-effects originated from the therapy, such as dizziness, low back pain and headache, disappeared in all patients 1-3 days post-procedure. A follow-up evaluation reported that 10 patients' conditions persisted stable for half a year or more, though in 14 cases patients conditions returned to previous status before the treatment, on average 3 months after the treatment. Another clinical trial was conducted to evaluate the viability, efficiency and toxicity of transplantation of UC-MSCs in patients diagnosed with SCA pathology. Sixteen patients received 4 consecutive intravenous and intrathecal injections of UC-MSCs at 1-week intervals. The first administration consisted of $4x10^7$ UC-MSCs intravenous injection. For the subsequent 3 treatments, $2x10^7$ UC-MSCs were infused via intravenous injection and $2x10^7$ UC-MSCs were administered by intrathecal injection, simultaneously. The transplantation was reported to be safe as no serious adverse effects were observed in all 16 patients after a follow-up of 12 months post-treatment. After one year of treatment, 7 patients showed continuous improvement of motor deficits for at least 6 months after receiving the therapy, although 5 patients suffered from disease aggravation. MSCs therapy was reported to be safe for SCA patients and suggested to delay progression of the disorder (Jin et al., 2013). Recently, another clinical trial using UC-MSCs (Miao et al., 2015) was performed in order to evaluate the technical difficulties underlying intrathecal UC-MSCs injection via lumbar puncture and assess the cells effects in several neurodegenerative disorders, including 8 spinocerebellar ataxias patients. The UC-MSCs were injected 4 to 6 times between the L4 and L5 interspace within an interval of 5-7 days. Two ml of cerebrospinal fluid (CSF) was

removed and replaced with 2ml of cell suspension. All patients were followed-up for more than 1 year for clinical status. Three of the eight patients showed motor function improvement after treatment. The authors concluded with this study that intrathecal UC-MSCs injection was safe and effective in treating neurological conditions. As conclusion, clinical trials have shown that the benefic effect promoted by treatment with MSCs was transient, as several patients returned to the stage previous of the treatment some time after the MSCs administration.

As suggested either by the pre-clinical or the clinical studies, mesenchymal stromal cells transplantation has huge potential for the treatment of neurological disorders, but their transient effect must be overwhelmed. Therefore, a stem cell therapy using repeated administrations of MSCs to treat Machado-Joseph disease may be an excellent approach and should be further evaluated.

OBJECTIVES

Despite there is some pre-clinical research in various types of ataxias, currently there are no pre-clinical studies using MSCs to study MSCs effects in MJD. It was previously observed by our group that the delivery of MSCs directly into the cerebellum or intracerebroventricular in a mouse model of MJD could promote a transient phenotype alleviation of both MJD phenotype and neuropathology (unpublished data).

The goal of the present project was to investigate whether repeated MSCs delivery through a less invasive way, as the intravenous route, could promote a sustainable relief of MJD symptoms. In order to achieve this goal, MSCs isolated from the bone-marrow of wild-type mice were periodically injected intravenously in the tail vein of a transgenic mouse model of MJD (the Tg-ATXN3-69Q mouse model). Subsequently, their behavioral performance through a serious of motor tests was assessed to evaluate MSCs effect on motor deficits. Besides motor performance, neuropathological analysis was also completed to estimate neuronal preservation within the cerebellum after MSCs repeated transplantation.

With this study it was expected to provide evidences that a recurrent treatment with MSCs through a less invasive route would become a potential cellular therapy approach for MJD patients, and possibly, other SCAs.

CHAPTER II – MATERIALS AND METHODS

2.1. MSCs isolation and expansion

MSCs have been previously isolated from the bone marrow of 6- to 8- weeks-old wild type mice with C57BL/6 background of both genders, adapted from a protocol described elsewhere (Miranda et al., 2014). Briefly, femurs and tibias were collected and the contents of bone marrow were flushed with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen) into a sterile petridish. The cell suspension was homogenized with a 20G syringe, centrifuged and the pellet resuspended in DMEM (not supplemented with serum). Finally, mononuclear cells were separated in a Ficoll (Sigma) density gradient and resuspended in DMEM/F12 (Gibco) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco), 20ng/ml epidermal growth factor (EGF, PeproTech), 20ng/ml fibroblast growth factor (FGF, PeproTech) and 2% B-27 (Gibco) and were incubated at 37ºC in 5% CO_2 air atmosphere. Forty-eight hours later, nonadherent cells were removed and adherent cells were expanded for approximately 2-3 weeks, with medium changes every 2-3 days, until sub-confluence was reached. In order to remove hematopoietic contaminants from MSCs cultures (Phinney et al. 1999), the elimination of the $CD45^+$ cells have been performed by fluorescence activated cell sorting (sorter: FCASAriaIII; data analysed with FACSDIva software, version 6.1.3.). MSCs were expanded in the conditioned medium described above and stocks of cells were frozen in FBS with 10% DMSO for later usage.

2.2. Production of GFP-lentiviral vectors and MSCs infection/expansion

Lentiviral (LV) vectors encoding green fluorescence protein (GFP), under the control of phosphoglycerate kinase promoter, were previously produced in HEK-293T cells with a four-plasmid system, as previously described (de Almeida et al., 2001).The lentiviral particles were ressuspended in 1% bovine serum albumin in PBS and viral particle contents determined by assessing HIV-1 p24 antigen levels (RETROTek, Gentaur, France). Viral stocks were stored at −80°C until usage. MSCs were incubated

with LV-GFP (80 ng virus/100,000 cells with 4 µl/ml of hexadimethrin bromid, Sigma), during an overnight period and medium changed in order to remove the lentivirus. Efficiency of transfection was determined by GFP positive gated events acquired in a FacsCalibur flow cytometer (Becton Dickinson). Data were analyzed by Cell Quest software (BD). In the present study, MSCs from these stocks have been thawed and expanded in the conditioned medium described in the previous section, until the required number of cells was achieved. In all assays, MSCs used were between passages 13-16.

2.3. Animals

Machado-Joseph disease transgenic mice (C57BL/6 background) expressing the N-terminal-truncated human ataxin-3, with 69 glutamine repeats and an N-terminal haemagglutinin (HA) epitope (Tg-ATAXIN-3-69Q), driven specifically in cerebellar Purkinje cells by the L7 promoter, as previously described (Torashima et al., 2008) were used. A colony of these transgenic mice was maintained at the Centre for Neuroscience and Cell Biology of the University of Coimbra by backcrossing heterozygous males with C57BL/6 females. Genotyping was performed by PCR. For this study, heterozygous males and females were divided into MSCs-treated or non-treated mice.

2.4. Intravenous injection of MSCs

The treatment used in the present study consisted on the intravenous injection (in the tail vein) of MSCs in Tg-ATXN3-69Q MJD mice, every two weeks, four consecutive times. Treated mice were injected with 4-8x10^7/kg MSCs ressuspended in 70-150ul of HBSS cells per injection. Non-treated mice received the vehicle - Hank's Balanced Salt Solution (HBSS) - instead of MSCs, forming the control group. Therapy began at 4-6.5 weeks of age.

2.5. Behavioral tests

Mice were trained in a serious of motor tests, starting before the first MSCs injection (week 0) and ending after the last MSCs administration (week 8), with the exception of week 6, which did not include behavioral assessments. Non-treated Tg-ATXN3-69Q MJD mice from the same littermates were used as controls. All tests were performed in the same room, with lights turned off and after 60 minutes minimum of acclimatization.

2.5.1. Rotarod

Motor coordination and balance were evaluated in a rotarod apparatus (Letica Scientific Instruments, Panlab). Animals were placed on the rotarod at a constant speed (5 rpm) during 5 min maximum. The latency to fall was registered. Rotarod test was performed for 3 consecutive days, for each time point. Mice were allowed to perform four trials per day. The mean latency to fall of all trials performed in the second day was used for analysis, as was considered to produce the most reproducible results.

2.5.2. Pole

The pole test was used to evaluate mice motor coordination and equilibrium. Animals were put horizontally at the end of the pole, which was then lifted to the vertical position letting mice to get down. It was recorded the time spent by the animal to turn down and walk along the pole, until they reached the floor. Similarly to the rotarod test, pole was performed for 3 consecutive days, four trials per day, for each time point and the mean latency to fall off all trials performed in the second day was used for analysis.

2.5.3. Swimming

Mice motor coordination was also assessed through the swimming test. Animals were put inside a tank full with water and with an escape platform at the end. The time that the animals spent to cross the tank and reach the platform was registered. In the swimming, only one day of test was performed. The first trial (of four) of each time point was eliminated for the analysis, as mice spent a lot of time to find the platform.

2.5.4. Footprint

The footprint test was used to evaluate the gait of Tg-ATXN3-69Q MJD mice. The front and the hind paws of mice were painted with different color, nontoxic ink, and the animals were allowed to walk along a corridor to a goal box over a fresh sheet of white paper, leaving a trail of footprints. The footprint track was measured for stride length, hind-base and front-base width, and fore and hind paw overlap. Measures were taken from six consecutive right and left footprints, excluding steps made at the beginning and end of the run.

2.6. Quantification of transaminases in the serum

At the end of the experiment (week 10), blood was collected after decapitation of mice under anesthesia in DNAse/RNAse free eppendorfs and maintained at 4ºC for approximately 2 hours. Blood samples were then centrifuged at 1000x*g* for 15 minutes. When necessary, a second or even a third centrifugations were performed in order to clean serum samples from cellular contaminants. Serums were kept at -20ºC for future analysis in the laboratory (Aeminium, Laboratório de Análises Clínicas, Lda, Coimbra, Portugal) of liver transaminases - aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

2.7. Immunohistochemical procedures

2.7.1. Tissue preparation

Animals were sacrificed through intracardiac perfusion and the tissues were fixed with 4% paraformaldehyde solution (PFA 4%). After perfusion, brains were removed from the skull and put in PFA 4% solution during 24 hours. Subsequently, brains were transferred to a 30% saccharose, 0.1 M PBS solution for dehydration and frozen at -80ºC. Brains were cut at a cryostat (Leica CM3050S, Leica Microsystems) in 35µm sagittal sections, which were collected to 48-multiwell plates filled with 0.1 M PBS-azide solution and stored at 4ºC.

2.7.2. DAB-labeling free-floating immunohistochemistry

Free-floating immunohistochemistry with a 3,3'-diaminobenzidine (DAB)-labeling system was performed to detect GFP signal in brain sections. Sections were incubated in 0.1 PBS containing 0.1% diphenylhydrazine for 30 min at 37ºC and then in a 0.1% Triton 10% NGS 0.1 PBS blocking solution for 1h under moderate agitation at room temperature. Sections were then incubated with primary antibody anti-GFP rabbit serum (Life Technologies; 1:1000), under soft agitation, at 4ºC, overnight. In the following day, sections were incubated with goat anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories Inc., CA, USA), diluted in the same blocking solution, under soft agitation, for 2h, at room temperature. After incubation with the secondary antibody, the ABC amplification system was used (Vectastain ABC kit, Vector Laboratories, West Grove, USA) and the 3,3′- diaminobenzidine tetrahydrochloride (peroxidase substrate kit, DAB, Vector Laboratories, CA, USA) was used as substrate. Sections were then mounted over microscope slides and led to dry at room temperature. Slides were then hydrated with milliQ H_2O for 30 seconds, and dehydrated by passing sections for 3 minutes through an increased degree of ethanol solutions (ETOH 75%, 96% and 100%) and finally, xylene. The mounting medium Eukittw (O. Kindler GmbH & CO, Freiburg, Germany) was then applied on the top of the dehydrated sections and coverslips covered all sections.

2.7.3. Fluorescence-labeling free-floating immunohistochemistry

In the fluorescence-labeling protocol, sections were incubated in a 0.3% Triton 10% NGS 0.1 PBS blocking solution for 1h under moderate agitation at room temperature. Sections were then simultaneously incubated under soft agitation at 4ºC overnight with the following primary antibodies, diluted in the blocking solution previously described: mouse anti-HA (InvivoGen; 1:1000) and rabbit polyclonal anti-PCP-4 (Santa Cruz Biotechnology; 1:500). After primary antibody incubation, sections were incubated with goat anti-mouse Alexa fluor 647 (Life Technologies; 1:200) and goat anti-rabbit Alexa fluor 568 (Life Technologies; 1:200) secondary antibodies, diluted in the previous blocking solution, under soft agitation for 2h at room temperature. Sections were then put in DAPI solution (1:5000) for 10min at room temperature and mounted on Moviol mounting medium.

2.7.4. Cresyl violet histological staining

Sagittal brain sections were stained with cresyl violet dye for 5 min, dehydrated by passing through 75%, 96%, 100% ethanol and xylene solutions for 3 min, and mounted with Eukitt.

2.8. Quantification of molecular and granular layers thickness and lobular volume

Images of the entire cerebellum of three sagittal sections (corresponding approximately to the following brain coordinates: 1.08 mm lateral to midline to 0.60 mm lateral to midline (Paxinos and Franklin, 2001)) stained with cresyl violet were acquired with a Zeiss Axio Imager Z2 microscope using a 20x objective. The same three sections were chosen in all animals. For each section, 3 measurements of the molecular and granular layers were performed in lobule X and the mean value of cellular layers' thickness was obtained from the 3 measurements in these 3 sections. Lobular volume of lobule X and lobules VI and VII (determined together) was quantified by measuring the area of lobules in each section and multiplying this value by the distance between sections (280 µm). Lobular volume and cerebellar molecular and granular layers thickness were measured using Zeiss Zen 2 (blue edition) software.

2.9. Quantification of Purkinje cells and mutant ataxin-3 aggregates

Fluorescence images of three sagittal sections of the entire cerebellum, stained with anti-HA, anti-PCP-4 and DAPI were acquired with a Zeiss Axio Imager Z2 microscope using a 20x objective and ApoTome 2. Selected sections corresponded to the same brain region described in point 4.8 were used for all animals. For each section, the number of mutant ataxin-3 aggregates and number of Purkinje cells were counted in lobule X. Results are represented as mean value of the number of aggregates or number of Purkinje cells of the 3 sections analyzed, respectively.

2.10. Statistical analysis

The effect of group (treated and non-treated MJD mice) and time (before, two, four and eight weeks after treatment) on all behavioral response variables was accessed through repeated measures ANOVA, followed by post-hoc Bonferroni pairwise comparisons to identify significant differences among groups and time periods. Normalization with body weight was used when the effect of the body weight was significant (continuous independent variable on all the response variables). All statistical analyses regarding behavioural testing were carried out in R (Version 3.01) (R Core Team 2015). Response variables were tested for normality (Q-Q plots) and homogeneity (Cleveland dotplots) before each statistical test and transformed when needed (Zuur et al. 2010). Statistical analysis of molecular and granular layers' thickness, lobular volume and number of aggregates or Purkinje cells was performed using Unpaired t-test with Welch's correction, using the GraphPad software (La Jolla, USA). Throughout the results, all values are presented as the mean ± SEM, unless otherwise stated. All analyses were performed assuming a significance level of $P < 0.05$.

CHAPTER III - RESULTS

In order to evaluate whether systemic transplantation of MSCs is able to sustainably alleviate the phenotype and neuropathology observed in the transgenic MJD mouse model used in the present study (the Tg-ATXN3-69Q model), when administered as a continuous treatment, we have performed systemic periodic administrations of MSCs. MSCs had been previously isolated in our group from the bone marrow of C57BL/6 wild-type mice with the same background as the transgenic mice used in this study. Therefore, MSCs used in the present work are denominated syngeneic, i. e., they are genetically identical and immunologically compatible. Since there is some risk that cultures of murine MSCs obtained by plastic adherence isolated from bone marrow are contaminated by hematopoietic cells (Javazon et al., 2004; Phinney et al., 1999b), MSCs have been purified by fluorescence activated cell sorting (FACS) in order to isolate only the CD45 negative population of cells and were afterwards characterized by flow cytometry. As expected, MSCs were negative for the hematopoietic markers CD11b and CD45 and highly positive for the mesenchymal markers CD73 (94.5%), CD105 (99.7%), Sca-1 (98.6%), CD29 (99.9%) and CD106 (95.4%), commonly used to characterize MSCs isolated from the bone marrow of mice (Baddoo et al., 2003). Moreover, MSCs were able to expand as a monolayer and presented fibroblast-like morphology, typical features of mesenchymal stromal cells (unpublished data). For the present work, MSCs originated from the same batch, previously infected with LV-GFP and frozen were thawed and expanded.

In order to test the efficacy of MSCs therapy, two groups of animals were used in the present study: the control or non-treated group, comprising 4 males and 4 females, which received HBSS, and the treated group, including 5 males and 9 females, which were transplanted with MSCs (Fig.4-A). In this last group, animals received four consecutive injections with a 2-week interval between them. The injections were intercalated with behavioral analysis that started before the first treatment (week 0) and ended after the last MSCs administration (week 8), with the exception of week 6, which did not include behavioral assessments. Animals were sacrificed 3 weeks after the last injection (week 10) (Fig.4-B). The number of cells infused in the 3 sets of animals at each time point ranged between $4x10^8$ MSCs/kg and $8x10^8$ MSCs/kg and is represented in Table 1. In order to find out what would happen to MSCs when they were injected via systemic route, after mice were sacrificed and brain tissue fixed and cut, we performed an immunohistochemistry with anti-GFP in eight central sagittal sections, as

MSCs used in this work express this protein. However, three weeks after the last treatment, we were not able to find the presence of MSCs in the cerebellum or in other brain region.

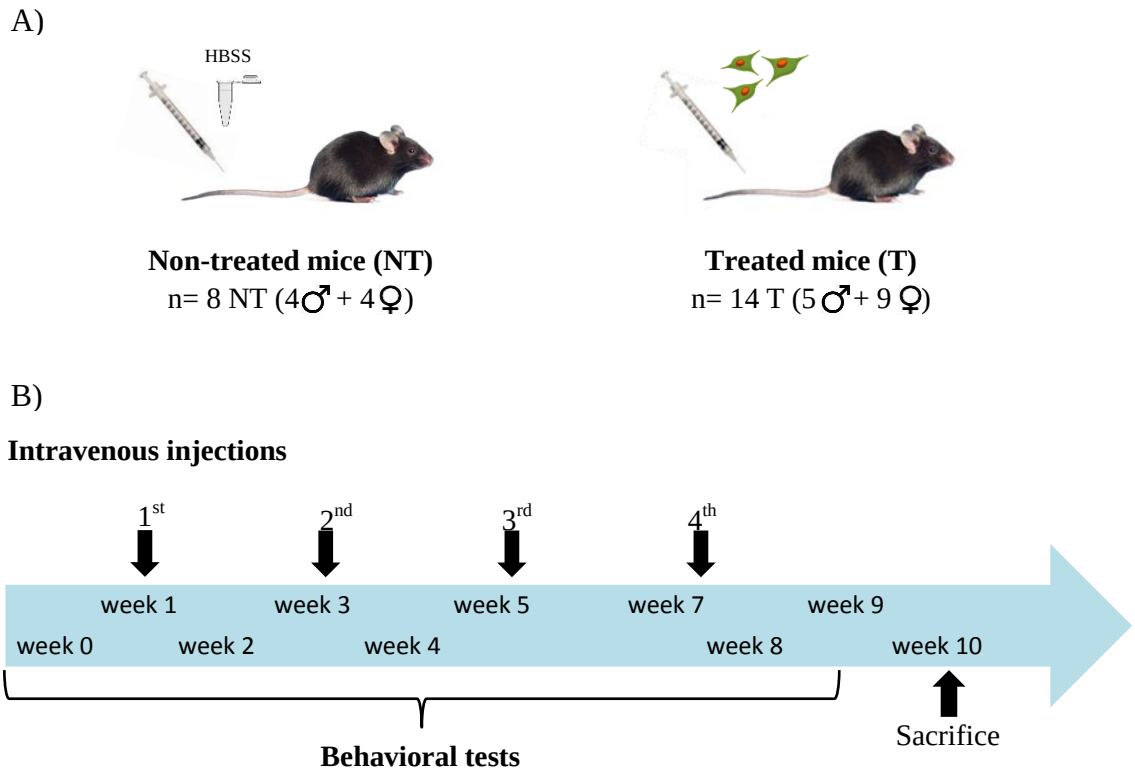


Figure 4: Schematic representation of the experimental timeline. A) Tg-ATXN3-69Q mice with 5-7 weeks of age were used at the beginning of the experiment. In the present study, mice were divided into two groups: 8 animals (4 females + 4 males) were injected with saline solution (HBSS) and comprised the non-treated or control group; another 14 mice (9 females + 5 males) were injected with MSCs, ressuspended in HBSS (vehicle), which formed the treated group. **B)** Four intravenous administrations (in the tail vein) were given to animals with 2 week-intervals; behavioral analysis was performed also every two-weeks between injections, starting one week before first treatment and ending one week after last treatment, except for week 6. Mice were sacrificed at 10 weeks after the beginning of experience (week 10), 3 weeks after last treatment.

Table 1: Number of MSCs/Kg injected in each set of animals in Tg-ATXN3-69Q mice

Number MSCs/Kg	1st administration	2nd administration	3rd administration	4th administration
Set I	$3.7\text{-}5.4x10^7$	$6x10^7$	$6x10^7$	$6x10^7$
Set II	$8x10^7$	$8x10^7$	$6.3x10^7$	$8x10^7$
Set III	$8x10^7$	$4x10^7$	$7.5x10^7$	$8x10^7$

Table 1: Representation of the number of MSCs per Kg of body weight injected in Tg-ATXN3-69Q mice. Animals were divided into three sets of experience and were administered four intravenous injections of MSCs. The number of MSCs/Kg infused in each treatment per set is described in this table.

3.1. Repeated systemic infusions of MSCs ameliorates phenotypic features observed in Tg-ATXN3-69Q mice of MJD

Previously, it was observed in our group that a single infusion of MSCs, either intracerebroventricularly or directly into the cerebellum, produced a transient effect in motor performance of treated mice. In this work, we intended to evaluate whether repeated administrations of MSCs would lead to sustained improvements in motor function of treated mice compared to the control group. For this purpose, treated and non-treated animals were subjected to various motor tests one week before the first treatment and one week after each administration (except for week 6 of the experimental assay).

The rotarod apparatus was used to assess mice motor coordination and balance. The test was performed for 3 consecutive days for each time point, at constant speed (5 rpm) for a maximum of 5 minutes, and the latency to fall was recorded. Just one week after the first treatment (week 2 of the experimental assay), motor performance of mice treated with MSCs (n=5 males) was significantly increased when compared to the control group (n=3 males) (week 2: 1069.83±267.62 sec*g *vs* 467.07±65.41 sec*g, p=0.04). Motor function continued to improve throughout time, showed by a gradual increase in the latency to fall off rotarod apparatus (week 4: 913.94±131.92 sec*g *vs* 410.60±78.27 sec*g, p=0.01; week 8: 1305.38±98.66 sec*g *vs* 505.64±170.32 sec*g, p=0.001) (Fig.5-A). The pole test was also used to evaluate mice motor coordination and equilibrium. The time spent to reach the floor by the animals was recorded and

comparisons between the treated group (n=5 males) and the non-treated animals (n=4 males) were made. Equally to the rotarod test, mice treated with MSCs showed a better performance in the pole test, as they reached the floor faster than the control group in the second and fourth week of the experiment (week 2: 317.80±27.9 sec*g *vs* 445.45±39.54 sec*g, p=0.03; week 4: 293.12±9.29 sec*g *vs* 394.83±37.06 sec*g, p=0.02). In the week eight, significant differences were not observed between the two groups; however a tendency to spend less time coming down from the pole was registered (Fig.5-B). Regarding the swimming (n=4 non-treated; n=5 treated, all males), despite the fact that it was not observed significant differences between the two groups of animals, treated mice spent less time to cross the tank and reach the platform at all time points after the first treatment (week 2, week 4 and week 8) (Fig.5-C).

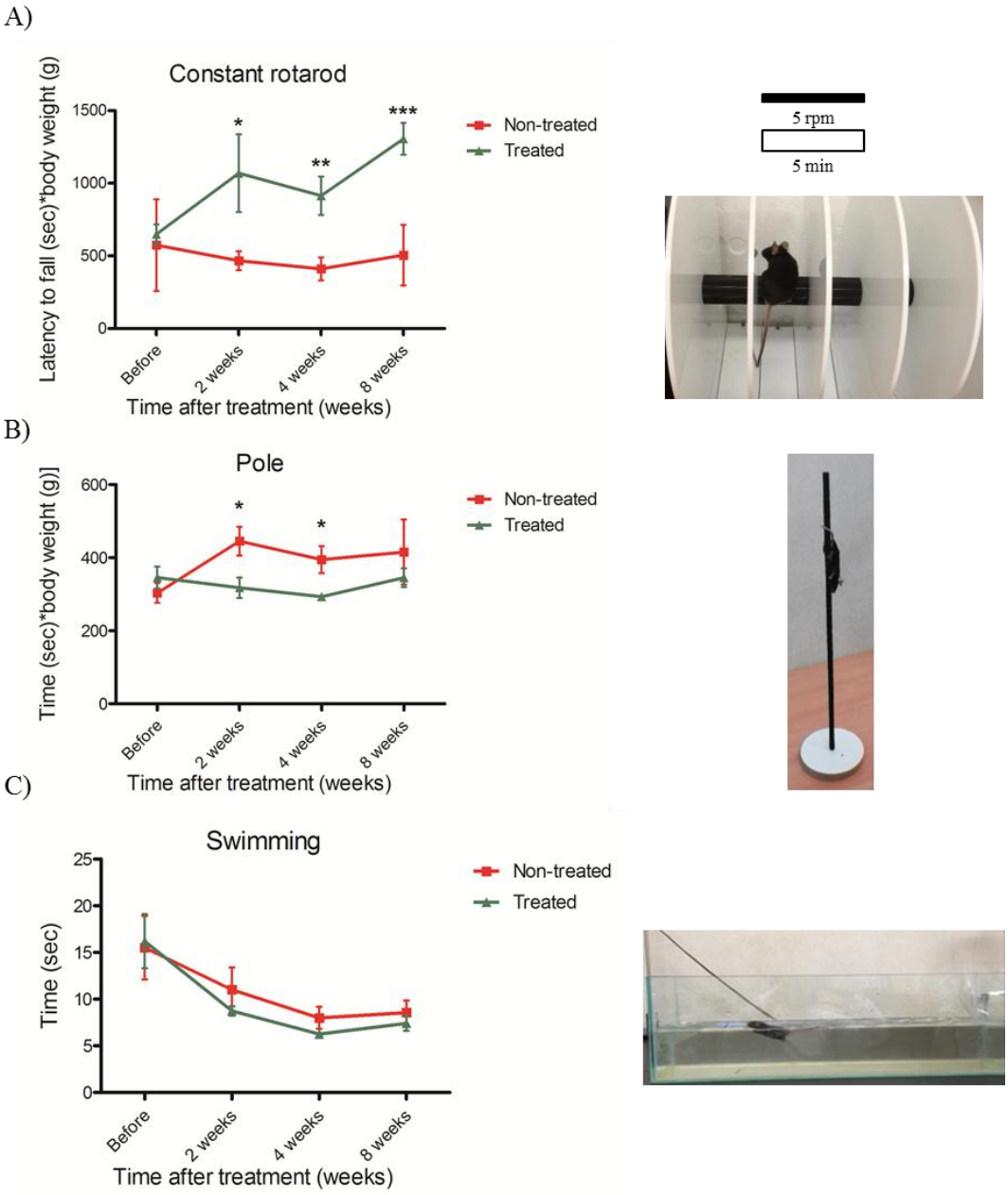
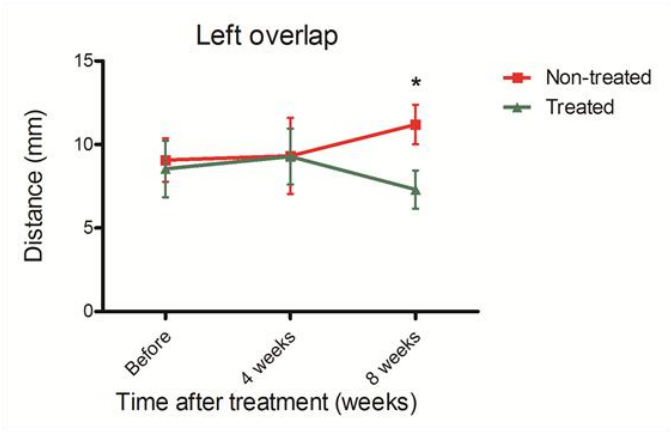


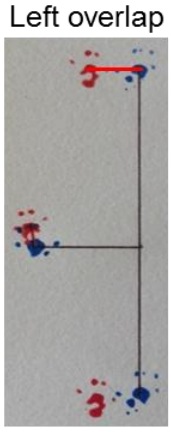
Figure 5: Analysis of motor function of Tg-ATXN3-69Q mice. Mice motor coordination and equilibrium were assessed through rotarod, pole and swimming test one week prior to first treatment (0 weeks) and a week after each administration, excluding week 6 (week 2, week 4 and week 8). **A)** The latency to fall off the rotarod apparatus (sec) normalized with mice body weight (g) was measured (rotarod test: 5 rpm for a maximum of 5 minutes). The latency to fall was significantly increased in treated group compared with non-treated animals at week 2, and continued to improve over the study time course (week 2, p=0.04; week 4, p=0.01; week 8, p=0.001). **B)** The time spent to turn and get down the pole was measured (sec) and normalized with body weight (g), and comparisons between the two groups were made. In agreement with rotarod test, treated animals spent less time getting down the pole than control group at 2 and 4 weeks after treatment (week 2, p=0.03; week 4, p=0.02), although no significant differences were observed at week 8. **C)** Also, treated mice showed a better performance in the swimming, as they presented a tendency to spent less time to cross the tank and reach the platform than non-treated group, though not significant. Values ate presented as mean ± SEM (Repeated Measures ANOVAs, followed by Bonferroni *post hoc* corrected tests, ***p <0.001, **p<0.01 and *p<0.05).

The gait of animals was also assessed through the footprint test. Differences between mice injected with MSCs and the control group were noticed at week 8 of experiment. At this time point treated mice (n=5 males) showed lower values in both overlap (right: 7.64±0.93 mm *vs* 11.98±1.26 mm, p=0.01; and left: 7.30±1.14 mm *vs* 11.20±1.18 mm; p=0.04) (Fig. 6-A and –B) and hind base width (27.48±0.66 mm *vs* 31.13±0.92 mm, p=0.009) (Fig.6-C) when compared to non-treated group (n=4 males). All tests above were also performed in females (n=4 non-treated; n=8 treated) but no significant differences were observed between the two groups (data not shown).

Overall, results obtained in behavior tests suggested that repeated MSCs treatment could rescue the phenotype observed in Tg-ATXN3-69Q MJD mice and lead to improvements in motor function.

A)





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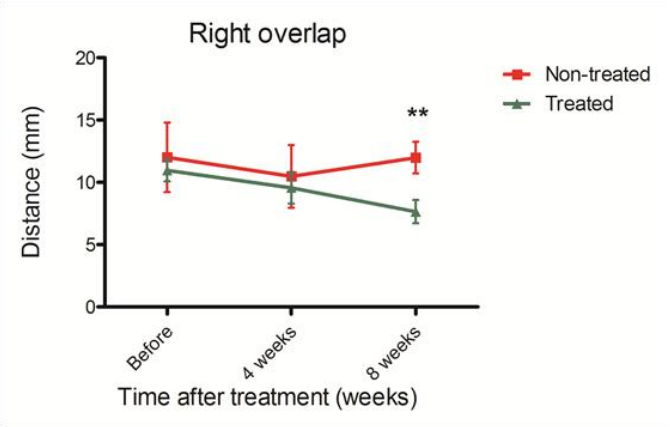
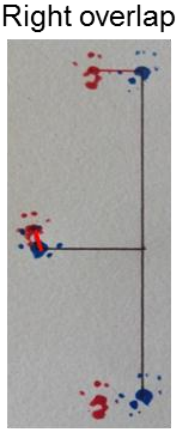
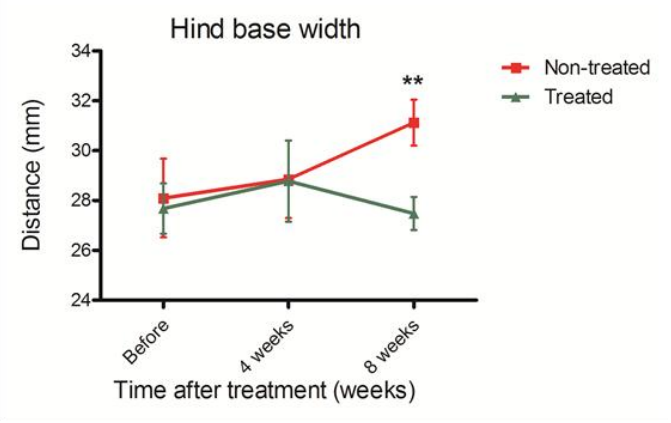




C)



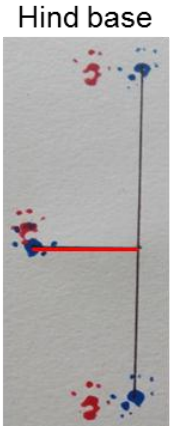


Figure 6: Gait analysis of non-treated and treated Tg-ATXN3-69Q mice. Mice footprints were measured for different parameters: hind base width and right and left overlap. Treated mice showed significant lower values for all measures when compared with the control group at the week 8 of the study for the Left overlap (p=0.04) (**A)**, Right overlap (p=0.01) (**B)** and Hind base width (p=0.009) **C)**. Values are presented as mean ± SEM (Repeated Measures ANOVAs, followed by Bonferroni *post hoc* corrected tests, ***p <0.001, **p<0.01 and *p<0.05).

3.2. Repeated systemic injections of syngeneic MSCs do not induce toxicity in Tg-ATXN3-69Q mice of MJD

We aimed next at investigating whether repeated infusions of syngeneic MSCs via the intravenous route would be safe and would not induce toxicity in treated animals. In order to do this, blood of mice sacrificed at week 10 of the experience (i.e., 3 weeks after the last infusion of cells), was centrifuged to separate blood cells and collect only the mice serum (Fig.7-A). Serum was analyzed for the prototypical three hepatic enzymes (transaminases) levels: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). When comparing treated animals (n=5 females) and non-treated mice (n=5 females) it was not observed an increase in any of those hepatic transaminases levels (Fig. 7-B, -C and –D). Though, a significant decrease of ALP was observed in treated mice compared to non-treated mice (70.20±4.96 vs 105.80±12.82, p=0.04), and compared to wild-type mice (WT) (70.20±4.96 vs 95.57±4.70, p=0.04) (Fig.7-D, any sign of their health condition was registered to be different). Moreover, spleen weights were similar between treated and non-treated groups (data not shown). Therefore, repeated doses of MSCs were considered safe for Tg-ATXN3-69Q MJD mice as no toxicity was detected in serum analysis.

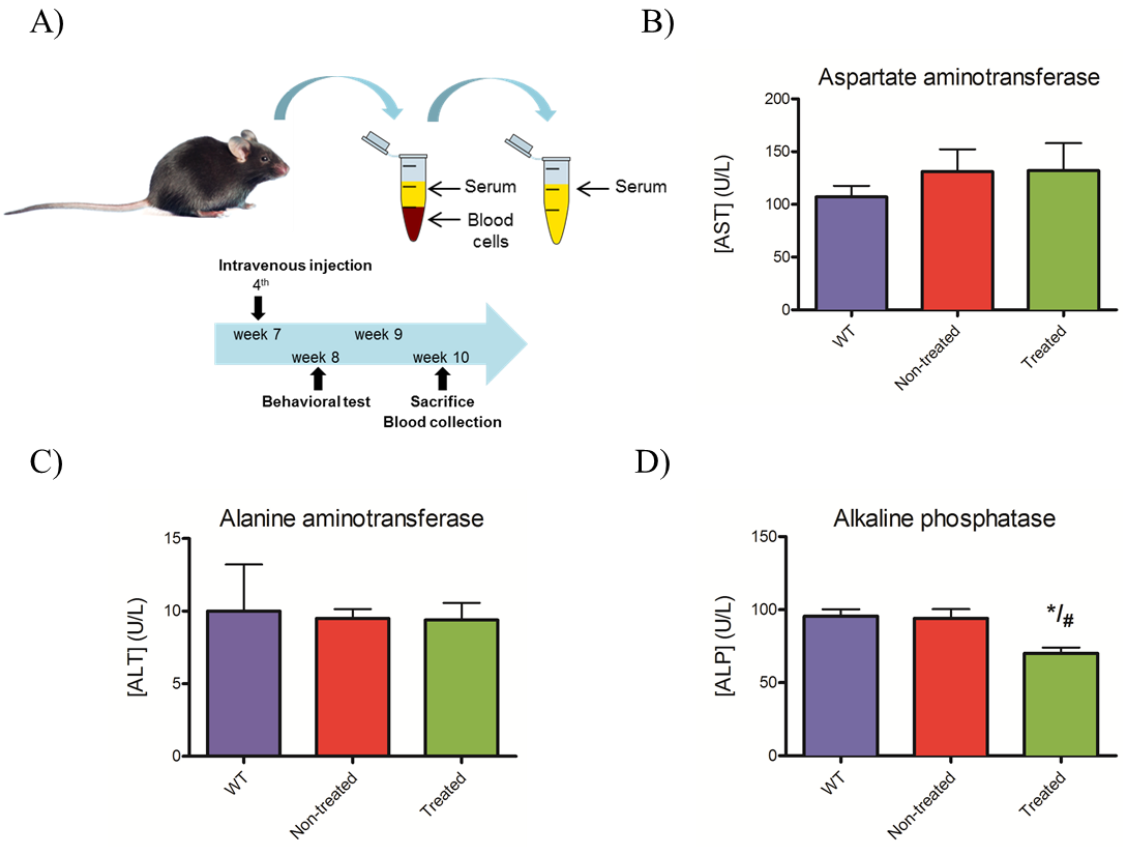


Figure 7: Repeated systemic MSCs administrations do not induce toxicity in MJD Tg-ATXN3-69Q mice. Hepatic enzyme levels were assessed in both treated and non- treated mice and in wild type controls in order to investigate possible toxic effects in transplanted mice. **A)** Mice were sacrificed at week 10, 3 weeks after last MSCs infusion, and blood was collected and centrifuged to separate blood cells from serum, which was then analyzed. Four MSCs intravenous injections did not promote an increment in the levels of the hepatic enzymes aspartate aminotransferase (AST) **(B)**, alanine aminotransferase (ALT) **(C)**, and alkaline phosphatase (ALP) **(D)** in the serum of treated females (n=5 non-treated vs n=5 treated). ALP is decreased in treated mice relatively to non-treated group (p=0.04) and was decreased in treated group relatively to WT mice (p=0.04). Values are presented as mean ± SEM. One-way ANOVAs, followed by Bonferroni *post hoc* corrected *t*-tests; ***p <0.001, **p <0.01 and *p <0.05 for non-treated and treated comparisons; ###p <0.001, ##p <0.01 and #p <0.05 for comparisons between WT and treated).

3.3. MSCs transplantation recues cerebellar neurodegeneration in Tg-ATXN3-69Q mice of MJD

As we have observed a phenotypic alleviation in transplanted MJD mice, we wanted to know if motor benefits would be correlated with neuropathologic alterations, so we decided to perform the histological analysis of the cerebellum, as it is the principal organ affected in Tg-ATXN3-69Q transgenic mice, used in this study. For this, after mice were sacrificed (3 weeks upon the last treatment or at week 10 of experiment), brains were removed and sliced into 35 µm sagittal sections. Three sections were selected to perform either cresyl violet or immunohistochemical staining.

Cresyl violet staining allowed us to distinguish the cerebellar cortex layers, so we could measure the thickness of both cellular layers. As lobule X is localized near the fourth ventricle, an intravenous treatment could induce neurologic improvements faster and more efficiently in this lobule. So we decided to focus our analysis in cerebellar lobule X and molecular and cellular layers thickness was measured in 3 regions of this lobule as represented in figure 8-A. It was observed a tendency to increase both molecular and cellular layer thickness in treated mice when compared to control group (non-treated: n=3 females + 2 males; treated: n=3 females + 1 male) (Fig. 8-B and -C). When both layers were measured together, a significant increase in layer thickness was registered in animals injected with MSCs (262.5±5.03 µm *vs* 237.3±5.67 µm, p=0.0160) (Fig.8-E). Moreover, we measured the volume of lobule X and lobule XI and XII in the same brain region in all animals. Accordingly, it was noticed a significant larger volume of lobule X in treated group (n=3 females + 2 males) compared to control group (n=3 females + 2 males) ($1.89x10^8 \pm 7.4x10^6$ μm^3 *vs* $1.47x10^8 \pm 2.6x10^6$ μm^3, p=0.0029) (Fig.9-B). Moreover, we observed that in some non-treated animals lobule VII did not fully developed and separated from lobule VI (Fig.9-A, right panel). As such, we have measured the volume of lobules VI and VII (together) and a significant volume increase in animals treated with MSCs was found (treated group: n=3 females + 1 males; non-treated group: n=4 females + 2 males) ($4.84x10^8 \pm 3.9x10^6$ μm^3 *vs* $4.53x10^8 \pm 1x10^7$ μm^3, p=0.0276) (Fig.9-C). These results suggest that MSCs transplantation via intravenous route could alleviate neuropathological defects in Tg-ATXN3-69Q transgenic mice of Machado-Joseph disease.

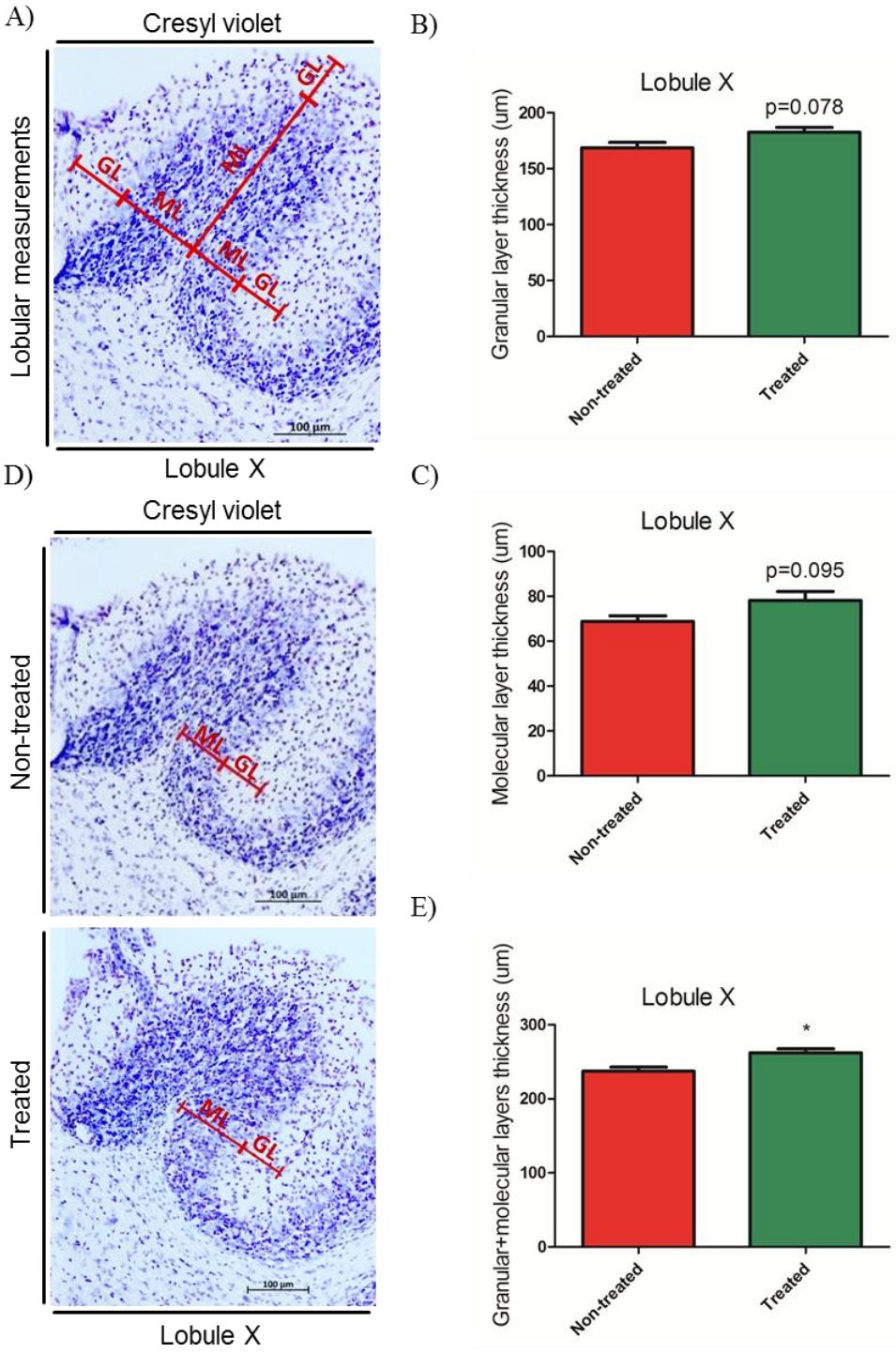


Figure 8: Repeated systemic MSCs administration recue cerebellar layers' atrophy in MJD Tg-ATXN3-69Q mice. A) Representative images of sagittal sections of lobule X of the cerebellum of non-treated (upper panel) and treated (lower panel) mice stained with cresyl violet, showing the molecular (ML) and granular (GL) layers. **B)** Analysis of granular layer thickness measures in lobule X. Treated mice showed a tendency to present a thicker granular layer when compared to control group (p=0.078). **C)** Molecular layer thickness analysis in lobule X. Similarly, animals treated with MSCs showed a tendency to increase molecular layer thickness comparing to non-treated mice (p=0.095). **E)** When measured together, treated animals showed a significant increase in cerebellar cellular layers compared to control group in lobule X (p=0.016). Values are presented as mean ± SEM (Unpaired Student's t-test, ***p <0.001, **p <0.01 and *p <0.05).

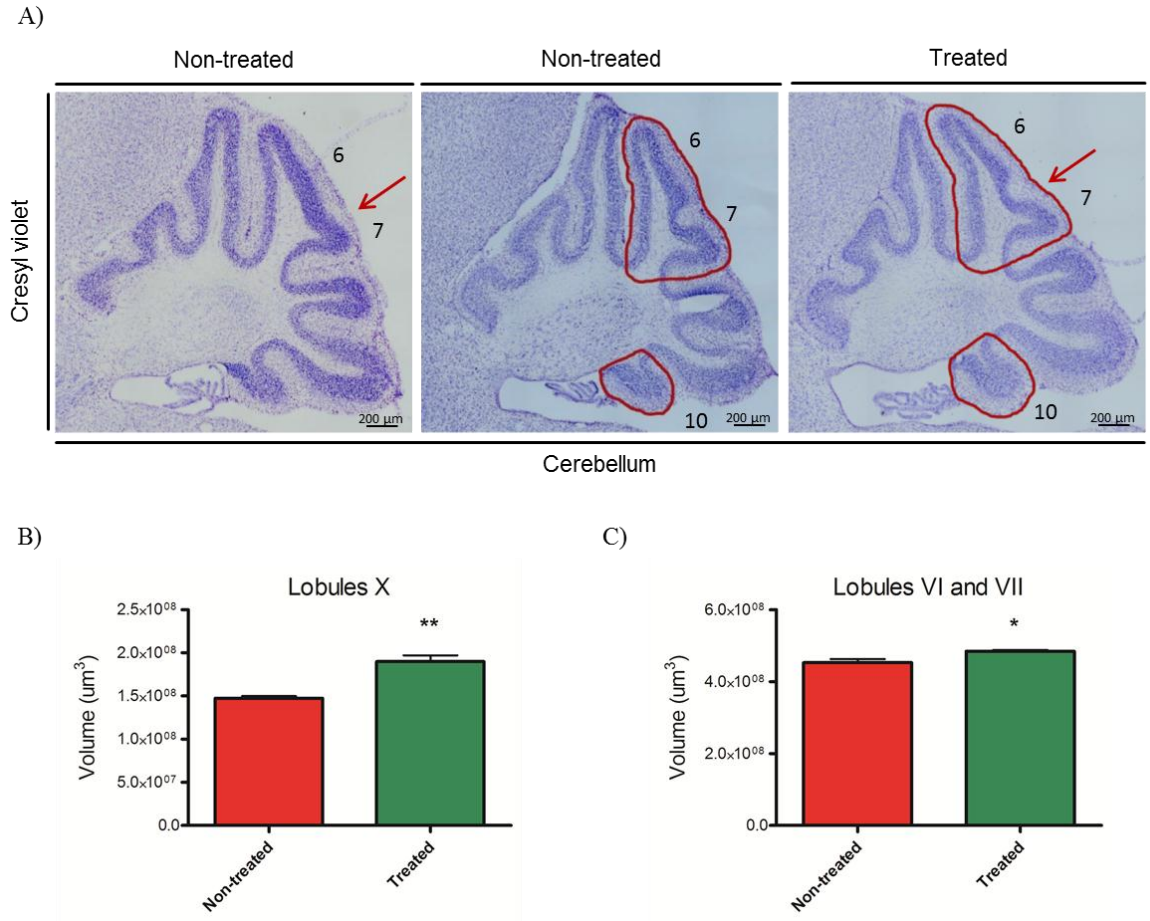


Figure 9: Repeated systemic MSCs transplantation improves lobular volume in cerebellum of Tg-ATXN3-69Q transgenic mice. A) Representative images of sagittal sections of the cerebellum of non-treated (left and middle panel) and treated (right panel) mice stained with cresyl violet, showing cerebellar lobules. **B)** Analysis of lobule X volume. Treated mice presented significant higher volume when compared to control group (p=0.0029). **C)** Lobules VI and VII volume analysis. Similarly, animals treated with MSCs showed a significant increase in lobules VI and VII volume comparing to non-treated mice (p=0.0276). Values are presented as mean ± SEM (Unpaired Student's t-test, ***p <0.001, **p <0.01 and *p <0.05).

The transgenic mice (Tg-ATXN3-69Q) used in this study has a L7 promoter that promotes expression of mutated ataxin-3 protein, containing an haemagglutinin (HA) epitope in the N-terminal region, specifically in Purkinje cells, present in the cerebellum. This mutated form of ataxin-3 leads to formation of aggregates in the cerebellum, a hallmark of neuropathology in this model. Thereby, we performed an immunohistochemistry using an antibody against anti-HA, in order to count the number of aggregates. For this procedure, we chose 3 sections per animal corresponding to the brain region referred previously and counted the number of ataxin-3 inclusions in all lobules of the cerebellum. In agreement with the cresyl violet staining results, differences in the number of there was a tendency to reduce the number of inclusions in treated animals (n=3 females + 2 male) when compared with non-treated group (n=3 females + 1 male) (30.20±2.25 *vs* 36.25±1.46, p=0.074) in lobule X (Fig.10-B). Furthermore, by using an antibody against Purkinje cell protein 4 (PCP-4), a protein present in the brain and highly expressed by Purkinje cells, we were able to label Purkinje cells. As the lobule X was the lobule that showed higher differences in previous analysis, we decided to count the number of Purkinje cells in this lobule within the same 3 sections mentioned before. However, no differences were observed between mice treated with MSCs (n=4 females + 2 males) and the control group (n=4 females + 1 male) (Fig.10-C).

Accounting all the histological results presented, we showed that repeated systemic administrations of MSCs can mitigate the neuropathologic features observed in Tg-ATXN3-69Q MJD mouse model, particularly in lobule X. This effect in lobule X may be due not only to its location, as it communicates directly with the fourth ventricle, where CSF circulates, but also due to its highly vasculated structure, enabling systemic therapies to reach in higher extent to that region.

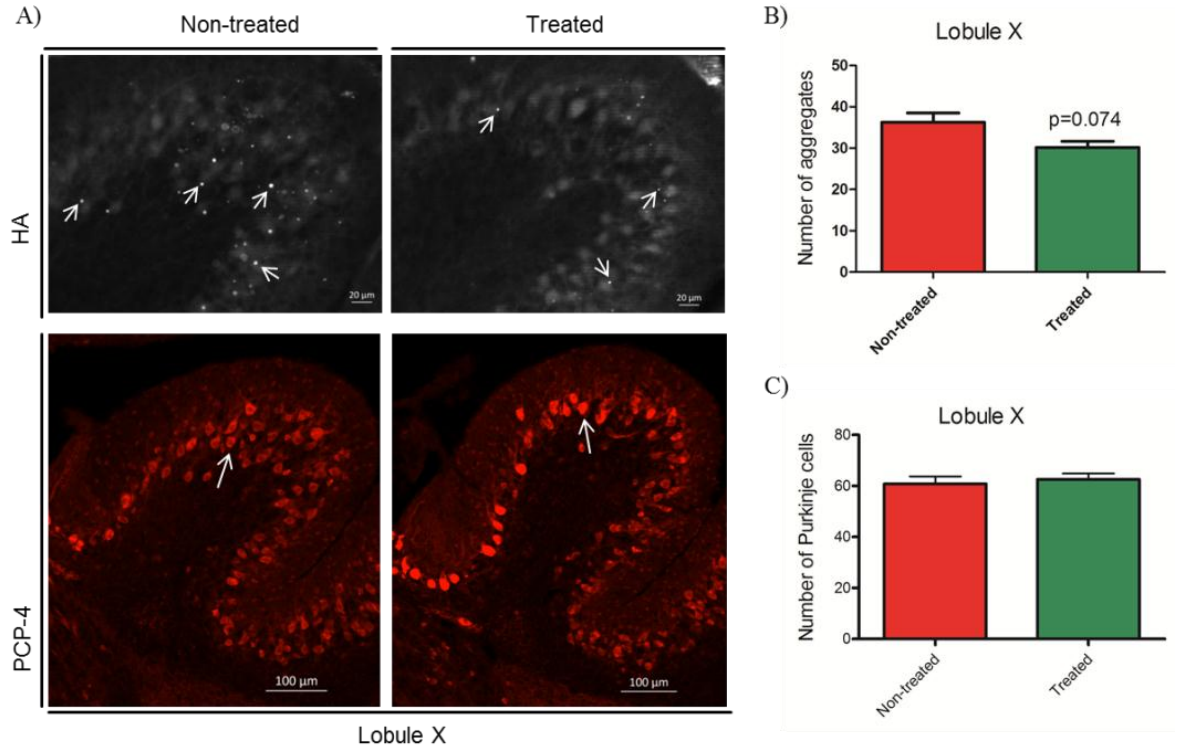


Figure 10: MSCs transplantation shows a tendency to decrease mutant ataxin-3 protein aggregates in cerebellum of Tg-ATXN3-69Q transgenic mice. A) Representative images of sagittal sections of the lobule X of non-treated (left upper panel) and treated (right upper panel) mice stained with primary antibody anti-HA, and non-treated (left lower panel) and treated (right lower panel) mice stained with primary antibody anti-PCP-4. **B)** Number of aggregates in lobule X. Treated mice presented decreased mutant protein inclusions when compared to the control group, though not significant (p=0.074). **C)** Number of Purkinje cells in lobule X. No differences were observed between the two groups. Values are presented as mean ± SEM (Unpaired Student's t-test, ***p <0.001, **p <0.01 and *p <0.05).

CHAPTER IV - DISCUSSION

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Previously, our group has observed that a single intracerebral administration of MSCs, either directly into the cerebellum or into the lateral ventricle of the brain, could alleviate phenotypic and neuropathologic features characteristic of the Tg-ATXN3-69Q transgenic mouse model of MJD. However, improvements observed were only temporary and afterwards animals returned to prior state, which is in accordance with the preliminary reports of clinical trials performed in SCAs. Therefore, the main goal of this study was to provide evidences that a continuous treatment with MSCs, via the intravenous route, would be able to produce sustainable benefic effects, being a safe and efficient therapy for Machado-Joseph disease.

In the present study, it was used the Tg-ATXN3-69Q transgenic mouse model of MJD which presents a severe early onset of cerebellar ataxic phenotype, showing imbalance and gait disturbances, as early as 3 weeks of age (Torashima et al., 2008). Mice treated with MSCs received 4 intravenous injections of cells (4-8x10^7 MSCs/Kg) spared two weeks from each other, starting at 4-6.5 weeks of age, i.e., after the phenotypic symptoms. Motor behavioral tests demonstrated that a periodic treatment with MSCs could improve motor function throughout time. Moreover, ataxic gait was mitigated as was shown by a decrease in the hind base width and both left and right overlaps, measures that are all increased in this transgenic model relatively to wild types. Similar results were also achieved in another model of ataxic mice induced by cytosine beta-D-arabinofuranoside (Ara-C) (Zhang et al., 2011). In this report, ataxic mice received an intravenous injection of 2x10^6 HU-MSCs once a week for three consecutive weeks. Eight weeks after the treatment, HU-MSCs transplantation significantly alleviated motor impairments in ataxic mice. In agreement, another research study that used a transgenic mouse model of SCA-2 compared the therapeutic effect of HU-MSCs administered directly into the intracranial space (3 infusions of 8.4x10^6 cells/Kg) or transplanted via systemic route (4 injections of 4.2x10^7 cells/Kg) (Chang et al., 2011), and concluded that intravenous infusion of HU-MSCs significantly improved motor function while intracranial transplantation did not produce neuroprotective effects on transgenic mouse model of SCA-2. Therefore, our results are consistent with pre-clinical studies in other spinocerebellar ataxias. However, in pre-clinical studies referred herein, treatment with MSCs was only administered for a small period of time and most probably, if motor function continued to be measured in later assessments, treated animals would probably return to previous disease status. So, our

results support the hypothesis that a continuous treatment with MSCs would be a more effective therapy for SCAs, in particular for MJD.

Clinical trials have also investigated the therapeutic potential/safety of MSCs in spinocerebellar ataxias. One of these clinical studies was conducted in fourteen patients diagnosed with SCA pathology and ten patients with MSA-C (Dongmei et al., 2011). A dose of $1x10^6$/kg UC-MSCs with 5mg dexamethasone were administered via intrathecal injection weekly during 4 weeks. One month after UC-MSCs transplantation, motor function was improved in all patients, except for one that showed no response to treatment. The study reported that the clinical condition of 10 patients persisted stable for half a year or more, though in 14 cases, patients' condition returned to the status previous to the treatment, on average 3 months after the treatment. This result supports our premise that a periodic, continued treatment with MSCs may be required for the treatment of SCA patients. Another clinical study assessed the therapeutic evaluation of UC-MSCs administration in sixteen patients with SCA (Jin et al., 2013). The treatment consisted on a first intravenous injection of $4x10^7$ UC-MSCs and 3 subsequent treatments with simultaneous intravenous and intrathecal infusions ($2x10^7$ UC-MSCs for both transplantations), all transplants separated by each other of one week. After one year of treatment, 7 patients exhibited improved motor function while 5 patients suffered from disease aggravation. Moreover, a clinical trial using UC-MSCs was also performed in 8 spinocerebellar ataxic patients (Miao et al., 2015). The UC-MSCs were intrathecally injected four to six times, within an interval of 5-7 days. After 1 year of follow-up for clinical status, only three of eight patients showed alleviation of motor deficits. Therefore, results from clinical studies using MSCs to treat SCAs show that MSCs effect seems to be lost during time. In agreement with these observations our results suggest that a repeated therapy with MSCs transplantation might be a good therapeutic strategy for the amelioration of motor impairments in Machado-Joseph disease.

Pre-clinical studies are of extreme importance, not only because they allow to address fundamental questions before applying a certain treatment/drug to human patients, but also because they help to understand the neuropathologic alterations that result from the administration of this treatment/drug. In the present work, mice were sacrificed 10 weeks after the beginning of the experience, i.e., 9 weeks after the first treatment, and brain sagittal sections were stained for different markers. We focused

mainly in the cerebellum, as it is the main organ affected in the Tg-ATXN3-69Q mouse model. MSCs promoted an increased preservation of the neurons of the cerebellar layers in lobule X as shown by the increased thickness of molecular and granular layers. Moreover, lobular volume of lobule X and lobules VI and VII was also significantly higher in mice treated with MSCs when compared to non-treated animals. These results suggested that multiple administrations of MSCs might alleviate the cerebellar atrophy characteristic of the transgenic mouse model of MJD. These observations are consistent with Zhang and colleagues' study mentioned above (Zhang et al., 2011), where they reported that repeated HU-MSCs injections led to improvements in cerebellar volume. Furthermore, as the presence of mutant ataxin-3 protein inclusions is the hallmark of neuropathology in the Tg-ATXN3-69Q transgenic mice used, we assessed the number of aggregates in all cerebellar lobules. According to the results obtained regarding the cerebellar volume and the layer's thickness, the number of aggregates showed a tendency to decrease in lobule X of animals treated with MSCs. However, alterations in the number of Purkinje cells in this lobule were not observed. These results might be explained by the fact that neuropathologic analysis was only performed in 2 males, in both non-treated and treated groups (along with 4 females in each group). In motor performance, significant differences were observed only in males (non-treated mice: n=4 males; treated mice: n=5 males), while when we have tested females alone, no phenotypic differences were observed. If the number of males were increased in the set of animals used for the neuropathologic evaluations, we could possibly obtain differences in the number of Purkinje cells in Tg-ATXN3-69Q transgenic mice treated with MSCs, as these neuronal cells are fundamental for motor performance.

MSCs mechanisms of action are still unknown. Homing for lesion sites and release of neurotrophic factors have been proposed to explain MSCs neuroprotective effects (Paul and Anisimov, 2013). In our study, we could not find MSCs in the cerebellum, or in any other brain regions. On the contrary, Zhang and colleagues observed that HU-MSCs transplanted intravenously were able to reach the cerebellum (Zhang et al., 2011). Furthermore, Chang's study also reported the presence of hMSCs in cerebellar space (Chang et al., 2011). As neuropathologic improvements were mainly observed in lobule X, a lobule localized near the fourth ventricle, MSCs benefic effect might be due to the release of neurotrophic factors rather than homing for the injured site and act locally. In fact, Zhang's study has reported that mice treated with MSCs had

enhanced expression of insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) in either the cerebellum or blood serum samples. Other experiments will be also performed to track MSCs after injected intravenously, such as *in vivo* Bioluminescence imaging. Bioluminescence is a light source based on the luciferin-luciferase reaction assay, that is widely used for in vivo cell tracking (Kim et al., 2015). For this experience, we will perform transfection of MSCs with luciferase and will inject intravenously these cells in mice. Afterwards, luciferin will be administered to animals and when catalyzed by luciferase, light will be generated. Imaging of MSCs will be performed at defined times by an appropriate CCD photon imaging system. As both phenotypic and neuropathological alleviation could be achieved in the present study, we concluded that multiple injections of MSCs showed to be a valuable treatment for MJD.

Repeated administrations of MSCs raises, however, the question of whether these cells are safe and do not induce toxicity for its receptors. Safety of MSCs has also been assessed in clinical trials with SCA patients. Dongmei and colleagues reported that after MSCs treatment, side-effects such as dizziness, low back pain and headache, disappeared in all patients 1-3 days post-procedure (Dongmei et al., 2011). Jin and collaborators' research work also noticed that after a follow-up of 12 months post-treatment, no severe adverse effects were observed in all patients (Jin et al., 2013). Taken these observations in consideration, both studies reported that MSCs were safe for clinical use. Consistently with these results, we observed that MSCs did not induce toxicity in treated mice, as hepatic aminotransferases levels were not increased when compared to non-treated and WT mice. Therefore, MSCs were suggested to be safe for the treatment of Machado-Joseph disease.

To sum up, in this study we observed that a repeated systemic treatment with MSCs was safe and could efficiently improve motor function deficits throughout time in Tg-ATXN3-69Q transgenic mice. Also, mitigation of cerebellar atrophy suggested that neuropathologic features were alleviated. So, in the present study, we suggest a periodic, continued treatment with MSCs as a possible therapeutic strategy for MJD and probably other SCA patients.

CHAPTER V – CONCLUSION AND FUTURE PERSPECTIVES

In this study, we provided evidences that a repeated and continued transplantation of MSCs can promoted sustainable benefic effects in motor performance and neuropathology of a transgenic mouse model of MJD. These results proved the therapeutic potential of MSCs for Machado-Joseph disease, but simultaneously showed that several treatments are necessary to achieve the desirable action of the cells at least in this particular context.

In the future, we intend to investigate the mechanisms of action by which MSCs exert neuroprotection in this disease. Particularly, we will perform quantitative PCR and western blot analysis of cerebellar tissue samples from Tg-ATXN3-69Q transgenic mice treated with MSCs intravenously and non-treated Tg-ATXN3-69Q transgenic mice (injected with HBSS) to search for altered levels of some trophic factors, such as BDNF, NGF, VEGF and GDNF. Moreover, in vitro studies will also be conducted to investigate the MSCs mechanism of action in MJD. For that, we will perform co-cultures of MSCs with neurons infected with lentiviral-mutant ataxin-3 in a transwell system. These systems include a transwell permeable support with micropores, allowing for the communication between cultured cells in the bottom and the cells localized in the supports, as it occurs the movement of small molecules between both cell types through the medium. ELISA and quantitative PCR assays will allow for the identification in a more controlled system of possible trophic factors released by the MSCs that will support the neurons survival.

With these experiments, we expect to identify potential candidates for MSCs beneficial effect in MJD that can finally lead to a more targeted and efficient therapy for MJD patients.

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