CHAPTER III

MATERIALS AND METHODS

Cultures and treatments of Schwann cells

To determine proliferative/cytotoxic effects of iron on cultured Schwann cells, Schwann cell line the D6P2T (ATCC, USA) was selected as an *in vitro* model for this study. This cell line derived from a N-ethyl-N-nitrosourea (ENU) induced rat peripheral neurotumor. D6P2T cells were plated at a density of $1x10^4$ cells/well in 96-well plates for MTT assay or $1x10^5$ cells/well in 24-well plates for RT-PCR or $4.5 x10^5$ cells/well in 6-well plates for FACs analysis and grown in 5% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM) for 24 h. Both were purchased from JRH, (USA). To begin the experiment, the medium in each well was completely and gently removed and added with freshly prepared 1% serum contain with DMEM 50,100, 250, 500, 1000 µg/ml ferric ammonium citrate (FAC) (Sigma, USA). Cells in 1% serum DMEM served as the untreated control. Times for treatments were indicated in each experiment (see below).

MTT cell proliferation/cytotoxic Assay

MTT assay is the measurement of cell proliferation/cytotoxicity. This method is based on the reaction of yellow tetrazolium salts MTT (3-(4, 5 dimethylthiazoly-2)-2, 5-diphenyltetrazolium bromide) by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The production of insoluble formazan is proportion to the activity of these enzymes and reasonably constant between cell types. Therefore, it can give an indication of the number of cells present in each well. The resulting intracellular purple of formazan can be solubilized and quantified by spectrophotometric means. D6P2T cells were plated at a density of $1x10^4$ cells/well in 96-well plates in 100 µl. Cells were incubated at 5 % CO_2, 37 °c under treated-conditions for 24 h. Then, 10 µl of 5 mg/ml

MTT reagent was added to each well, including blanks (DMEM without cells), and plate was returned to the incubator for 2 h. When the purple precipitate was clearly visible inside the cells under the microscope, DMEM and MTT reagent were removed and 100 µl of DMSO was replaced to each well, including controls, Plate was swirled gently and covered in the dark for 2 h. Absorbance in each well was measured, including the blanks, at 570 nm using a microplate reader (Multiskan EX).

Trypan blue dye exclusion assay

Trypan blue staining was used to quantify viable cell number. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. D6P2T cells were plated at a density of $1x10^4$ cells/ well in 96- well plates and then they were incubated at 5 % CO_2, 37 c under treated-conditions. After 24 h, D6P2T cells were trypsinized, gently centrifuged, and resuspened in 50 µl of serum free DMEM. An aliquot of 0.4% trypan blue and cell suspension were mixed and incubated at room temperature for 5 min. The number of unstained (viable) cells was counted on a hemocytometer. The percentage of proliferation of control was calculated using the formula below.

% proliferation of control = (viable cells of treated conditions/viable cells of untreated control) x100

Flow cytometry

Flow cytometry was used to determine whether D6P2T express TfR and to use TfR as indicator for iron entry into the cells. D6P2T cells were plated at a density of $4.5x10^5$ cells/ well in 6-well plates and incubated under FAC treated-conditions for 24 h. Then, the cells were trypsinized and transferred to 15 ml steriled conical tube and re-suspended in PBS/BSA solution containing 4% paraformaldehyde solution to fix cells for 30 min. The fixative was removed and the fixed cells were stained with saturating

concentration of labeled-mouse monoclonal antibodies; which is an anti-rat TfR (CD71) (Serotec, UK) at recommended dilution according to the manufacture protocol for 1 h. at room temperature. The stained cells were washed twice (5 min each) with PBS/BSA solution, centrifuged at 400 x *g* for 5 min to collect the cell pellet and resuspended in 0.2 ml of PBS/BSA before fluorescence measuring through the Flow Cytometer (BD Biosciences, USA) using 30,000 cells.

Western blot analysis

An immunoblot analysis of iron storage protein ferritin was included in this study to demonstrate that iron added into the cell culture medium could enter the cells and was sufficient to induce cellular response of Schwann cells. The expression of ferritin in the presence of iron was determined using rabbit anti rH-ferritin antiserum (a gift from proffessor James R Connor, College of Medicine, The Pennsylvania State University, USA). The specificity of this antiserum was previously determined by western blot analysis (Cheepsunthorn et al., 1998). Briefly, following 24 h treatments, total cells from each sample were collected and lysed in lysis buffer containing 1% Triton-X 100. Total protein content in each sample was quantified using BCA protein assay kit (Pierce, USA). Then, equal amounts (10 µg) of total protein from each sample was mixed with sample buffer containing beta-mercaptoethanol, boiled for 10 min, and separated on 12% SDS-PAGE. Then, proteins were transferred to PVDF membranes, blocked in 5% dry milk for 1 h at room temperature, rinsed with TBS, and incubated overnight at 4 °c in the primary antibody (1:700). Subsequently, the membranes were washed with TBS containing 1% Tween-20 and incubated in the secondary antibody (1: 10000) goat anti-rabbit IgG peroxidase conjugate (Sigma, USA) for 1 h at room temperature. Bound antibodies were visualized with the supersignal solutions (Pierce, USA) and detected band with film explosion. See appendix B for buffer preparations.

RNA Isolation

To elucidate the effect of iron on the mRNA expression of P0 and MBP, D6P2T were plated at a density of $1x10^5$ cells/ well in 24-well plates. After 24 h treatments, total RNA from each condition was isolated using Trizol reagent (Invitrogen, USA). Briefly, the medium in each well was completely removed. The cells were washed once with phosphate-buffered saline (PBS), pH 7.4. Then, Trizol reagent (300 µl) was added into each well. The homogenates were placed on a low speed rotator for 5 min at room temperature. This is to allow the complete dissociation of nucleoprotein complexes. Next, the homogenates were transferred into steriled 1.5 ml tubes. Then, chloroform (60 µl) was added into each tube followed by vortexing vigorously for 15 sec. The cells were allowed to stay at room temperature for 5 min before centrifugation at 12,000 x g for 15 min at 4°c. RNA in the colorless upper aqueous phase was carefully transferred to a fresh tube. Then, isopropanol (150 µl) was added into each tube. Each sample was stored at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4°c. RNA was precipitated and forms a white pellet at the bottom of the tube. The supernatant was carefully removed before adding iced-cold 75% ethanol (300 µl) into each tube to wash RNA pellet for 5 min. Subsequently, the pellet was centrifuged at 10,000 x g at 4°c for 5 min. At the end of procedure, the RNA pellets were allowed to air dry. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. The RNA pellets were dissolved in RNase free water. The amount of RNA obtained by spectophotometry was calculated using the formula below.

RNA (µg) = Absorbance at 260 nm x 40 x dilution factor

The solution containing RNA sample can be stored at -80°c until use.

Reverse transcription (cDNA synthesis)

A volume of 2 µg total RNA from each sample was added into the reaction mixture containing 10x reaction buffer, 25 mM $MgCl_2$, dNTPs, random primer, Ribonuclease inhibitor, AMV Reverse transcriptase and RNase free water. All reagents were purchased from Promega, (USA). Each sample was kept at room temperature for 10 min incubated at 42 °c for 60 min followed by inactivation at 99 °c for 5 min. Then, distilled water was added into each sample to a final volume of 100 µl. At this point, each sample can be kept at -20 °c until use. See appendix C for preparing the reaction mix.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 µl. A 5 µl of cDNA (as described above) was use as templates for subsequent PCR reaction in the geneAmp PCR system 9700. PCR reaction was composed of distilled water, PCR buffer, dNTP mix; Taq DNA polymerase (Promega, USA) and primer pairs (see Table 3). To ensure there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water was replaced the RNA samples in the PCR protocol. Subsequently, amplified PCR products were visualized by gel electrophoresis. Briefly, a 10 µl of each PCR product was mixed with 2 µl of loading buffer, vortexed, spined, and then loaded on to 1.5 % agarose gel containing ethidium bromide. The gel was run at 95 volts until front dye reaches the end of gel. Estimate size of PCR products was compared to the DNA marker (100 bp ladder, Fermentas, USA). No PCR products were observed in any control reactions (data not shown). See appendix C for preparing the reaction mix.

Table 3 Specific primers for P0, MBP and GAPDH

Gene	Primer sequences (5′ → 3′)	bp.	PCR Protocols
P0	GCC CTG CTC TTC TCT TCT TT CCA ACA CCA CCC CAT ACC TA	410	1
MBP	ACT GCG GAT AGA CAG G GAT GGT GAC CTT CGG C	368	2
GAPDH	AAG CTC ACT GGC ATG GCC TTC C TTG GAG GCC ATG TAG GCC ATG AG	300	3

Protocol 1 Pre denature at 94°c for 4 min followed by 30 cycles of 94°c for 1 min, 59°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 2 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 2 min, 48°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 3 Pre denature at 94°c for 4 min followed by 30 cycles of 94°c for 1 min, 59°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Statistical analysis

All data were presented as means and SD or standard error of means (mean ± SEM). One way analysis of variance (ANOVA) followed by LSD test was used to compare the significance between treatment. The p-value of less than 0.05 was set for the significant difference.