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Experimental Paper

Neurotherapy: Regeneration of Myelin Sheath by Utilizing Stem Cell Therapy

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system. It affects the brain and spinal cord nerves that send messages throughout the body. In MS, the immune system attacks myelin, a sheath-like membrane that covers and protects your nerves. It also can damage the actual nerves. The method used to treat Multiple Sclerosis is Qualitative method because it depends more on observation in the Microscope and *in vitro* tests. Multiple Sclerosis causes the immune cells to differentiate to B-Cells to plasma cells to release antibodies against myelin sheath cells and Oligodendrocytes. In this case, the solution is to decrease B-Cells activity and commence to differentiate pluripotent stem cells to Oligodendrocytes which releases myelin sheath cells to regenerate sclerosis parts. Utilizing Ocrelizumab which is a type of drug called a monoclonal antibody drug which is approved by the FDA. Then to identify oligodendrocytes amino acid and glycoprotein sequence to culture new stem cells with the same receptors. Consequently, utilizing and applying specific protocols such as Adenosine *in Vitro* Test, CNPase *in Vitro* Test and Glutamate *in Vitro* Test to make sure that the patient is an MS patient. Inject adenosine only with different concentrations and frequently check neuron and myelin sheath morphology and function *in vitro*, then apply it *in vivo*. Then adding immunosuppressant drug or Ocrelizumab before and after therapy. After PCR and Probe implementation, whilst culturing the grown self-stem cells.

Prepare the protocol to differentiate them to oligodendrocytes, and then re-inject them again in sclerosis parts as can be seen in CT scan or MRI. Then re-scan and make comparison with the pre and post-treatment scans.

Introduction

Multiple Sclerosis became nowadays increasing dramatically in recent demographic results. Proven Estimates claim that worldwide prevalence "range from 1 million to more than 2.5 million cases with an uneven geographical distribution. Nearly 400,000 people living in the United States have Multiple Sclerosis and approximately the same number of patients resides in Europe" (Rotella, A., 2014). However, the disease is much less common in Asia and Africa. Concerning gender, Multiple Sclerosis disease is more active in females rather than males and they are the ones with a very high chance to transmit Multiple Sclerosis disease to offsprings, comparing to males, males have low chance apparently because of Sex Linkage. Multiple Sclerosis disease called auto-immune disease due to self-response of the immune system, that neuron's myelin sheath covering cell is recognized as a foreign/non-self-antigen, which results with adverse influences on neuron's function. The issue that till nowadays there are no specific cures to Multiple Sclerosis because whenever any therapy has done leads to immune system rejection. Not only in therapy but also an issue in neuron's oligodendrocyte genesis CNS medications or remission injections does not affect 100 percent. Majority of researchers emphasize on drug discovery for remission, yet the focus that indeed leads to advantageous consequences is focusing on the perks side of treatment that is going to benefit the end user the patients and to decrease cons as much as possible. Instead of solving and curing Multiple Sclerosis symptoms focus on the roots that exposed and risen MS. Being curious and asking many times is one of the solutions for example: What causes Multiple Sclerosis? The Hypothesis of this research is to regenerate the oligodendrocyte function by the re-planting process. As the number of functioned cells increase, sclerosis parts decrease and as immunosuppressant drugs intake increases, immune response decreases which leads to the blood-brain barrier to re- construct.

Multiple Sclerosis is Divided into the Following

• ***Relapsing-Remitting*** Multiple Sclerosis (RRMS). This is the most common form of multiple sclerosis. About 75% of people with Multiple Sclerosis are initially diagnosed with RRMS. People with RRMS have temporary periods called relapses, flare-ups or exacerbations when new symptoms appear

• ***Primary-Progressive*** Multiple Sclerosis (PPMS). This type of MS is not very common, occurring in about 10% of people with Multiple Sclerosis. PPMS is characterized by slowly worsening symptoms from the beginning, with no relapses or remissions

• ***Secondary-Progressive*** Multiple Sclerosis (SPMS). In SPMS, symptoms worsen more steadily over time, with or without the occurrence of relapses and remissions recognized in about 7%. Most people who are diagnosed with RRMS will transition to SPMS at some point

• ***Progressive-Relapsing*** Multiple Sclerosis (PRMS). A rare form of Multiple Sclerosis (3%), PRMS is characterized by a steadily worsening disease state from the beginning, with acute relapses but no remissions, with or without recovery

• Acute allergic ***Encephalomyelitis*** (EAE) developed a model for Multiple Sclerosis.

Background

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system. It affects the brain and spinal cord nerves that send messages throughout the body. In MS, the immune system attacks myelin, a sheath-like membrane that covers and protects your nerves. It also can damage the actual nerves.

People Who Have Multiple Sclerosis Can Experience Symptoms That Include

• Blurred or double vision
• Tingling, numbness, or pain
• Extreme fatigue
• Loss of bladder control
• Memory problems
• Lack of concentration
• Difficulty walking

Symptoms that are consistent with Multiple Sclerosis have been studied for decades. Researchers have come a long way in determining the causes and progression of the disease. There has also been considerable headway on treatment options that help people with Multiple Sclerosis live healthy and fulfilling lives.

Adverse consequences of Multiple Sclerosis disease on neuron's function, the starting point HDA-LR2 genome become activated when the immune system develops molecular mimicry by chemokines. This happens by accident as a result of brain blood circulation which triggers to deteriorate Blood-Brain Barrier wall because the immune system detects non-self-antigen which is hidden, so it triggers more white blood cells by exocrine histamine from mast cells, hence immune system T-cells detect non-self-antigens and digest collagen by matrix metalloproteinasis on in particular MMP-9, therefore, directly attacking oligodendrocytes. Myelin is the target of a cascade of events in the pathology of multiple sclerosis. MS, demyelination of neuron oligodendrocytes is the disease hallmark. Axons become denuded and the excitatory nerve transmission is compromised. Often, neurons become axotomized, resulting in permanent neurological deficits. The immune system attacks on myelin are mediated by autoreactive T cells that respond to a genetic and environmental factor and move from the systemic circulation into the central nervous system. Autoreactive T cells penetrate into the CNS via the action of matrix metalloproteinase and intracellular adhesion molecule 1 and vascular cell adhesion molecule 1. Interaction with antigen presenting cells via the MHC complex and T cell receptor activates T cell and stimulates the information of T-helper cell 1. TH1 cells secrete pro-inflammatory cytokines and factors such as interferon gamma, tumor necrosis factor alpha, interleukin 1, or interleukin 12. These cytokines, in turn, activate Macrophages and another group of T helper Cell 2 population.TH2 cells secrete a separate repertoire of cytokines interleukin 10, interleukin 4, transforming growth factor beta, and interleukin 13. Most of the TH2 secreted cytokines act to curb the inflammatory response, with the exception of interleukin 13 involved in B-Cell recruitment, which may play a role in the inflammatory response.

Recent studies show that Multiple sclerosis is an infectious disease that is influenced by Chlamydia pneumonia parasite, which resides inside the body it starts to activate by the induction of biochemical signals from other subsidiary cells on the body. According to recent research, the main influence on the disease to increase its exacerbation; is nutrition, as the patient reduces animal-based diet and shifts to a plant-based diet. The patient's recovery increases and the disease intensity decreases. That is all due to the effect of processed food on our body.

Description

The method used to treat Multiple Sclerosis is Qualitative method because it depends more on observation in the Microscope and *in vitro* tests. One of the main treatments that can help to treat M.S is to focus on the immune system cells especially the mediated cells B-Cells. In deep pathology and immunology about M.S figured out that T-Cells are the cells responsible to stimulate other cells. That causes to cells to differentiate B-Cells to plasma cells to release antibodies against myelin sheath cells and Oligodendrocytes. In this case the solution is to decrease B-Cells activity and commence to differentiate pluripotent stem cells to Oligodendrocytes which releases myelin sheath cells to regenerate sclerosis and in the meantime to decrease B-Cells activity until to make sure that the BBB (Blood Brain Barrier) and GTW (Gut Wall) is regenerated by Mitosis to regulate entrance of immune cells again. This idea will be formed by the extraction of Embryonic Stem Cells from M.S patient spleen, thus add those stem cells in a petri dish with suitable temperature 2-8 degrees Celsius and equivalent amount of nutrients, hence this produces as a product a specialized stem cell mature which mean that it is the time to undergo tests to change from specialized to Oligodendrocytes to be replanted in the CNS using MOG and MBP as a therapeutic target.

Approved Tested Drugs by The FDA

Utilization of "Ocrelizumab is a type of drug called a monoclonal antibody. This means that it specifically targets one substance. The substance ocrelizumab targets and binds to is called the CD20 protein, which is found on B cells. When ocrelizumab binds to CD20-positive B cells, the B cells burst and die" [1].

This is helpful because experts believe that B cells might play an important role in MS by:

- Activating other immune cells to attack the body's nerve cells
- Increasing inflammation in the brain and spinal cord

By destroying certain B cells, Ocrelizumab helps to reduce inflammation and reduce attacks by your immune system on your nerve cells. This is mainly emphasized on Primary Progressive Multiple Sclerosis.

Chemical compounds have been tested and resulted with negative results of symptoms which the mechanism commence to attack cell division and avoids it by inhibiting the cell cycle which is called Proliferation, such as in the following:

- Methotrexate
- TNF alpha inhibitor

• Azathioprine

• Cyclosporin

• Glatiramer Acetate

Methodology

Therapy and Treatment Phase 1

Cell Targeting

After Stem cells are cultured, a PCR will be utilized to devote a probe or a fluoresce substance during denaturation, annealing and elongation cycle with a valid temperature in each phase. Culturing new cells with probes, then this will help us to determine stem cell place *in vivo* in phase 2.

Another procedure will be tested is to identify oligodendrocytes amino acid sequence to culture new stem cells with the same receptors because during cell signaling this assures that the cell will be attracted towards signals from other cells in different places. This will help us also to discover immune system action to recognize self-cells as non-self, yet to know oligodendrocytes specific chemicals one of them is Cytokine substance that triggers demyelination.

These procedures will be done upon positive results of the following test below:

Adenosine *in Vitro* Test

Recommended single-reagent assay for multi-well plate format:

CellTiter-Glo® 3D Cell Viability Assay, Promega Corporation, Cat.# G9681, G9682, G9683

CellTiter-Glo® 3D Cell Viability Assay combines the enhanced penetration and lytic activity required for efficient lysis of 3D cell culture with the generation of the stable ATP-dependent luminescent signal. This thereby reduces the complexity of processing multiple assay plates and HTS applications [2].

ATP Assay Preparation

CellTiter-Glo® 3D Cell Viability Assay is provided as a ready-to-use solution and no additional preparation is required. The reagent should be equilibrated to room temperature before use. For stability and storage conditions please refer to the manufacturer's guidelines (www.promega.com).

To perform the assay on microtissues cultured in 96-well GravityTRAP™ ULA Plates, mix 1:1 the required volume of CellTiter-Glo® 3D Cell Viability Assay (20 µl per well) and PBS without calcium and magnesium (e.g. PAN-Biotech, Cat.# P04-36500)

ATP Assay Protocol

Equilibrate GravityTRAP™ ULA Plates with cultured micro-tissues to room temperature.

Prepare 96-opaque well microplate, hereinafter referred to an assay plate (e.g. Greiner Bio-One, Cat.# 675075), by pipetting into dedicated wells (Figure 3, lower panel):

Blank - 40µl of diluted CellTiter-Glo® 3D Cell Viability Assay

Optional: Standard curve - depending on the type of microtissue and the detection range of luminometer available, mix 20µl of CellTiter-Glo® 3D Cell Viability Assay with 20µl of 1µM ATP (e.g. for human liver microtissues of ~ 300µm diameter) or with 5µM ATP (e.g. for more metabolically active or bigger microtissues), and with corresponding ATP dilutions.

Optional: To check background interference of the compound tested in the cytotoxicity assay, pipet 5µl of a culture medium from wells containing microtissues treated with the highest concentration of the compound into wells on the assay plate containing 20µl CellTiter-Glo® 3D Cell Viability Assay and 20µl of 1µM ATP.

Gently remove the culture medium from the GravityTRAP™ ULA Plate by placing the pipette tip at an inner edge of the well, leaving intact the microtissues in the remnant volume of the medium in the V-shaped bottom of the well.

Dispense 40µl of diluted CellTiter-Glo® 3D Cell Viability Assay into each well of the GravityTRAP™ ULA Plate.

Mix and transfer the content of each well from the GravityTRAP™ ULA Plate into the corresponding well on the assay plate.

Protect the lysate from light by covering the assay plate with aluminum foil or with aluminum plate sealer (e.g. Greiner Bio-One, Cat.# 67609).

For effective MT lysis keep the plates on an orbital shaker for 20 min at room temperature.

Record luminescence with a microplate luminometer using a program recommended by the manufacturer.

CNPase *in Vitro* Test

Three-day-old Wistar rats were given an intraperitoneal injection of lipopolysaccharide to induce microglial activation, and the rats were killed at different time points. Along with this, primary cultured microglial cells were subjected to lipopolysaccharide treatment, and expression of CNPase was analyzed by real-time reverse transcription PCR and immunofluorescence. Additionally, siRNA transfection was employed to downregulate CNPase in BV-2 cells. Following this, inducible nitric oxide synthase, IL-1β and TNF-α were determined at mRNA and protein levels. Reactive oxygen species and nitric oxide were also assessed by flow cytometry and colorimetric assay, respectively. In parallel to this, CNPase expression in activated

oligodendrocyte was also investigated in adult rats subjected to fluid percussion injury as well as middle cerebral artery occlusion.

Immunofluorescence staining of Oligodendrocyte cells *in vitro*

Primary Oligodendrocyte cells and BV-2 cells were plated on poly-L-lysine coated coverslips in a 24-well plate. For immunofluorescence labeling, the cells subjected to different treatments of varying durations were fixed with 4% paraformaldehyde for 20 minutes at room temperature and blocked with 5% normal goat serum for 1 hour. The cells were then separately incubated overnight at 4˚C with mouse anti-human CNPase monoclonal antibody (1:100; Chemi-Con; Cat. No. MAB326), rabbit anti-mouse TNF-α polyclonal antibody (1:100, Millipore Bioscience Research Reagents, Billerica, MA, USA; Cat. No. AB2148P), rabbit anti-mouse IL-1β polyclonal antibody (1:100, Millipore Bioscience Research Reagents; Cat. No. AB1413), and mouse anti-mouse inducible nitric oxide synthase (iNOS) monoclonal antibody (1:100, BD Pharmingen, San Jose, CA USA; Cat. No.640432), followed by Cy3-conjugated sheep anti- mouse IgG (1:200, Sigma-Aldrich; Cat. No. C2181) or Cy3-conjugated sheep anti-rabbit IgG secondary antibodies (1:200, Sigma-Aldrich; Cat. No. C2306) incubation for 1 hour at room temperature. The cells were then washed three times with PBS and mounted using a fluorescent mounting medium (Sigma-Aldrich; Cat. No. F6057). Cellular localization was then examined under a confocal microscope (Fluoview 1000; Olympus) with the same exposure settings for each comparison group.

Silencing of CNPase with siRNA

Two constructs of mouse CNPase-specific siRNA (Ambion, Foster City, CA, USA; siRNA ID: s64160 & s64161; Cat. No. 4390771) was used for CNPase silencing. Nonspecific scramble siRNA (Ambion; Cat. No. 4390846) was used as control siRNA. To achieve a higher siRNA knockdown efficiency, the reverse transfection method was adopted for silencing according to the manufacturer's instructions. Briefly, sub-confluent, early passage BV-2 cells were harvested by trypsinization, centrifuged and resuspended in optimum (GIBCO, Invitrogen, Carlsbad, CA, USA; Cat. No. 31985070) and plated in 6-well plates at a density of ~3 × 105 cells/well. This was followed by adding 500µl optimum with 5µl siRNA (10µM) and 4µl Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen; Cat. No. 13778075) dropwise in the above well. The cells were incubated with the siRNA transfection mixture for 8 hours and then the medium was replaced with DMEM with 10% FBS without antibiotics and incubated for another 16 hours for RNA extraction to detect the knockdown efficiency by reverse transcription (RT)-PCR. Oligodendrocyte cells were subjected to LPS treatment for 6 hours at 42 hours after transfection. After that, cells were either fixed for immunofluorescence staining, or mRNA and protein extracted for real-time RT-PCR and western blotting, respectively.

Cell Viability Analysis of BV-2 Cells

The effect of siRNA transfection on the viability of BV-2 cells was evaluated by CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Fitchburg, WI, USA; Cat. No. G3580). The cell viability of non-transfected BV-2 cells, control siRNA transfected BV-2 cells, and CNPase siRNA transfected BV-2 cells were measured. The dye solution was added to the cells at 24 hours post-transfection and incubated for

up to 4 hours at 37˚C in a humidified, 5% CO2 incubator. The absorbance was read at 490nm using the Tecan 2000 microplate reader. Cell viability was expressed as a percentage of non-transfected BV-2 cells.

Cell Cycle Analysis

For each experiment, control siRNA and CNPase siRNA transfected cells were harvested at 48 hours post-transfection and pelleted by centrifugation. The cell pellet was resuspended in 0.5ml PBS and fixed in 4.5ml 70% cold ethanol overnight at 4˚C. Ethanol-fixed cells were washed twice with PBS before resuspending with 200µg/ml propidium iodide solution containing 1mg/ml RNAse A, PBS and Triton-X. Cells were incubated at room temperature for 30 minutes to allow the DNA content of cells to be stained before analysis by flow cytometry.

Real-Time Reverse-Transcription PCR

The total RNA was extracted from primary Oligodendrocyte cells and BV-2 microglial cells subjected to various treatments (LPS, control, and CNPase siRNA transfection) using RNeasy Mini kit (Qiagen, Valencia, CA, USA). The concentration of RNA was quantified with Nanodrop Spectrophotometer (Thermo Scientific; Model No. ND1000). RNA (1µg) was reverse transcribed to cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen; Cat. No 18080-051) according to the manufacturer's protocol. The resulting cDNA was diluted and used as a template for real-time reverse transcription PCR using an ABi 7900HT Fast PCR system (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. Primer pairs for *CNPase, TNF-α, IL-1β, iNOS,* and *β-actin* were designed using the primer design program (Primer 3 software version 1.0, Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The primer sequences for the genes are listed in Table 1. The RT-PCR was carried out in a 10µl final volume containing the following: 5µl 2 × SYBR Green fast master mix (Invitrogen); 1µl 5µM forward primer and 1µl 5µM reverse primer; and 3µl diluted cDNA. After an initial denaturation step at 95˚C for 15 minutes, temperature cycling was initiated. Each cycle consisted of denaturation at 94˚C for 15 seconds, annealing at 60˚C for 25 seconds, and elongation at 72˚C for 20 seconds. In total, 45 cycles were performed. Mouse β- actin was amplified as the control for normalizing the quantities of transcripts of each of the genes mentioned above. The differences in expression for *CNPase, TNF-α, IL-1β,* and *iNOS* between the control and treated cells were calculated by normalizing with the β-actin gene expression according to the following formula [3]:

$$\text{Fold change} = 2^{-[Ct(control)gene\times-Ct(control)actin]-[Ct(activated)gene\times-Ct(activated)actin]}$$

Table 1.1: *CNPase Nucleotide sequence*

CNPase	Forward	gcaggaggtggtgaagagat
	Reverse	cagatggcttgtccagatca

Glutamate *in Vitro* Test

Materials

L-Glutamate, L-glutamine, 6-diazo-5-oxo-L-norleucine (DON), MSO, 2-deoxy-D-glucose, and rotenone were purchased from Sigma-Aldrich (St. Louis, MO). L-[^{3}H]glutamate and L- [^{3}H]glutamine were acquired from PerkinElmer-New England Nuclear (Waltham, MA). All cell culture media and sera were obtained from Invitrogen (Carlsbad, CA). All salts, buffers, solvents, and other reagents were from Sigma-Aldrich unless otherwise specified.

Preparation of Primary Oligodendrocyte Cultures

Confluent primary Oligodendrocyte cultures were prepared from brain cortical tissue of 1- to 2- day-old Sprague-Dawley rats. All animal procedures were approved by the Albany Medical Center Institutional Animal Use and Care Committee. Pups were euthanized by rapid decapitation. The cerebral cortices were dissected from the meninges, hippocampi, and basal ganglia and transferred to ice-cold OptiMEM medium (Invitrogen). Cortical tissue collected from four animals was minced and transferred into 10ml of a solution of the recombinant protease TrypLE (Invitrogen), which was diluted 1:1 (vol: vol) with OptiMEM. Cells were extracted at 37˚C using three 10-min incubations with TrypLE additionally supplemented with bovine pancreatic DNAse I (Sigma). The first extraction was discarded, while the second and the third extractions were combined with minimal essential medium (MEM) containing 10% heat- inactivated horse serum (HIHS) and 50U/ml penicillin plus 50μg/ml streptomycin (Pen-Strep). After each extraction cells were sedimented by brief centrifugation (1,000g for 1.5 min) and then resuspended in MEM-HIHS. Dissociated cells were seeded on poly-L-lysine-coated T75 culture flasks (Techno Plastic Products, TPP, Trasadingen, Switzerland) at the density of 250,000 cells/flask. Cultures were grown for 2-3 wk in MEM-HIHS + Pen-Strep at 37˚C in a humidified atmosphere of 5% CO2-95% air. The culture medium was changed twice a week. Culture purity was periodically verified by staining with an antibody recognizing specific astrocytic marker, glial fibrillary acidic protein (GTAP, Sigma); ≥98% of cells were GFAP positive. Confluent cells were replated as necessary on 6- or 12-well tissue culture plates (TPP) or 18-mm square coverslips (Carolina Biological, Burlington, NC).

Assay of Glutamine Synthetase Activity

The activity of GS was quantified as the intracellular conversion of L-[^{3}H]glutamate to L- [^{3}H]glutamine. Because the GS and the subsequent GLNase assays are the subjects of the present methodological paper, we describe them in a step-by-step manner with brief comments on the significance of each step.

Oligodendrocyte grown in six-well plates were washed from the culture media three times with HEPES-buffered basal solution of the following composition (in mM): 135 NaCl, 3.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 10 D-glucose, 10 HEPES (pH = 7.4). This was necessary to remove extracellular amino acids, particularly 2mM glutamine that is present in cell culture media. All the subsequent steps were performed at 37˚C in an air atmosphere in a water-jacketed incubator.

Cells were preincubated at 37°C in basal medium for 40 min with the irreversible GLNase inhibitor 1mM DON [4]. At this concentration and duration of treatment DON irreversibly blocked GLNase activity by >75% and prevented the reverse conversion of glutamine to glutamate. As seen in RESULTS, this degree of inhibition was sufficient for specific measurements of GS activity. DON could not be present in the subsequent steps because it strongly interferes with the transport of amino acids (see RESULTS).

Cells were washed from DON two times with 2ml of basal solution and transferred into 2ml of the GS reaction medium that was prepared on the basis of basal with addition of 2μCi/ml of L- [^{3}H]glutamate (final concentration adjusted to 2μM with unlabeled L-glutamate) and 100μM of $(NH_4)2SO_4$([NH_4+/NH_3] = 200μM). Ammonium sulfate was added to provide sufficient NH4+ levels for the GS reaction. Cells were incubated in this reaction mix for 30 min at 37°C.

The reaction was terminated and extracellular isotope was removed by three consecutive washes with 2ml of ice-cold basal solution.

One milliliter of milliQ H_2O was added to each well to lyse Oligodendrocyte; cells were scraped and sonicated for 4 min using Branson 200 Ultrasonic Cleaner. Lysates were clarified by rapid centrifugation (4 min × 12,100g, room temperature).

Each cell lysate (1ml) was added onto activated AG 1-X8 Polyprep column (Bio-Rad, Hercules, CA) for anion exchange separation of L-[^{3}H]glutamate and L-[^{3}H]glutamine. Column content was eluted with three 2-ml volumes of milliQ H_2O, followed by three 2-ml volumes of 0.1M HCl. Water elution removed uncharged L-[^{3}H]glutamine, while subsequent acid elution extracted negatively charged L-[^{3}H]glutamate as well as its metabolites [such as α-ketoglutarate and other intermediates of the mitochondrial tricarboxylic acid cycle (TCA)].

Eluent fractions were collected into scintillation vials and 4ml of scintillation cocktail (Ecoscint A, National Diagnostics, Atlanta, GA) were added to each vial. ^{3}H content was measured in a Tri-Carb 1900TR Liquid Scintillation Analyzer. The GS activity was calculated as percent conversion of L-[^{3}H]glutamate into L-[^{3}H] glutamine, which was normalized to the total ^{3}H recovered from each sample. This was done using a simple formula: % conversion = [(dpms in "glutamine" fractions nos. 1-3)/(dpms in "glutamine" fractions nos. 1-3) + (dpms in "glutamate" fractions no. 4-6)] × 100%.

Assay of Glutaminase Activity

The activity of GLNase was quantified as the intracellular conversion of L-[^{3}H]glutamine to L- [^{3}H] glutamate.

Confluent Oligodendrocyte cultures grown in six-well plates were initially washed from culture media three times with HEPES-buffered basal solution. All the subsequent steps were performed at 37°C in an air atmosphere in a water-jacketed incubator.

Cells were then pre-incubated at 37°C in basal medium for 40 min with the irreversible inhibitor of GS 1mM MSO [3]. At this concentration and duration of treatment, MSO irreversibly blocked GLNase

activity by >95% and prevented the reverse conversion of glutamate to glutamine. This is a particularly critical treatment because of the very high GS activity in glial cells. MSO could not be present in the subsequent steps because it interferes with the transport of amino acids (see Results).

Cells were washed from MSO two times with 2ml of basal solution and transferred into 2ml of the GLNase reaction medium that was prepared on the basis of basal with addition 4 μCi/ml of L-[^{3}H]glutamine (final concentration adjusted to 2μM with unlabeled L-glutamine). Cells were incubated with this reaction mix for 30 min at 37°C.

The reaction was terminated and extracellular isotope was removed by three consecutive washes with 2ml of ice-cold basal solution.

One milliliter of milliQ H_2O was added to each well to lyse astrocytes; cells were scraped and sonicated for 4 min. Lysates were clarified by rapid centrifugation (4 min × 12,100g at room temperature).

Each cell lysate (1ml) was added onto AG 1-X8 Polyprep column, and L-[^{3}H]glutamate was separated from L-[^{3}H]glutamine by subsequent H_2O and 0.1M HCl elutions as described above.

Eluent fractions were collected into scintillation vials, and ^{3}H content was determined as described in the GS assay section. The GLNase activity was calculated as percent conversion of L-[^{3}H]glutamine to L-[^{3}H] glutamate, which was normalized to the total ^{3}H recovered from each sample. This was done using the following formula: % conversion = [(dpms in "glutamate" fractions nos. 4-6)/(dpms in "glutamine" fractions nos. 1-3 + dpms in "glutamate" fractions nos. 4-6)] × 100%.

HPLC Assay of Intracellular Amino Acid Content

For determination of intracellular amino acid content, cells were treated under identical conditions as in the enzymatic assay experiments with the exception of ^{3}H-labeled compounds. Confluent cell cultures grown in six-well plates were pre-incubated in basal HEPES-buffered medium for 40 min with or without inhibitors of GS and GLNase, as indicated in figure legends. They were then washed from inhibitors two times with 2ml basal medium and transferred to media containing 2μM glutamate plus 100μM $(NH_4)2SO_4$ or 2μM glutamine to mimic enzymatic assay conditions for GS and GLNase, respectively. After 30 min incubation at 37°C, experimental media were aspirated, cells were washed three times from extracellular amino acids, and 1ml of a solution containing 5mM HEPES and 1mM EDTA was added to each well. Cells were scraped and sonicated for 4 min at room temperature. Aliquots (100μl) of cell lysates were taken for protein assays, and the remaining lysates were clarified by rapid centrifugation (4 min × 12,100g, room temperature). Amino acid levels in each supernatant were determined by a reverse-phase HPLC using an Agilent 1200 HPLC setup and Eclipse XDB-C18 column (4.6 × 150mm, 5μm particle diameter). Precolumn derivatization of amino acids was performed with a freshly prepared mix of o-phthaldialdehyde and 2-mercaptoethanol in 0.4M sodium tetraborate buffer (pH = 9.5). The amino acid derivatives were eluted with a solvent containing 30mM NaH_2PO_4, 1% tetrahydrofuran, 30mM sodium acetate, 0.05% sodium azide, and increasing concentration of HPLC grade methanol (10-30%). Fluorescence signals were registered using a programmable 1200 series fluorescence detector (Agilent). Amino acid standards

(L-alanine, L- aspartate, L-glutamate, L-glutamine, taurine), which were processed in the same fashion, were used to locate amino acid peaks and calculate concentrations of individual amino acids in the samples.

H-labeled Amino Acid Uptake Assay

To determine rates of amino acids accumulation, confluent Oligodendrocyte cultures grown in 12-well plates were washed three times from serum-containing media with 1ml of HEPES-buffered basal medium and incubated for 5-40 min at 37˚C with basal medium containing additionally 2μCi/ml of L-[^{3}H]glutamine plus 2μM of unlabeled L-glutamine or 1μCi/ml of L- [^{3}H]glutamate plus 2μM of unlabeled L-glutamate. Amino acid uptake was terminated by three washes with 1ml of ice-cold basal medium. Cells were then lysed in 1ml of 2% SDS plus 8mM EDTA. Lysates were transferred into scintillation vials and ^{3}H content was determined as described above. Rates of amino acid accumulation were then normalized to a specific activity of each radiolabeled compound and average protein content. Protein content was determined using bicinchoninic acid (BCA) assay kit (Thermo Scientific-Pierce, Rockford, IL) according to the manufacturer's instructions. Optical density was read at 590nm using an ELx800 plate reader (Bio-Tek Instruments, Winooski, VT).

L-[^{3}H] Glutamine Release Assay

To measure L-[^{3}H]glutamine release, cultured astrocytes were grown on 18-mm square coverslips were pre-incubated for 40 min in basal medium with the GLNase inhibitor 1mM DON to minimize conversion of L-[^{3}H]glutamine to L-[^{3}H]glutamate. Coverslips were then washed from DON and placed into a fresh portion of basal medium containing additionally 4μCi/ml of L-[^{3}H] glutamine and 2μM of unlabeled L-glutamine. After 30 min loading, cells were washed from extracellular isotope and transferred to a Lucite perfusion chamber. This chamber had a depression precisely cut in the bottom to accommodate the coverslip and a Teflon screw top leaving space above the cells of around 150-200μm in height. Cells were superfused at a flow rate of 1.2ml/min in an incubator set at 37˚C with a basal medium as indicated in figures. One-minute perfusate fractions were collected and analyzed for ^{3}H content. At the end of each experiment, Oligodendrocyte on coverslips was lysed with 1ml of 2% sodium dodecyl sulfate (SDS) plus 8mM EDTA to calculate remaining isotope content. Fractional isotope release for each time point was calculated by dividing radioactivity released in each 1-min interval by the radioactivity left in the cells.

Therapy and Treatment Phase 2

In Vitro testing on differentiated stem cells with extracted infected oligodendrocytes with sclerosis. Then to inject and plant differentiated stem cells in sclerosis part and check duration and function. Consequently, apply Phase 1 tests and then compare between both phases and to take the best.

Therapy and Treatment Phase 3

Inject adenosine only with different concentrations and frequently check neuron and myelin sheath morphology and function *in vitro*, then apply it *in vivo*.

Therapy and Treatment Phase 4

The preparation of differentiated stem cells to Oligodendrocytes:

Initially, Extract Somatic pluripotent stem cells from M.S patient spleen. Secondly, grow them in a petri dish containing (BFGF) basic fibroblast growth factor for 2 months then remove (BFGF) and add (PDGF) platelet-derived growth factor plus (TH) thyroid hormone for 5 days then (GC) Gemcitabine cells will be produced and especially 30% are GC+ then take 2% of GC Cells and culture them in (BFGF) again, so more Oligodendrocytes cells can be produced in the future or if there is oligodendrocyte cells shortage by transferring specialized cells into (PDGF) plus (TH). The differentiated cells from stem cells to oligodendrocytes will be incubated until it is executed in 2-8 C to make sure the cell is dormant. The therapy will be in the following after cell differentiation and CT Scan of CNS, detect sclerosis parts in the CT scan Image and mark their places according to patient brain size and make sure the patient under full anesthesia injection and M.S patient is given immunosuppressant drug or Ocrelizumab before and after therapy and record the neuron function and CT scan to observe clearance of sclerosis comparing to previous scan results. Whilst cells are differentiated implement probes to cell's DNA by using PCR to determine cell place during *in vivo* trial. These probes, as a result, they will emit radioactive waves which can be detected by x-rays.

Therapy and Treatment Phase 5

After PCR and Probe implementation, whilst culturing the grown self-stem cells prepare the protocol to differentiate them to oligodendrocytes, and then re-inject them again in sclerosis parts as can be seen in CT scan or MRI. Self-Stem cells utilized to avoid immune system rejection. Furthermore, a valid dose of immunomodulatory cortisol or azathioprine. Consequently, add appropriate dose concentration of adenosine to enhance neuron and oligodendrocyte function. It is recommended to utilize azathioprine because during purine metabolism converts adenosine to inosine by ADA (adenosine deaminase), therefore, as product hypoxanthine and increase in uric acid level which plays a very crucial role to act as a neuroprotective [5-33].

Conclusion

To conclude, this idea can change Multiple sclerosis rate upside-down due to the belief in research pathophysiology and future consequences in treating Multiple sclerosis. The aim is to strike the 2.5 million people suffering and living with MS and to free this world from Multiple Sclerosis. The therapy is going to be applied accordingly in the aforementioned phases with the supervision of a nutrition plan to the patient that is a plant-based diet. The research disadvantages are in the following treatment duration which takes about 6 to 8 months, under regular follow-up and medical restrictions and legislation. However, the advantages are that the therapy is not expensive, not major, has very low risk and side effects. Furthermore, no immune system rejection because it's made of self-cells. My study is more related to enhance pharmacotherapy and by devoting a new therapy which merely trying as much as possible to achieve balance in the pharmaco-economic category to become more affordable to patients. This is a project, which requires funding from resolute organizations and a laboratory to commence the tests and to record the results that can be further discussed in the near future.

Bibliography

1. Brown, M. (2005). Ocrelizumab for MS: Benefits for Multiple Sclerosis and Side Effects.

2. Steinman, L. (1999). Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron., 24*(3), 511-514.

3. Yang, L., Kan, E. M., Lu, J., Wu, C. & Ling, E. A. (2014). Expression of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and its roles in activated microglia *in vivo* and *in vitro*. *Journal of Neuroinflammation, 11,* 148.

4. Mongin, A. A., Hyzinski-García, M. C., Vincent, M. Y. & Keller, R. W. (2011). A simple method for measuring intracellular activities of glutamine synthetase and glutaminase in glial cells. *American Journal of Physiology-Cell Physiology, 301*(4), C814-C822.

5. Albrecht, J. & Norenberg, M. D. (1990). l-methionine-DL-sulfoximine induces massive efflux of glutamine from cortical astrocytes in primary culture. *Eur J Pharmacol., 182*(3), 587-589.

6. Albrecht, J., Sonnewald, U., Waagepetersen, H. S. & Schousboe, A. (2007). Glutamine in the central nervous system: function and dysfunction. *Front Biosci., 12,* 332-343.

7. Alcaro, M. C. & Papini, A. M. (2006). Contribution of peptides to multiple sclerosis research. *Biopolymers, 84*(4), 349-367.

8. Barres, B. A. & Raff, M. C. (1993). The proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature, 361*(6409), 258-260.

9. Baas, D., Legrand, C., Samarut, J. & Flamant, F. (2002). Persistence of oligodendrocyte precursor cells and altered myelination in optic nerve associated with retinal degeneration in mice devoid of all thyroid hormone receptors. *Proc Natl Acad Sci USA., 99*(5), 2907-2911.

10. Belachew, S., Yuan, X. & Gallo, V. (2001). Unraveling oligodendrocyte origin and function by cell-specific transgenesis. *Dev Neurosci., 23*(4-5), 287-298.

11. Chofflon, M. (2005). Mechanisms of action for treatments in multiple sclerosis. Does a heterogeneous disease demand a multi-targeted therapeutic approach? *Biodrugs, 19*(5), 299-308.

12. Cavaletti, G. (2006). Current status and future perspective of immuno-intervention in multiple sclerosis. *Curr Med Chem., 13,* 2329-2343.

13. Cubelos, B., Gonzalez-Gonzalez, I. M., Gimenez, C. & Zafra, F. (2005). Amino acid transporter SNAT5 localizes to glial cells in the rat brain. *Glia, 49*(2), 230-244.

14. Darlington, C. (2002). Overview multiple sclerosis and gender. *Curr Opin Investig Drugs., 3,* 911-914.

15. Hyzinski-Garcia, M. C., Vincent, M. Y., Haskew-Layton, R. E., Dohare, P., Keller, R. W., Jr, Mongin, A. A. (2011). Hypoosmotic swelling modifies glutamate-glutamine cycle in the cerebral cortex and in astrocyte cultures. *J Neurochem., 118*(1), 140-152.

16. Kijanska, M. & Kelm, J. (2016). *In vitro* 3D Spheroids and Microtissues: ATP-based Cell Viability and Toxicity Assays.

17. Kondo, T. (2000). Oligodendrocyte Precursor Cells Reprogrammed to Become Multipotential CNS Stem. *Cells, 289*(5485), 1754-1757.

18. Lutterotti, A. & Martin, R. (2008). Getting specific: monoclonal antibodies in multiple sclerosis. *Lancet Neurol., 7*(6), 538-547.

19. Lublin, F. (2005). History of modern multiple sclerosis therapy. *J Neurol., 252*(Suppl 3), iii3–iii9.

20. Zhao, C., Fancy, S., Kotter, M. R., Li, W. W. & Franklin, R. J. (2005). Mechanisms of CNS remyelinatin: the key to therapeutic advances. *J Neurol Sci., 233*(1-2), 87-91.

21. McFarland, H. F. & Martin, R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol., 8*(9), 913-919.

22. Nistor, G., Totoiu, M., Haque, N., Carpenter, M. & Keirstead, H. (2004). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia, 49*(3), 385-396.

23. Noseworthy, J. H., Lucchinetti, C., Rodriguez, M. & Weinshenker, B. G. (2000). Multiple sclerosis. *N Engl J Med., 343*(13), 938-952.

24. Neuhaus, O., Archelos, J. J. & Hartung, H. P. (2003). Immunomodulation in multiple sclerosis: from immunosuppression to neuroprotection. *Trends Pharmacol Sci., 24*(3), 131-138.

25. Oleszak, E. L., Kuzmak, J. & Good, R. A. (1995). Immunology of Theiler's murine encephalomyelitis virus infection. *Immunol Res., 14*(1), 13-33.

26. Pozzilli, C., Martinelli, F., Romano, S. & Bagnato, F. (2004). Corticosteroids treatment. *J Neurol Sci., 223*(1), 47-51.

27. Pilz, G., Wipfler, P., Ladurner, G. & Kraus, J. (2008). Modern multiple sclerosis treatment: what is approved, what is on the horizon. *Drug Disc Today., 13*(23-24), 1013-1025.

28. Rizvi, S. A. & Bashir, K. (2004). Other therapy options and future strategies for treating patients with multiple sclerosis. *Neurol., 63*(Suppl 6), S47-S54.

29. Rudick, R. A., Cohen, J. A., Weinstock-Guttman, B., Kinkel, R. P. & Ransohoff, R. M. (1997). Management of multiple sclerosis. *N Engl J Med., 337*(22), 1604-1611.

30. Sadovnick, D. A. (2002). The genetics of multiple sclerosis. *Clin Neurol Neurosurg., 104*(3), 199-202.

31. Sobocki, P., Pugliatti, M., Lauer, K. & Kobelt, G. (2007). Estimation of the cost of MS in Europe: extrapolations from a multinational cost study. *Mult Sclerosis., 13*(8), 1054-1064.

32. Steinman, L. & Zamvil, S. S. (2005). Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. *Trends Immunol., 26*(11), 565-571.

33. Sriram, S., Stratton, C., Yao, S., Tharp, A., Ding, L., Bannan, J. & Mitchell, W. (1999). Chlamydia pneumoniae infection of the central nervous system in multiple sclerosis. *Annals Of Neurology, 46*(1), 6-14.