IMPACT OF MESENCHYMAL STEM CELL TRANSPLANTATION WITH CO-THERAPY OF NICOTINAMIDE ON NEUROLOGICAL AND PERIPHERAL DEFICITS IN THE R6/2 MOUSE MODEL OF HUNTINGTON'S DISEASE

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ABSTRACT

IMPACT OF MESENCHYMAL STEM CELL TRANSPLANTATION WITH CO-THERAPY OF NICOTINAMIDE ON NEUROLOGICAL AND PERIPHERAL DEFICITS IN THE R6/2 MOUSE MODEL OF HUNTINGTON'S DISEASE

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Huntington's disease (HD) is a fatal neurodegenerative disease characterized by psychiatric and cognitive symptoms, motor impairment, and weight loss that results ultimately death. There is currently no treatment for HD beyond palliative care. Due to the complex etiology and wide-ranging symptoms of the disease, it seems unlikely that a monotherapy will emerge in the near future that is able to address the multifaceted HD-induced dysfunctions that result in degeneration and, ultimately, death. Therefore, this study employed a combinational treatment using mesenchymal stem cell (MSC) transplantation into the striatum in conjunction with subcutaneous administration of the B3 complex supplement, nicotinamide. Results showed that this combination conferred a synergistic effect on metabolic function in the striatum. These results suggest nicotinamide supplementation may be a viable adjunct supplement to cell transplantation therapies to address some of the symptoms of HD.

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INTRODUCTION AND REVIEW OF LITERATURE

Huntington's disease (HD) is a progressive inherited neurodegenerative disease characterized by psychiatric and cognitive symptoms, progressive motor impairment, weight loss, and, ultimately, death. HD is caused by an expanded polyglutamine stretch of the huntingtin protein (HTT), with CAG repeat lengths beyond 40 in the gene encoding HTT, resulting in the disease state (Zuccato, Valenza, & Cataneo, 2010). The precise role of HTT is uncertain, but due to its structure, it likely subserves protein-protein interactions (Zuccato, Valenza, & Cataneo, 2010), and its mutation (mHTT) results in a broad array of cellular impairments, including transcriptional dysregulation, mitochondrial dysfunction, neurotrophic factor insufficiency, impaired cell signaling, as well as impaired synaptic transport and activity (Maglione et al., 2010). There is currently no treatment for HD beyond palliative care, and due to the complex etiology and multifaceted dysfunction observed in this disease, the advent of an effective monotherapy in the near future seems unlikely. Therefore, combinatorial approaches may offer a timelier route to an effective regimen capable of delaying the onset and progression of this devastating disease.

The use of cell therapies has become increasingly popular as a potential treatment option for a number of disorders, including neurodegenerative diseases, with over 2500 clinical trials in various diseases over the past 10 years (Culme-Seymour et al., 2012). With continuing research, cell therapies may someday offer a treatment for several, heretofore, untreatable diseases. Mesenchymal stem cells (MSCs) in particular, offer a number of advantages for potential therapeutic applications and have been tested in several neurodegenerative diseases, including HD, and have shown promise in ameliorating symptoms. MSCs can be harvested from a number of sources, including bone marrow, adipose tissue, and umbilical cord blood, making them readily available. MSCs are also easily expandable in culture and can survive for a relatively long period of time (Wyse, Dunbar, & Rossignol, 2014). In addition to these pragmatic qualities, MSCs, when transplanted, are immunosuppressive and release trophic factors, both of which may help to support cell function and survival near the transplantation site (Lescaudron et al., 2012). Together, these characteristics help make MSCs an ideal candidate for cell transplantation therapies. Unfortunately, while MSC transplantation into the brain shows promise in addressing the neurological deficits observed in HD, this targeted treatment is unable to address several critical factors, including peripheral metabolic dysfunction, that results from the ubiquitous expression of mHTT.

Therefore, adjunct systemic therapies may help to improve MSC therapy outcomes by addressing the metabolic dysfunctions which result in both peripheral and neurological symptoms.

One substance that has shown efficacy in a number of metabolic and neurodegenerative disorders is nicotinamide (NAM; Nicolson, 2014; Herskovits & Guarente, 2013; Hathorn et al., 2011; Hankes et al., 1991). NAM is part of the vitamin B3 complex, along with nicotinic acid, known as niacin. However, the biological pathways for salvage, as well as bioavailability, differ significantly between nicotinamide and nicotinic acid (Belenky et al., 2006; Hankes et al., 1991). NAM has been shown to be more bioavailable (Hankes, et al., 1991), and does not induce the vasodilation seen with administration of nicotinic acid. NAM is a key component of nicotinamide adenine dinucleotide (NAD+), which is crucial for all metabolic processes. In addition to its key role in cellular metabolic function, NAD+ plays an important role in regulating the histone deacetylases, SIRT1 particularly, which have been found to function in the regulation of stress response, apoptosis, and DNA repair (Naia et al., 2016; Nicolson, 2014; Yang et al., 2014; Herskovits & Guarente, 2013; Belenky et al., 2006). Taken together, these wide-ranging effects may offer a treatment solution that not only addresses the neurological and peripheral metabolic dysfunctions of HD, but also the apoptotic and transcriptional dysregulation, making NAM an enticing substance for further research.

Therefore, the goal of the present study was to employ a combinational therapy in an attempt to address both the neurological and peripheral symptoms seen in HD. MSC transplantation into the striatum and subcutaneous nicotinamide supplementation were employed individually as monotherapies, as well as in conjunction with one another, in the R6/2 murine model of HD. Behavioral and histological analyses was performed to evaluate the efficacy of each treatment alone, and when combined.

MATERIALS AND METHODS

Animals

Thirty-two hemizygous R6/2 (HD) mice and 7 of their wild-type (WT) littermates were included in the study and assigned to one of the following groups: (1) WT-Control (n = 7), where animals received a subcutaneous osmotic pump containing PBS; (2) HD-Control (n = 9); where animals received a subcutaneous osmotic pump containing PBS; (3) HD-NAM (n = 7), where animals received subcutaneous osmotic pump containing NAM; (4) HD-MSC (n = 8), where animals received MSC transplantation; and (5) HD-MSC+NAM (n = 8) in which animals received both MSC transplantation and subcutaneous pump with NAM. All mice were weaned at three weeks of age, and housed in numerical birth order in groups of mixed genotypes with same-sex littermates in polypropylene tubs. Housing was lined with aspen chip bedding, supplemented with a single paper towel for enrichment. Mice had access to food and water, *ad libitum*, in an environmentally controlled room on a twelve-hour light/dark cycle. At three weeks of age, mice will be handled daily to habituate them to handling. All animal tests and procedures were performed in accordance to the Guide for Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at Central Michigan University.

Mesenchymal Stem Cells

WT mice at 10 months of age were sacrificed by cervical dislocation, after which the tibia and femur were harvested, and washed with ethanol. Under sterile conditions, extraction of total bone marrow was conducted by breaking the tibia and femur bones in half and removing marrow from bones with a 25-gauge needle. As bone marrow was collected, it was placed in a 15-mL tube (Falcon) containing 10 mL of MSC medium (α-MEM: Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS: Invitrogen), 10% horse serum (HS: Invitrogen), and 1% penicillin-streptomycin (Sigma: St. Louis, MO, USA). Upon obtaining the maximal marrow, the cell-containing media was centrifuged for 7 minutes at 220 g relative centrifugal force (RCF) at 4°C. The supernatant was aspirated and precipitated cells were suspended in 1 mL MSC medium and counted with the use of a hemocytometer. Cells were then placed in a vented 25-cm^2 tissue culture plate (Techno Plastic Products, Trasadingen, Switzerland; TPP)

and incubated in a water jacket incubator at 37°C and 5% CO^2. After 24 hours, the medium was removed and replaced, while all non-attached cells were discarded. Upon reaching 70% - 90% confluency, the supernatant was aspirated then discarded, and the plate was rinsed twice with 10 mL of phosphate buffer saline (PBS). The cells were then detached from the flask using 5 mL of 0.25% Trypsin-ethylenediaminetetraacetic acid (Trypsin/EDTA; Invitrogen) for 5 minutes at 37°C. Trypsin/EDTA was then deactivated with equal parts of medium, cells and medium were aspirated, placed in a 15-mL tube, and centrifuged for 7 minutes at 220 g RCF at 4°C. The supernatant was then removed. Obtained cells were counted and replated in a new plate with the MSC medium. Each time a cell underwent this process it was considered a single passage. Cells used for transplantation had undergone approximately 40 passages.

Nicotinamide (NAM)

The NAM treatment was delivered via mini osmotic pumps (Alzet, model 2004) implanted subcutaneously mid-scapularly. Pumps delivered a dosage of 250 mg/kg per day for a duration of two weeks. The initial pump implantation occurred concurrently with MSC transplantation. At the end of the second week following implantations, the pumps were removed and replaced with new pumps containing the same dosage. In total, three pumps were implanted for a total treatment duration of 6 weeks.

Surgery

Surgical procedures were conducted at postnatal week five. Mice were anesthetized throughout the procedure via inhalation of 0.5-3 % isoflurane (Halocarbon Products Corporation, Rivers Edge, NJ) and 500 mL-1 L O^2 gas. The scalps of mice were shaved with an electric pet grooming trimmer then disinfected with chlorehexidine (Molnlycke Healthcare, Brunswick, ME, USA). The mice were maintained in a stable level position with placement of ear bars and tooth bar on a stereotaxic device (David Kopf Instruments, Tejunga, CA). Upon positioning in the stereotaxic device, artificial tears, a sterile ophthalmic ointment (Butler AHS, Dublin, OH), was applied to the eyes of mice. Animals were provided with supplemental heat throughout the surgery via a heating plate, thermostatically controlled by a rectal probe thermometer. A single midline incision was made on the scalp to expose the cranial surface. Two burr

holes (0.5 mm) were drilled directly above the neostriatum as identified by relative coordinates from bregma: anterior +0.5 mm; lateral - 1.75 mm; and −2.5 mm ventral to the cranial surface. Hoechst-labeled MSCs or HBSS (Hanks balanced salt solution: Life Technologies) was loaded into a 10 µL Hamilton syringe (Hamilton, Reno, NV), and two administrations of 200,000 cells were made at 0.33 µL/minute for three minutes per striatum in each hemisphere. The second administration was -1.5 mm ventral from the cranial surface. Following each administration, the needle was left in place for three minutes to allow for cell diffusion and to reduce withdrawal of cells along the needle track. Upon the completion of bi-lateral injections, a solution of viscous 2% topical lidocaine was applied to the site of the incision which then was closed using sterile wound clips

Body Weight

Beginning with baseline behavioral testing, animals were weighed three times per week until the termination of the study. Animals that began to show excessive or rapid weight loss were monitored daily.

Rotorod

At four weeks of age, a time which precedes significant motoric deficits in the R6/2 mice, all mice were trained to traverse a rotorod apparatus (SDI Rotor-Rod; San Diego Instruments, San Diego, CA, USA). This task required mice to walk on a beveled, 3-cm diameter, variable-speed rotating rod. If mice are unable to maintain their balance, they fall onto a foam pad placed below the rod. During the initial training session, the number of bar rotations per minute (RPM) was set to a low speed (1-2 RPM). When mice were able to maintain balance on the bar for a 60-second trial, the speed of the bar was gradually increased in 2 RPM increments until reaching 15 RPM. Upon the completion of a 60-second trial, the mice were removed from the bar and allowed to rest for 1 minute. Training sessions were completed daily for no more than 30 min per session until reaching the criterion of two consecutive sessions at 15 RPM without falling.

A baseline recording was conducted on the day prior to surgery. Testing was completed once weekly for 7 weeks, beginning when animals were six weeks of age. The testing procedure included 3 trials with a 1-minute inter-trial-interval at 5, 10, and 15 RPM. The latency to fall from the rod was

recorded and the mean of the three trials for each animal, at each speed, was used for statistical analysis.

Clasping

R6/2 mice exhibit limb dyskinesia when held by their tails. When picked up, the R6/2 mice often retract their limbs to their torso. This dyskinesia increases as the mice age. Initially, animals clasp only a single limb briefly, but progressively all limbs become affected and the animals may clasp all four limbs (Mangiarini et al., 1996). For this study, a clasp was defined as the rigid retraction of a limb towards the torso for a minimum period of 1 second when mice were suspended by the tail at a height of 50 cm. Clasping assessment began concurrently with rotorod testing, and was assessed immediately following the completion of each rotorod testing session. A trial was terminated when 30 seconds has elapsed, or if all four limbs were clasped. Each trial was scored by the number of limbs clasped; ranging from 0 to 4. The clasping score was recorded and the mean of three trials per session for each animal was utilized for statistical analysis.

Open Field

Open-field testing was conducted weekly following rotorod testing sessions. Mice were placed into clean empty rat polypropylene tubs (approximately 47.5 cm x 25.5 cm) and the tubs were placed in frames containing two 16-beam and one 7-beam infrared arrays for positional tracking along X and Y axes and rearing detection (Hamilton-Kinder; Poway, CA, USA). Ambulatory movements are recorded when a series of beams are interrupted in succession, or fine-motor movements are recorded when only beams immediately surrounding the mouse are interrupted. The system was attached to a computer with a Windows 10 operating system and Hamilton-Kinder MotorMonitor (Build: 05266-10) software recording all motor movements. Open-field movements were recorded for 1 hour, with no lighting or ambient noise in the experimental room. Total distance travelled by each animal was recorded and used to assess overall motor behavior from week to week.

In addition to total distance travelled, time spent exploring the center of the field was recorded. Increased exploration of the unprotected center area is an indicator of reduced anxiety in mice (Bailey

and Crawley, 2009). Total time spent in the center was recorded weekly for each animal and used to assess anxiety behavior between groups.

Forced Swim Test (FST)

The forced-swim test (FST; also called the behavioral despair test or Porsolt test) is a widely-used test for screening antidepressants with good predictive validity (Castagne et al., 2011). When rodents are forced to swim in a small space from which they cannot escape they become immobile after an initial period of vigorous activity, except for necessary movement to keep their heads above water (Castagne et al.; Mortazavi et al., 2005). Castagne and collegues (2011) indicates the forced-swim protocol can be employed effectively to assess depression or despair in studies using transgenic animals (rats and mice).

Automated FST was conducted using the Hamilton-Kinder forced swim test device (Hamilton-Kinder; Poway, CA, USA). The device consists of a clear water-filled cylinder, equipped with two 4X4 photo-beam arrays that monitor swimming and climbing behaviors. The device is connected to a computer with Windows 10 operating system and Hamilton-Kinder MotorMonitor software. Testing was done once when animals are 9 weeks of age. Animals were placed individually in the device for a total of 6 minutes. The first 2 minutes were considered a habituation period (Kurtuncu et al., 2005). The duration of time spent inactive (floating) during the final 4 minutes for each animal was recorded and used for statistical analysis.

Perfusion

Mice were transcardially perfused, after which their brains were collected, frozen, and sectioned. At 90 days of age, all mice were intraperitoneally administered 80 mg/kg sodium pentobarbital. When unresponsive to paw pinch, mice were perfused transcardially using a 21-gauge needle with 25 mL PBS followed by 25 mL of ice cold 4% paraformaldehyde (PFA). Animals were decapitated and the brains harvested. A tail snip was also taken and stored at -20°C for genotype confirmation. Following harvesting, brains were placed in a 4% PFA solution for 48 h at 4°C, then placed in PBS with 30% sucrose for 24 hours or until they sank, at 4°C. The brains were then flash-frozen by placement for 2.5 minutes in a

beaker filled with 99% anhydrous 2-methylbutane (Sigma) that had been surrounded by powdered dry ice for at least 10 minutes. Immediately following flash-freezing, whole brains were transferred and stored at -80°C, until sectioned. Brains were sliced on the coronal plane at 30-µm thickness using a cryostat (Vibrotome UltraPro 5000; Sim Co Ltd, Denizli, Turkey) and serially moved into separated well plates filled with PBS. Sections were stored in well plates at 4°C for no longer than 2 weeks.

Histology

Tissue designated for cytochrome c oxidase (CYO) analysis was submersed in a solution of 800 µg of sucrose, 4 mg of cytochrome C, and 1 mg of DAB dissolved in 20 mL of phosphate-buffer for 4 hours at room temperature. The tissue was then transferred to deionized H_2O, mounted onto positively charged glass slides, and coverslipped using Depex (Electron Microscopy Sciences, Hatfield, PA, http://www.emsdiasum.com/microscopy).

Imaging and Image Analysis

CYO labelled tissue was scanned using Nikon ScanPro (Nikon, Melville, NY, USA). Images were captured from tissue sections obtained from 0.5 mm anterior to bregma to 0.58 mm posterior to bregma, approximately 180 µm apart. Optical densitometry was performed using ImageJ (https://imagej.nih.gov/ij/). Densitometric measures were taken from the striatum and the average intensities were normalized to the corpus callosum of each section.

Statistical Analysis

All statistics were performed with the Statistical Package for the Social Sciences (SPSS) v. 23 with an alpha level of equal to 0.05. Repeated measures analysis of variance (ANOVA) was used to assess any statistical significance of body weight change, latency to fall on the rotorod, clasping behavior, and distance travelled in the open field over the 6-week period between groups. One-way ANOVA was used to assess any statistical significance of rest time between groups on the forced-swim task and on measures of densitometric analyses of histology, and for between group differences at specific times

points in the aforementioned behavioral measures. Tukey Honestly Significant Difference (HSD) post hoc tests were conducted, when appropriate.

Age in Weeks	Day 1	Day 2	Day 3	Day 4	Day 5
5	Rotorod Train Weight	Rotorod Train	Rotorod Train	Rotorod & Open Field Baseline	Surgery Weight
6	Rotorod/OF Weight, Clasping		Weight		Weight
7	Rotorod/OF Weight, Clasping		Weight		Weight
8	Rotorod/OF Weight, Clasping	Forced Swim Test	Weight		Weight
9	Rotorod/OF Weight, Clasping		Weight		Weight
10	Rotorod/OF Weight, Clasping		Weight		Weight
11	Rotorod/OF Weight, Clasping		Weight		Weight
12	Rotorod/OF Weight, Clasping		Weight		Weight

Table 1. Behavioral testing schedule.

RESULTS

Weight

Repeated-measure ANOVA, analyzing percentage of body weight compared to baseline, showed significant changes in weight over time, $F(2,66) = 27.88$, $p < 0.001$. Additionally, there was a significant interaction between groups over time, $F(8,66) = 10.55$, $p < 0.001$ (Figure 1). One-way ANOVA of data from week 10 indicates no significant between-group differences in weight change, $F(3,38) = 1.71$, $p = 0.171$, but by Week 12 significant between-group differences in weight change $F(4,37) = 4.84$, $p = 0.003$ were observed (Figure 2). Tukey HSD analysis revealed that the HD Control and HD MSC group had lost significantly more weight than the WT Control group ($p = 0.003$ and $p = 0.020$ respectively). Percentage of weight change in both HD groups treated with NAM did not differ significantly from WT (HD MSC+NAM $p = 0.057$, HD NAM $p = 0.551$).

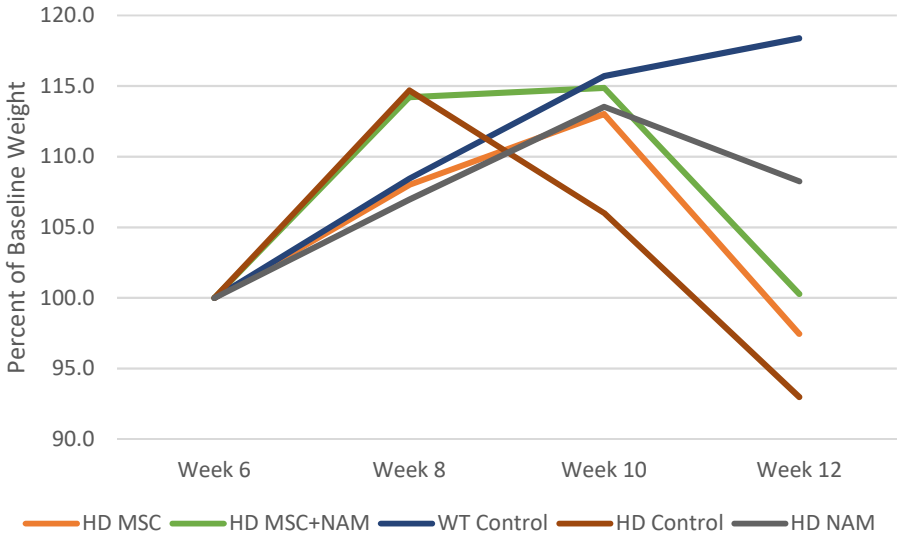

Figure 1. Percentage of weight change from baseline week 6 through week 12.

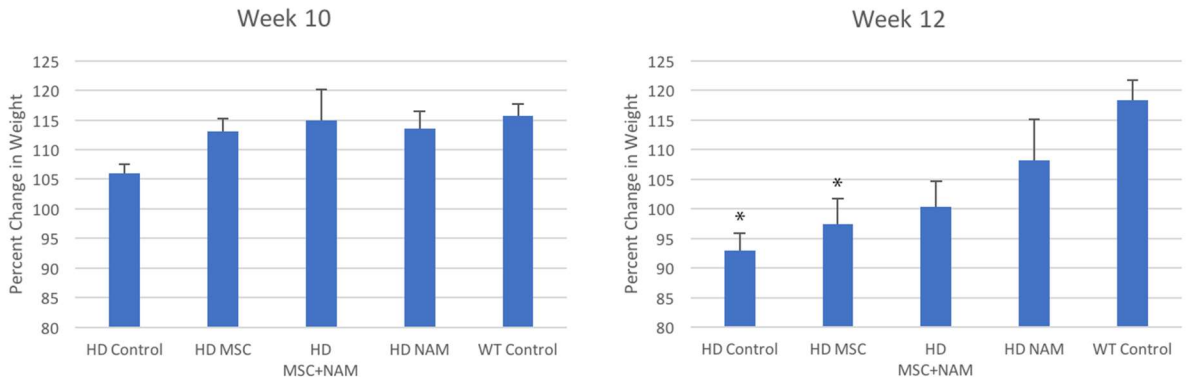

Figure 2. Percentage of weight change from baseline at 10 and 12 weeks of age. At 10 weeks of age there were no significant differences in weight change between groups. However, by 12 weeks of age HD Control and HD MSC groups had lost significantly more weight than WT Controls. Weight change in both groups treated with NAM did not differ significantly from WT. Bars represent SEM and asterisks represent $p < 0.05$, relative to WT Control mice.

Clasping

Repeated measure ANOVA revealed significant between-group differences in clasping behavior $F(1,34) = 112.33$, $p < 0.001$ (Figure 3). Change in clasping behavior was also significant over time, $F(1,34) = 46.99$, $p < 0.001$, and there was also a significant interaction, $F(12,102) = 7.84$, $p < 0.001$. Tukey HSD analysis revealed there was not a significant difference between the WT Control group and the HD MSC group. One-way ANOVA performed on data from week 10 revealed significant between-group differences, $F(4,38) = 8.02$, $p < 0.001$. Tukey HSD revealed that both groups of animals that received MSC transplantation performed similarly to the WT group, suggesting a delay in clasping behavior with MSC transplantation, whereas HD animals that received only NAM or vehicle clasped significantly more limbs on average than the WT group ($p = 0.010$ and $p < 0.001$ respectively; Figure 4).

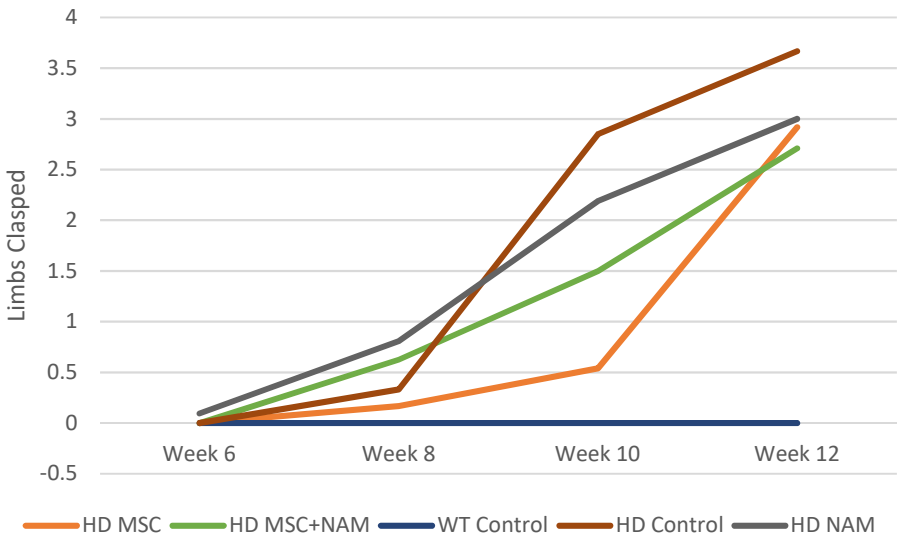

Figure 3. Clasping. Average number of limbs clasped by each group from 6 weeks of age to 12 weeks of age.

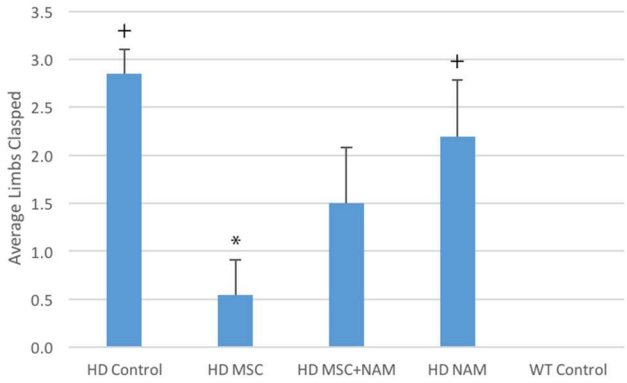

Figure 4. Average number of limbs clasped at 10 weeks of age. The HD MSC group clasped significantly less limbs on average than the HD Control animals (*). HD Control and HD NAM animals clasped significantly more limbs than WT Controls (+). There was not a significant difference in clasping behavior at week 10 between WT animals and both groups that received MSC transplantations. Bars represent SEM, asterisks represent $p < 0.05$ relative to HD Control, and plus signs indicate $p < 0.05$ relative to WT Control.

Rotorod

Repeated measures ANOVA revealed significant change in latency to fall on the rotorod at 5 rpm during the course of the study, $F(3,102) = 74.29$, $p < .001$, as well as a significant interaction between groups over time, $F(12,102) = 4.87$, $p < 0.001$. There was also a significant between-group effect in rotorod performance, $F(4, 34) = 13.67$, $p = <.001$. Tukey HSD analysis revealed that the WT group had

longer latency to fall than all HD groups, with no differences in performance between treated and sham HD groups. Results of analysis of data at 10- and 15- rpm were similar, with no significant difference in performance between HD animals, regardless of treatment.

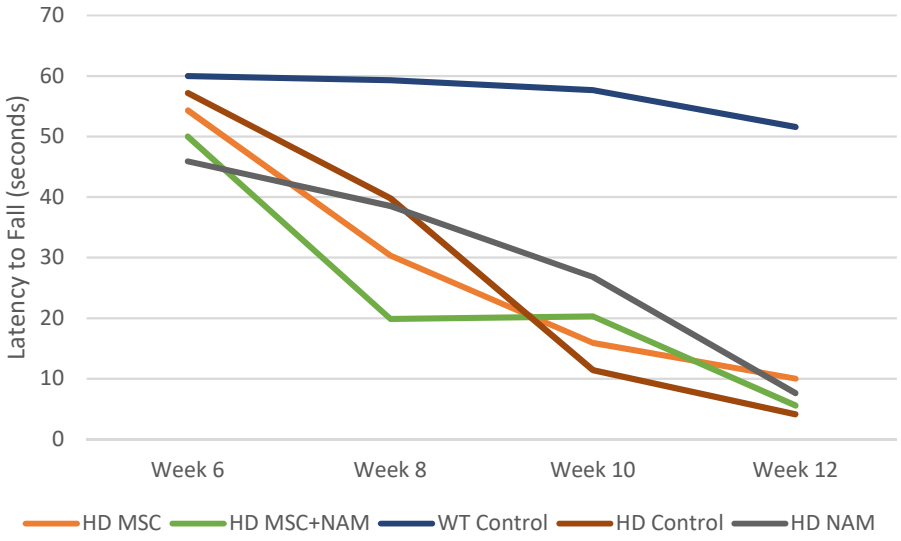

Figure 5. Rotorod latency to fall at 5-rpm. All HD groups performed significantly worse than WT. Results were similar at 10- and 15- rpm.

Open Field

Repeated measures ANOVA revealed a significant change over time in the total distance travelled during the hour in the open-field task, $F(3,102) = 12.67$, $p < 0.001$. There was also a significant interaction between groups over time, $F(12,102) = 2.33$, $p < 0.011$. One-way ANOVA for activity at week 12 revealed that there were significant between-group differences, $F(4,38) = 5.55$, $p = 0.002$ (Figure 6). Tukey HSD showed that there was not a significant difference in total distance travelled between WT and MSC+NAM groups ($p = 0.193$). However, the MSC+NAM group did not differ significantly from the other HD groups either, suggesting an intermediate treatment effect. Repeated measure ANOVA showed no significant differences in any respect in the amount of time spent in the center of the open field.

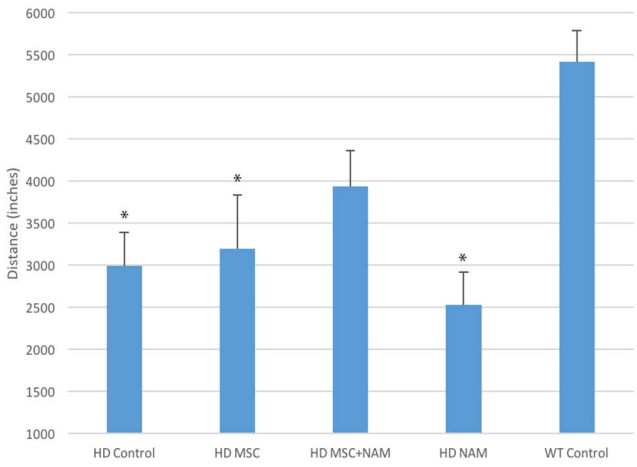

Figure 6. Total distance travelled in the-open field at 12 weeks of age. HD Control, HD MSC, and HD NAM groups distance travelled in the open-field differed significantly from WT animals. Mice that received co-therapies of MSC and NAM did not perform significantly different from WT animals in the open-field. Bars represent SEM and asterisks represent $p < 0.05$, relative to WT Control mice.

Forced Swim Test

One-way ANOVA indicated significant group differences in rest time in the forced swim test, $F(4,35) = 2.879$, $p = .039$. Tukey HSD analysis revealed that the HD MSC treated group had significantly shorter rest time than all other groups, including WT animals, suggesting less despair in this learned helplessness task. Climbing behavior in the FST was also assessed using one-way ANOVA, but there was no significant difference in climbing behavior between groups $F(4, 15) = 2.10$, $p = 0.104$.

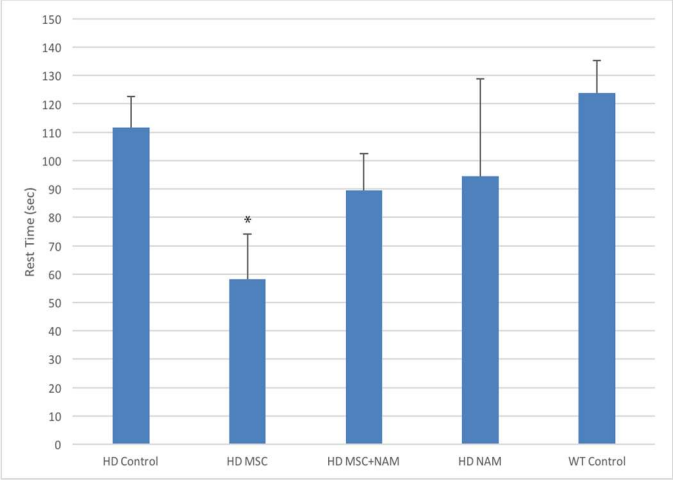

Figure 7. Time spent resting in the forced swim test. The HD MSC group had a significantly reduced rest time when compared to WT Control animals.

CYO Densitometry

One-way ANOVA analysis of striatal tissue labelled with CYO (Image 1) revealed significant between-group differences in optical densitometry, $F(4,14) = 7.07$, $p = 0.006$ (Figure 8). Tukey HSD analysis showed that animals that received MSC transplantation in conjunction with NAM had significantly increased densitometry values when compared to most of the other groups ($p < 0.050$) with the MSC alone group being only marginally significantly lower ($p = 0.056$). However, there was no significant difference noted between any other groups, including the WT and HD Control groups, suggesting a lack of genotype effect.

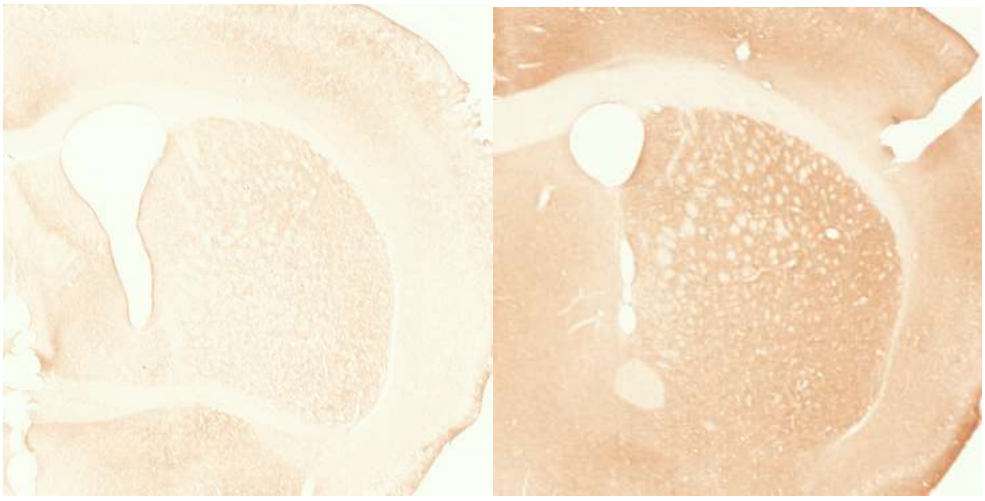

Image 1. Examples of CYO DAB stained tissue. The image on the left is striatal tissue from the HD MSC group and the image on the right is from the HD MSC+NAM group. Densitometric analysis revealed that the HD MSC+NAM group had significantly higher densitometric readings than all other groups. There were no significant differences between any of the other groups.

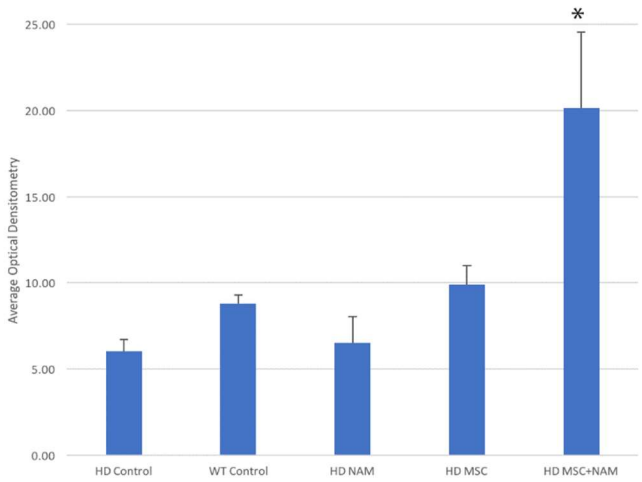

Figure 8. CYO Densitometry. Histological analysis of striatal tissue showed higher levels of optical densitometry of CYO labelling in HD animals that received co-therapies of MSC transplantation and NAM. Bars represent SEM and asterisks represent $p < 0.05$, relative to all other groups.

DISCUSSION

The aim of this study was to assess the ability of co-therapies of MSC transplantation and nicotinamide to reduce both the neurological and peripheral deficits observed in the R6/2 murine model of HD. The behavioral results supported the hypothesis that this combinatorial treatment can reduce some of the symptoms of HD observed in the R6/2 model, with densitometry measures of CYO activity suggesting a possible synergistic effect between the two treatments in the brain. Results of clasping and weight, neurological and peripheral pathologies respectively, both showed trends toward delayed onset, as MSC-treated animals clasped fewer limbs on average at week 10 than other HD animals and NAM-treated R6/2 mice experienced weight changes more similar to that of WT animals.

Analysis of weight loss revealed that there was not a significant difference in weight change between animals treated with NAM and WT animals at 10 weeks of age, suggesting a sparing of weight loss in the treated R6/2 mice. She and colleagues (2011) found that the weight loss observed in the R6/2 model results from an increased energy expenditure, coupled with loss of muscle mass, without altered food intake. Supplementation with NAM has been found to upregulate peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), the master regulator of mitochondrial biogenesis, in the brains of the R6/1 mouse model of HD (Hathorn, Snyder-Keller, & Messer, 2011; Naia et al., 2016), but this was not assessed in peripheral tissue. The results of the present study suggest that NAM may have a beneficial impact on metabolic processes in all tissues and warrants further exploration as a potential treatment beyond its impact on the brain. Interestingly, animals that received co-therapy of MSC transplantation and NAM did not have the same significantly delayed weight loss that was observed in the NAM-alone group. However, the co-therapy group was spared from the significant decrease in locomotor activity that was observed in all other HD mice at the end of the study (see Figure 6). In addition to a loss of muscle mass, She and colleagues (2011) also found an increase in adiposity in the R6/2 model. Coupled with the fact that the average percentage of baseline weight maintained in the HD MSC+NAM group was still greater than that observed in the MSC-alone and both control HD groups (i.e. sham surgery and vehicle-only pump), the lack of comparable weight maintenance in the HD MSC+NAM may just be the result of a slight loss of adiposity due to the increased locomotor activity observed. Again,

further analysis of body composition following supplementation of MSCs and/or NAM is needed to discern the exact cause(s) of the observed treatment-induced sparing effect on weight loss in HD.

Analysis of behavioral outcomes revealed that both groups receiving MSC transplantation had a delayed onset of clasping behavior. Clasping is a functional motor behavior anomaly of the visual placing response observed in many transgenic mouse models and is hypothesized to involve the cortico-striato-pallido-reticular pathway (Miedel et al., 2017; Lalonde & Strazielle, 2011). The debate continues regarding whether or not there is significant striatal cell loss in the R6/2 model, so it is likely that the behavioral results shown are due more to a functional improvement of this pathway than to cell replacement. These improvements may have potentially been imparted by the immunomodulatory and anti-inflammatory impact of the MSCs on the striatal environment (Rossignol et al., 2015), by the contributions of trophic factors released by the MSCs, or a combination thereof.

Unfortunately, no significant effect of any of the treatments was observed in the measures of rotorod performance. However, we have observed that the number of passages the MSCs have undergone prior to transplantation can affect the behavioral outcome (Rossignol et al., 2015). In addition, we found that R6/2 animals who received transplantation of cells harvested from 10-month-old animals performed poorly on the rotorod when compared to animals that received cells harvested from 5 week or 6-month-old donors (unpublished data). The cells employed in the current study were harvested from animals that were 10 months of age, which may offer some explanation for the lack of significant outcomes in the rotorod task. The MSCs used in this study were, however, at a relatively high passage (approximately 40 passages), which may have allowed them to impart at least some behavioral benefit, as was observed in the delay of clasping behavior. Surprisingly there was no behavioral impact of NAM treatment by itself, which has previously been shown to impart improvements in rotorod performance in transgenic HD models (Hathorn et al., 2011; Naia et al., 2016). However, the previous investigations of NAM in rodent models of HD used the R6/1 and YAC 128 transgenic models, both of which have a much slower disease progression than that observed in the R6/2, which may allow for more observable delays in motor deficits.

Analysis of time spent resting in the forced swim test revealed a significant reduction in rest time in the MSC transplantation group. However, there was no genotype effect observed, with WT animals

actually displaying the longest amount of times spent at rest. Increased rest time in this behavioral task is indicative of depression/learned helplessness, thus suggesting the WT animals performed the poorest, or not significantly different than HD animals. One possible explanation for the results observed was that all the vehicle and NAM treated animals were implanted with mini-osmotic pumps, whereas the MSC transplantation group were not implanted with pumps. The buoyancy of the pumps was most likely the confounding variable in the presents study since the presence of the pumps did not seem to impact rotorod performance, with HD MSC animals performing similarly to animals that received pump implants. Additionally, testing was performed when animals were 9 weeks of age. Ciamei, Detloff, and Morton (2015) found that the R6/2 model recapitulated the onset and progression of depression similar to what is observed in humans, in that depression is significant prior to the onset of motor impairment, but fades as other symptoms progress. Therefore, the lack of a genotype effect observed in the current study is in line with the R6/2 phenotype, and the reduction in rest time observed in the MSC-alone group is likely due to the absence of the osmotic pump buoyancy requiring these animals to exert more effort to stay afloat.

Finally, optical densitometry measures taken from the striatum revealed that animals receiving a combination therapy of MSC transplantation and NAM supplementation had significantly higher values than all other groups except the MSC transplantation alone group. This increase suggests a treatment-induced increase in metabolic activity in the striatum of the MSC+NAM mice, with an intermediate effect observed in the MSC-alone group. However, the lack of a significant difference between HD Control and WT groups suggests that this interesting effect was not the critical factor in the observed decreases in HD-induced deficits produced by the co-therapy. Unfortunately, due to leakage of the Hoechst label into the surrounding tissue (please see: Iwashita, Crang, & Blakemore, 2000; Scharenberg, Harkey, & Torok-Storb, 2002; Mohorko et al., 2005; or Lin, Xin, Dai, & Lue, 2013) it was not possible to accurately count the transplanted MSCs. It is hypothesized however, that the slight increase in CYO densitometry observed in the MSC alone group is the result of either the metabolic activity of the additional cells, or some metabolic benefit imparted by the MSCs on the surrounding tissue, while the dramatic increase seen in the MSC+NAM group results from NAM increasing the metabolic output of all cells or, more likely given the steep increase, a synergistic effect of the benefits imparted by MSCs and the effect of NAM on native tissue.

Taken together, results of this study suggest that a co-therapy of NAM supplementation with MSC transplantation reduces the neurological and peripheral deficits observed in R6/2 mice than either treatment alone. Treatments, such as MSC transplantation, that directly target the brain and show great promise, are unable to address the impact of the ubiquitous expression of mHTT on the rest of the body. Therefore, using NAM as a systemic adjunct to MSC transplantation may be able to help address the metabolic disruption which contributes to the weight loss and muscle wasting observed in HD. In addition to addressing peripheral symptoms, systemic co-therapies may also prove to be synergistic, enhancing the benefits of MSC transplantation. While it may not be directly related to symptom reduction, the increase in CYO optical densitometry outcomes suggests that synergistic effects of combinatorial treatments for HD are plausible and that such a regimen is able to address the multifaceted dysfunction observed in this terrible disease.

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