

**CD34 negative non-hematopoietic human umbilical cord blood stem cell
Amelioration of Ischemic Brain Injury: Mechanisms of Action**

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ABSTRACT

Background: Previous work in the laboratory has demonstrated that a novel cell population of CD34 negative non-hematopoietic umbilical cord blood stem cells (nh-UCBCs) can be isolated from the human umbilical cord blood (UCB). These cells when intravenously delivered into a transient or permanent middle cerebral artery occlusion (MCAO) rat model of stroke, 2 days after injury, helped alleviate the stroke induced behavioral deficits and reduced the infarct size. In addition, injection of nh-UCBCs directly into the brain near the site of ischemic injury also ameliorated limb placement deficits.

Purpose: The central goal of this project was to explore the mechanism(s) by which CD34 negative nh-UCBCs help ameliorate the neurological deficits when injected systemically into a rat model 48hrs after inducing stroke (acute period), and when directly injected into the brain at 3 months after ischemic injury (chronic period). The improvement in behavior of the rat model was evaluated based on the neurological severity score (NSS) derived from performing various functional tests. The first aim of the project was to check whether the nh-UCBCs has an elevated level of anti-inflammatory cytokines which helps in reducing the inflammation that results from stroke and thus helps in the behavioral improvement of the animal model during the acute period after an ischemic stroke. In order to test this aim, the cytokine levels in the nh-UCBCs and nh-UCBS cell conditioned media were tested using qRT-PCR and cytokine protein array, respectively. The next aim was to check whether the nh-UCBCs that are injected intravenously into the rat model can modulate the phenotype of immune cells in the brain. To test this aim we analyzed the expression of various M1 and M2 macrophage/microglia markers in the BV2 cells (murine microglial cell line) cultured in different conditions by qRT-PCR. We expect to see a shift from M1 type macrophages/microglia phenotype to M2 type macrophage/microglia phenotype which is responsible for eliminating inflammation and help in wound healing.

During the chronic period after a stroke, the inflammatory environment has most likely subsided, and the restorative effects of injecting nh-UCBCs directly into the brain parenchyma is most likely not due to the modulation of the inflammatory environment.

We postulate that it might be due to the induction of fiber sprouting by neurons from the non-damaged hemisphere towards the site of injury. In order to test this trophic action of the neurons and the sprouting of fibers in the presence of the non-hematopoietic population of human umbilical cord blood, an *in vitro* tissue culture model is used. The plausible mechanism in this model is that the nh-UCBCs can induce neural plasticity and therefore enhance the connectivity between the interhemispheric neuronal connections.

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INTRODUCTION

Stroke is the fourth leading cause of death in the United States; approximately 795000 people have a new or recurrent stroke each year ^[1]. 85% of strokes are caused by a blockage of a blood vessel in the brain and are called ischemic strokes. It is one of the most economically and emotionally devastating diseases ^[2]. The incidence of stroke is expected to dramatically rise as the population ages, as stroke risk increases with age ^[1].

Stroke occurs when the blood flow to the brain stops due to a blockage formed by a clot. Due to this, the brain cells are deprived of the necessary oxygen and nutrients and hence are prone to cell death. Approximately 1.9 million neurons die every minute and though some brain cells are viable, they are dysfunctional due to the low flow state during ischemic stroke ^[3]. Intravenously administered Ateplase (recombinant tissue plasminogen activator [rt-PA]) is the only Food and Drug administration approved pharmacologic treatment for acute cerebral infarction in the United States. Administering rt-PA within 3 hours of symptoms results in a decrease in mortality as it is a thrombolytic agent that restores the cerebral blood flow by dissolving the clot in the brain ^[4]. But due to the lack of awareness of the common symptoms of stroke, a majority of the ischemic stroke patients are unable to access the acute therapy. Beyond the 3- 4.5 hours' time window, the three acceptable methods of treatment for the acute ischemic stroke are intra-arterial (IA) administration of t-PA (up to 6 hours), use of MERCI retrieval device (up to 8 hours)^[5] and penumbra aspiration system (up to 8 hours) ^[44] for revascularization of the vessel occlusion in patients. But the efficacy of using these mechanical devices for clot removal has never been proven to be superior to rt-PA. Moreover there is no effective therapy that is available for chronic stroke. Hence developing new therapeutic strategies is critical for stroke treatment.

Stem cell based approaches are novel therapies for stroke that have expanded substantially over the past decade. The animal experimentation data obtained from treatment with stroke are promising and several clinical trials are currently being carried out. Studies focusing on the optimal dose and the route of administration of the stem cells are also being investigated ^{[6][51]}. Factors such as mode of action, immunogenicity,

harvesting, proliferative capacity, tumorigenicity and the overall feasibility of use of these stem cells should also be considered before the use of the stem cells for stroke treatment ^[7].

The nature of stroke and the mode of action of the stem cells are both diverse. Hence special consideration is to be given for the use of stem cell derivatives that are used to treat stroke conditions ^[7]. The possible sources of stem cells for stroke therapy are neural stem cells from human fetal brain, pluripotent cells generated from blastocysts (ES cells) or from somatic cells [induced pluripotent stem cells (iPS cells)] that are capable of being expanded and differentiated into specific cell types ^[6]. Moreover, recent studies have also shown the generation of specific types of neurons by direct conversion of fibroblasts (iN cells) for use in stroke therapy ^[56]. The adult stem cells like bone marrow-derived stem cells such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), very small embryonic like stem cells (VSELs) and neural stem cells (NSCs) are also being investigated as a possible stem cell based approach for stroke recovery. Transplantation of umbilical cord blood derived and bone marrow derived hematopoietic stem cells into animal models of stroke also have demonstrated functional recovery, reduced infarct size and higher expression of neuroprotective factors, such as BDNF and VEGF ^{[6][7]}.

1. Human umbilical cord blood stem cells

Human umbilical cord blood stem cells are primitive adult stem cells and have the ability to repopulate the different blood lineages ^{[8] [9] [10]}. It is a mononuclear fraction, which includes many cell types like hematopoietic progenitors, lymphocytes, monocytes and mesenchymal stromal cells and is a desirable potential source for transplantation ^{[11] [12]}. Due to their heterogenic nature, these stem cells are considered to be immunologically immature and earlier studies with human UCBS cells have shown that these cells have an influence in modulating the immune response and reduce the level of pro-inflammatory cytokines in response to the inflammation which results from stroke ^[8]. Moreover in comparison to bone marrow transplantation, transplantation of cord blood into an allogeneic recipient does not require a perfect human leukocyte antigen (HLA) matched

donor ^[57]. The chances of acute and chronic graft versus host diseases (GVHDs) is also lesser in UCB transplantation compared to bone marrow transplantation ^{[13][14]}.

1.1 CD34 negative non-hematopoietic umbilical cord blood stem cells (nh-UCBCs)

Flow cytometry, clonogenic assays and the expression of CD antigens coupled with self-renewal proliferation assays are used in order to distinguish between the hematopoietic stem, progenitor cells and other components of the cord blood ^[58]. CD34 is a type 1 transmembrane glycoprotein that is used as a phenotypic marker capable of recognizing the cell surface components in a heterogeneous substrate such as cord blood in order to identify and sort the different cell types present ^[45]. Previous work in the laboratory has demonstrated that a novel cell population of CD34 negative non-hematopoietic umbilical cord blood stem cells (nh-UCBCs) can be isolated from the human UCB by culturing the mononuclear fraction and cell sorting for the specific CD antigen. These nh-UCBCs that were isolated from the human UCB, exhibited properties of self-renewal and expressed high levels of pluripotency markers like OCT-4, REX-1, SOX-2 and NANOG that are typically expressed in stem cells ^[15]. In addition, these cells also expressed specific surface markers like SSEA-3, SSEA-4 and tumor rejection antigens like TRA1-60 and TRA1-80 that are usually used to characterize human embryonic stem cell lines ^{[16][17][18][19]}.

Moreover, the CD34 negative nh-UCBCs when intravenously delivered into a rat of transient or permanent middle cerebral artery occlusion model (MCAO) of stroke at two days after injury, helped alleviate the stroke induced behavioral deficits and reduced the infarct size ^[15]. In addition, injection of these cells directly into the brain near the site of ischemic injury also ameliorated limb placement deficits. The mechanism(s) of action, however, are yet to be determined.

The focus of the thesis was therefore to explore the mechanism(s) as to how the CD34 negative nh-UCBCs help in ameliorating the neurological deficits when injected systemically into a rat model 48hrs after induced with stroke (acute period), and when directly injected into the brain at 3 months after the ischemic injury (chronic period).

2. Mechanism of action for the recovery process in stroke by using stem cell based approach

The theories for the mechanism of action by which the use of a non-neuronal stem cell based approach helps in improving the functional behavior of an animal model induced with stroke is based on strategies other than cell replacement ^[6]. The main effects that are possible in mediating the restorative effects in the stroke induced animal model are “neuroprotection” which helps in preventing the damaged neurons from undergoing cell death within the ischemic penumbra in the acute phase of cerebral ischemia and “neurorepair” process in which the broken neuronal networks are repaired in the chronic phase of cerebral ischemia. The other mechanisms that can be involved are the modulation of the inflammation which occurs after stroke and the promotion of angiogenesis.

The mechanism of action by which the CD34 negative nh-UCBCs helps in improving the behavioral deficits in the animal model of stroke as shown in the previous study in the laboratory ^[15] could be due to one of the mechanisms above mentioned or a combination of the two mechanisms.

2.1 SPECIFIC AIM 1: CD34 negative nh-UCBCs express elevated levels of anti-inflammatory cytokines.

Stroke induced by middle cerebral artery occlusion (MCAO) which leads to the loss of oxygen and glucose to the cerebral tissue results in post- ischemic inflammation (figure 1). This henceforth triggers the pathological ischemic cascade which causes neuronal injury in the ischemic core within minutes after the arterial occlusion ^[20]. Earlier studies have shown that, cells of the ischemic penumbra region of the brain do not completely die due to the damage caused by the ischemic injury and hence this offers a promise that if proper therapies are devised, the brain tissue can be rescued and can help reduce post stroke disability ^[21].

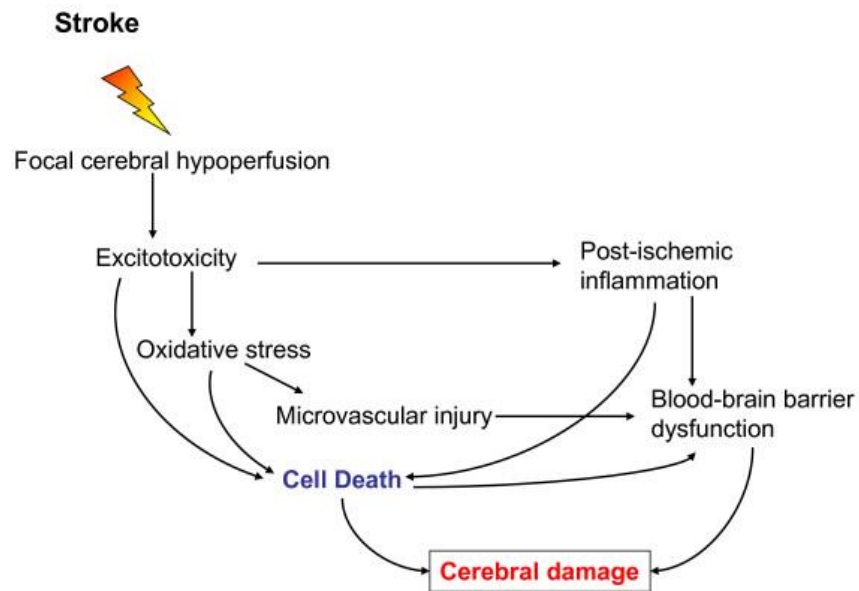


Figure 1: **Ischemic cascade of events leading to cerebral damage post injury** ^[21]. Ischemic stroke causes cell death of the neurons in the region of the brain affected due to the loss of blood supply which causes the region to be deficient of glucose and other nutrients. This hence forth triggers a series of events like excitotoxicity, oxidative stress, microvascular injury, blood-brain barrier dysfunction and post-ischemic inflammation ultimately leading to cerebral damage.

The immune response and inflammation are important events that occur as a result of stroke ^[21]. Reducing the inflammatory response by the administration of an anti-inflammatory agent is a possible mechanism for stroke recovery as it reduces the severity of post-ischemic injury. Cytokines are a group of glycoproteins that act as mediators and are produced in response to an antigen that help in the regulation of the innate and adaptive immune systems. In the brain, cytokines are expressed by both by the cells of the immune system and the resident brain cells like neurons and glial cells ^[22]. Peripherally derived cells like the mononuclear phagocytes, T lymphocytes, NK cells and polymorphonuclear leukocytes are also known to secrete cytokines that are involved in brain inflammation ^[23] and thereby potentiating the neurotoxicity within the CNS. It has been shown in earlier studies, that the ischemic damage can be decreased in animal models by blocking leukocyte adhesion to the endothelia with antibodies to ICAM-1 ^[24]. Alternatively, the ischemia induced inflammation can be controlled by modulating the pro-inflammatory cytokine level produced in the CNS ^{[25] [26]}.

The most studied cytokines related to inflammation in acute ischemic stroke includes tumor necrosis factor- α (TNF- α), the interleukins (IL), IL-6, IL-10, IL-20, IL-1 β , IL-2, IL-4 etc. and transforming growth factor (TGF)- β . While IL-1 β and TNF- α are the important pro-inflammatory cytokines which are responsible for exacerbating the cerebral injury and inflammation after the induction of stroke (figure 2), the cytokines like TGF- β and IL-10 are known as the anti-inflammatory cytokines which may be neuroprotective ^[27] ^[28]. Research in the past to study the mechanism of human UCB treatment in mediating the recovery of rat model of stroke showed that the intravenous delivery of human UCB decreased the pro-inflammatory cytokines such as TNF- α , IL-1 β , and IFN- γ and increased the production of anti-inflammatory cytokines like IL-10 and TGF- β in the brain of the stroke induced animal. The up-regulation of the anti-inflammatory cytokines and the simultaneous down-regulation of the pro-inflammatory cytokines helps in reducing the T-cell proliferation that results in a stroke induced animal. The human UCB treatment reduced the presence of both activated microglia and astrocytes in the brain ^[29]. Moreover the growth factors such as FGF, EPO and G-CSF help in promoting functional recovery and earlier studies show that administering such growth factors into an animal model 24 hours after stroke for 7 days enhances the motor skill of the animal model ^[16] ^[17] ^[18]. There is increasing evidence that the intravenous transplantation of CD34 positive HUCB cells transfected with the glial cell line- derived neurotrophic factor (GDNF) gene exerts therapeutic benefits in a stroke induced by MCAO in rats ^[30]. The GDNF modified CD34+ cells transplantation into the stroke affected animals, showed significant increase in the GDNF protein production levels in the infarcted hemisphere of the brain, which in turn is responsible for reducing the brain infarction volume helping in the functional recovery of the stroke induced animal model ^[30]. In some cases, certain cytokines might act either as pro- inflammatory cytokines or as anti-inflammatory cytokines depending on the environment. For example, certain cytokines that are believed to be pro-inflammatory agents responsible for inflammation that results after stroke like IL-8, MCP-1 and IL-1 β have been implicated to be the first line of defense in the inflammatory reaction. They are produced more extensively in the brain compared to other chemokine's in the brain. On the other hand, certain chemokine's like MCP-1 and macrophage inflammatory protein (MIP) -1 α are seen to be

elevated in the ischemic brain as observed in an in-vivo study ^[31] and are believed to play an important role in the infiltration of monocytes into the CNS under pathological conditions ^[32].

Hence in the first part of our study we hypothesize that the nh-UCBS cells that are injected intravenously into the stroke model, induce recovery by secreting anti-inflammatory cytokines that decrease the inflammation in the brain and thereby help enhance neuroprotection. For this we examine the gene expression for the various pro-inflammatory and anti-inflammatory cytokines in the nh-UCBCs and the protein expression for the cytokines in the nh-UCBS cell conditioned media.

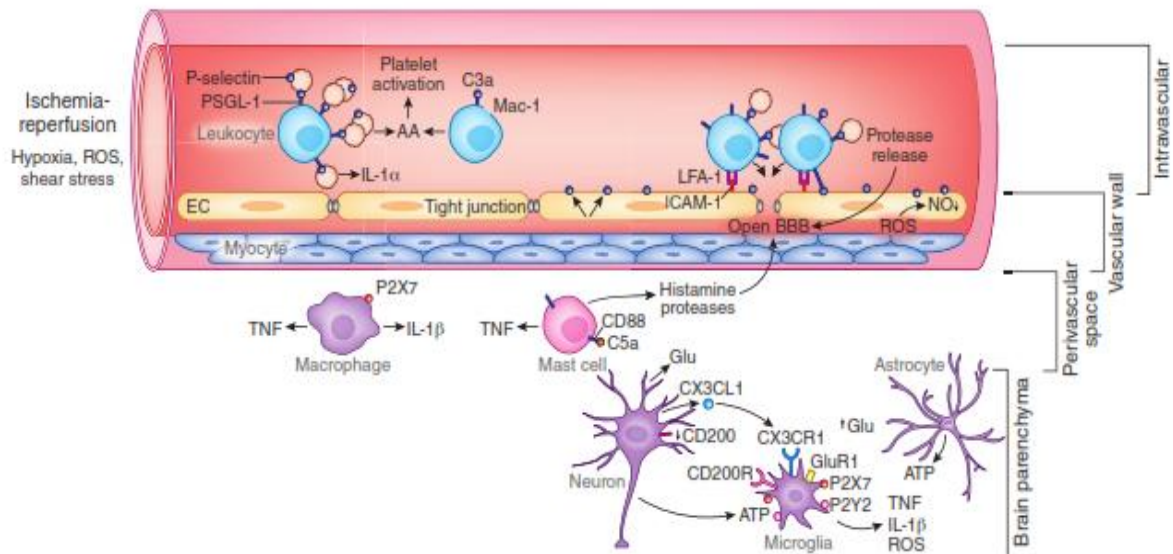
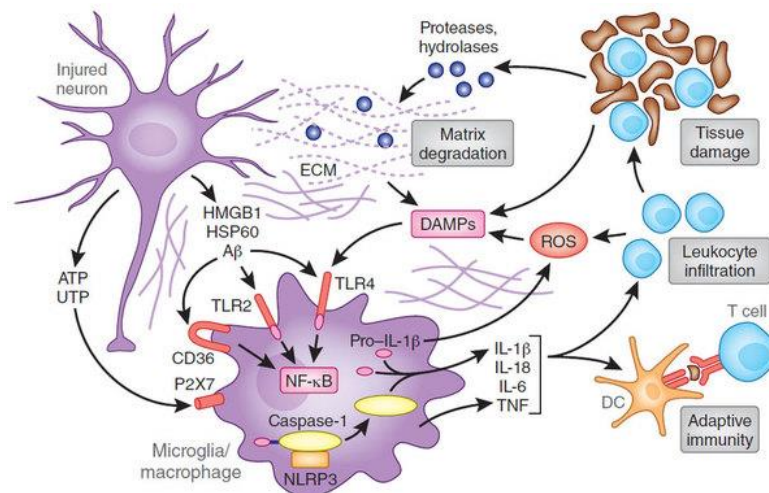


Figure 2: The early vascular, perivascular and parenchymal events triggered by stroke and reperfusion ^[21]. Ischemic stroke leads to blood clotting and hence causes the release of pro-inflammatory cytokines like IL-1 α . The MMP activation leads to blood brain barrier (BBB) breakdown and matrix proteolysis which contributes to BBB leakiness. The release of cytokines like TNF and IL-1 β from the mast cells and perivascular macrophages guides leukocyte migration across the vessel wall.

2.2 SPECIFIC AIM 2: nh-UCBCs are responsible for activating the immune cells from in brain, and other immune/peripheral organs of the body that help in decreasing the inflammation and participate in tissue remodeling after injury.

Microglial cells are resident immunocompetant macrophages of the brain [21]. These immune cells in the brain can provide immunosurveillance but can also be toxic [33] (figure 3a, 3b). Research in the past has shown that in a stroke model, the depletion of the proliferative microglia exacerbates the injury [34] and the injection of microglia into the ischemic brain helps in ameliorating the injury [35]. Hence after the induction of ischemic stroke, the microglial cells that are activated can transform into phagocytes which can release many cytotoxic and/or cytoprotective substances [21].

a)



b)

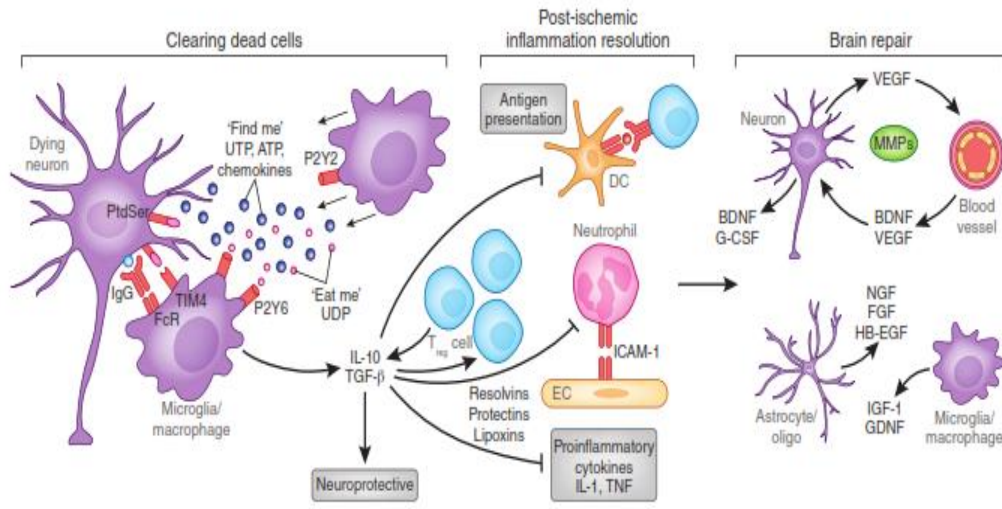


Figure 3: **The cytotoxic and cytoprotective role of the microglia in the brain** ^[21]. a) **Cytotoxic effect:** Microglial cells contribute to post-ischemic inflammation by the production of TNF, IL-1 β , potential cytotoxic molecules including NO, ROS, prostanoids and other proinflammatory cytokines b) **Cytoprotective effect:** Microglial cells contributing to the resolution of inflammation and tissue repair by the production of cytokines IL-10, TGF- β and several growth factors including IGF.

In order to study the role of microglia in neuroprotection, a large number of animal models would be required. In our study it was observed that the isolation of the inflammatory cells from bone marrow of the rat resulted in low yield of macrophages which limited further analyses. Hence in order to study the immune cells and their contribution to the effect of neuroprotection post nh-UCBS cell treatment, we used BV2 cell line which is an immortalized murine microglial cell line that is used as a substitute for primary microglia ^[59]. Using this cell line has advantages compared to primary culturing of the microglial cells from the rat brain tissue affected with stroke, as it reduces the necessity of continuous cell preparations and animal experimentation. A major impediment with primary culturing is that microglial cells are difficult to isolate in large numbers. This cell line also reproduces the in-vivo situation of the primary microglial cells with high fidelity ^[36]. Their response to lipopolysaccharide (LPS) is comparable to the response of the microglial cells which get activated after ischemia *in-vivo*.

Peripheral microglial cells show two distinct activation patterns otherwise known as the microglial subsets i.e. the classically activated pro-inflammatory M1 Phenotype (induced by IFN and LPS) and the alternatively activated M2 cells (induced by IL-4 or IL13) activation ^[37]. The high M1/M2 macrophage ratio has significant implications for CNS repair ^[38]. M2 phenotypical cells help in decreasing the inflammation caused due to stroke and also facilitate wound healing by recruiting scavenger receptors and matrix degrading enzymes that enhance phagocytosis thus helping in the tissue remodeling process ^[39]. The immune cell (Microglia and/or infiltrating macrophages) mediates the repair process by orchestrating signal cross talk between the injured site and the macrophages/microglial cells that are activated (figure 3b). The site of injury produces signals like UTP and ATP that attract microglia and

macrophages through P2Y2 receptors. The UDP signal released by the phagocytic cells acts on the P2Y6 receptors which stimulates the microglial phagocytosis and hence phosphatidylserine (PtdSer) protein is translocated to the outermost layer of the plasma membrane of apoptotic cells. T-cell immunoglobulin, TIMP-4 and EGF-8 which are the PtdSer binding proteins present on the surface of the immune cells are involved in the clearance of dead cells. The immune cells like the macrophages and microglial cells when activated; phagocytize by engaging Fc receptors on the phagocytic cells. This action of Phagocytosis promotes the secretion of the anti-inflammatory cytokines like IL-10, TGF- β which in-turn is responsible for the suppression of the antigen presenting cells, promotes T- reg cells formation, inhibits the expression of adhesion molecules in endothelial cells and reduces the secretion of the pro-inflammatory cytokines ^[40]. Previous studies of human UCB treatment for stroke, demonstrated that the cell survival was increased and the release of nitric oxide by the resident microglia (an innate inflammatory response) was seen to be reduced back to normal levels following the incubation with the HUCB cells ^[41] ^[42]. It was also shown that the HUCB cell transplantation into a stroke model impaired the secretion of IL-1 β by microglial cells in the brain. Moreover the viability of the microglia was also decreased following the treatment ^[43].

A previous study in the lab demonstrated that when CD34 negative nh-UCBCs were administered intravenously, these cells were not entirely present in the brain. Hence we hypothesize that the cells injected are being carried by the blood to the brain and other peripheral/immune organs like the spleen, bone marrow and lymph nodes in the body from where these organs are responsible for the activation the immune cells which secrete the necessary anti-inflammatory cytokines in response to the inflammation that results when stroke is induced. We believe that the microglial cells when activated in a stroke model undergo transition from M1 phenotype to M2 phenotype in the presence of the nh-UCBCs thus down regulating inflammation and facilitating the wound healing process. Hence, we expect to see higher expression of M2 phenotype markers compared to M1 phenotype markers in the microglial cells that are activated and co-cultured with the nh-UCBS cell conditioned media

2.3 SPECIFIC AIM 3: nh-UCBCs injected into the brain parenchyma are responsible for the trophic action of endogenous neurons/ sprouting of nerve fiber from the unaffected area of the brain to the site of injury thus helping in the tissue repair process.

The other possible mechanism of recovery after stroke using a stem cell based approach is neuroplasticity^[7]. Neuroplasticity refers to the organizational changes in the brain that help the individual endogenous neurons or neuronal networks to adapt to their function. Stem cells can help in the augmentation of this process of neuroplasticity by recruiting various mechanisms of recovery including sprouting and unmasking^[7]. There is increasing evidence that the axonal sprouting and rewiring in the adult central nervous system contributes to the functional plasticity and behavioral recovery after ischemic stroke^[54]. The treatment with bone marrow MSCs into an animal model of stroke demonstrated the axonal plasticity of long distance inter- and intra-cortical connections between the primary motor and pre motor areas of the brain facilitating the recovery of the brain from stroke^[55].

The concept of sprouting of the endogenous nerve fibers has been observed previously in our lab when CD34 negative nh-UBSCs were injected directly into the brain parenchyma of a rat model induced with stroke. This led to a 50% reduction in the lesion volume compared to the saline treated controls^[15]. Four weeks after grafting, by anterograde tracer experiments using biotinylated dextran amines (BDA) injected at the site of the injury, it was demonstrated that the grafted nh-UBSCs in the collateral side of the brain, facilitated the sprouting of nerve fibers towards the site of injury. In the chronic period after a stroke; the inflammatory environment is mostly subsided, and the restorative effects of injecting nh-UCBSCs directly into the brain parenchyma is most likely not due to the modulation of the inflammatory environment. Hence we postulate that it might be due to the induction of fiber sprouting by neurons from the non-damaged hemisphere towards the site of injury. In order to test this trophic action of the neurons and the sprouting of fibers in the presence of the non-hematopoietic population of human umbilical cord blood, an in-vitro tissue culture model is used in our study. The hypothesis here is that the nh-UCBCs help increase the axonal and dendritic lengths, and the number

of branch points in the neurons compared to the neurons cultured in the other culture conditions.

Previous Studies in the Lab

Isolation and phenotype analysis of nh-UCBCs from Human umbilical cord blood stem cells

It was shown that isolation of a novel population of stem cells known as the CD34 negative UCBCs was possible from human umbilical cord blood ^[15]. After 7 days in culture, these cells doubled in number and at 14 days, attached to the surface of the fibronectin coated tissue culture plate. These CD34 negative nh-UCBCs were seen to be elongated and also formed spindle like cells. Expression of the stem cell transcription factors like Oct4, Rex-1 and Sox-2 in the nh-UCBCs were confirmed by RT-PCR. In order to confirm that they are non-hematopoietic in nature, the cell surface antigens on the nh-UCBCs were studied by flow cytometry. It was seen that the cells were negative for the hematopoietic markers like CD34, CD45 and MHC class II antigens but showed high level of CD13 and CD44, and a moderate level of CD90, CD49, CD10, and MHC Class I antigens.

Systemic Administration of nh-UCBSCs in Acute Stroke Reduces Infarct Volume and Enhances Neurological Function

In the acute stroke model, where the nh-UCBSCs or saline was administered 48 hours after the induction of injury by MCAO, no significant differences were observed based on the modified neurological severity score (mNSS) and stepping up test between the experimental groups at day 2 and day 7 after the ischemic injury. NSS test and stepping up test at Day 14 and Day 28 showed a gradual increase in the NSS and improved performance in the stepping up test in the animals which received nh-UCBSCs which confirms their progressive recovery over time from 1day to 28 days after MCAO. The NSS score of nh-UCBSCs injected rat was significantly different from that of the saline control animals at Day 14 and 28.

Moreover systemic administration of nh-UCBSCs into ischemic rat model also significantly reduced the infarct volume by 50% (figures 4 and 5).

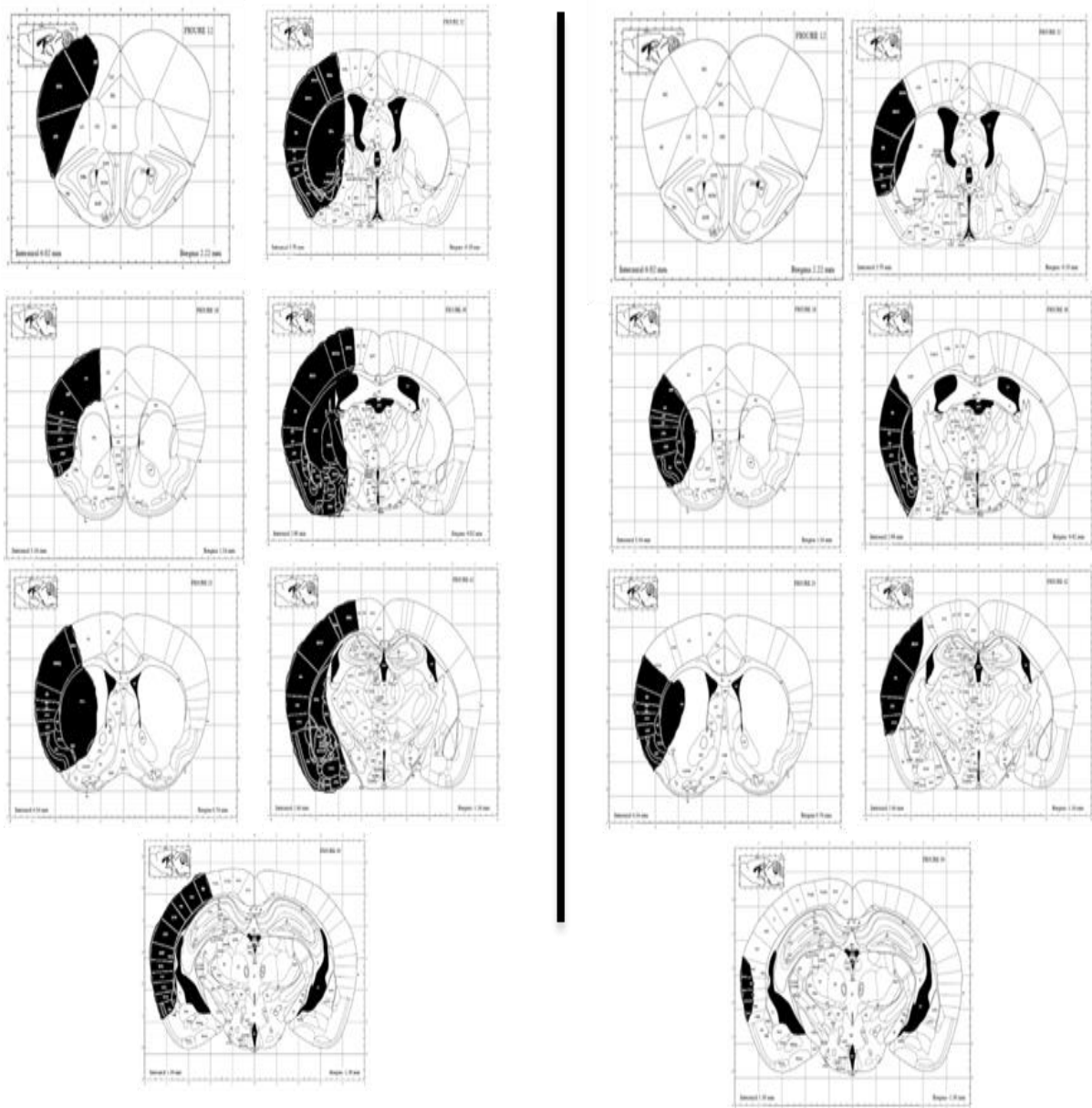


Figure 4: Systemic nh-UCBSC administration in ischemic rats reduces infarct volume.

Left panel – cross sections of brain showing areas of damage in untreated rats.

Right panel – cross sections of brain showing areas of damage in high passage nh-UCBSC treated rats (From L. Stone).

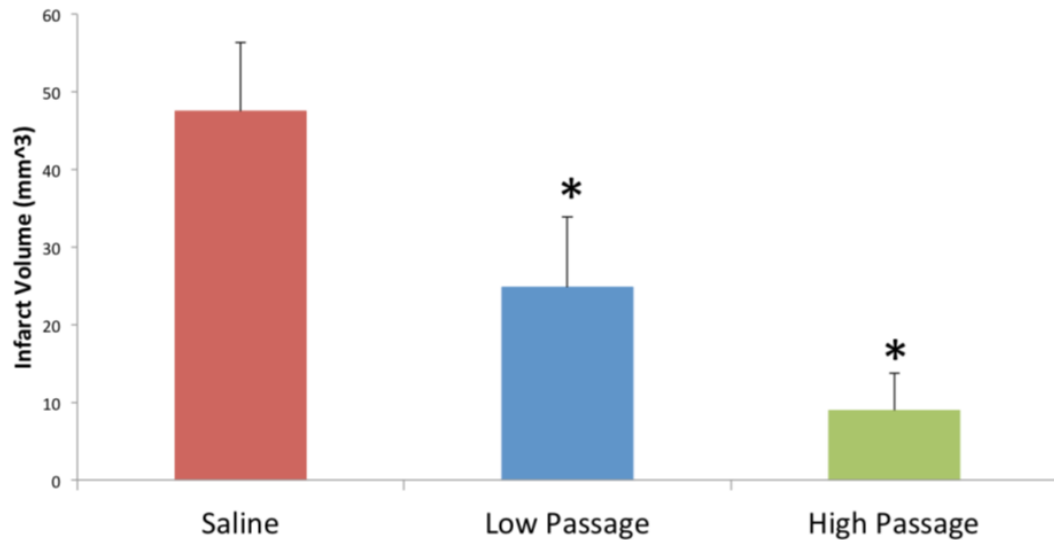


Figure 5: **Quantification of ischemia-induced brain infarct volume.** Shows the quantification of the infarct volume in the brain of the saline (Red) treated rats, and rats treated with low passage (Blue) and high passage (Green) nh-UCBSCs (From L. Stone).

Localized Intraparenchymal Transplants of nh-UCBS cells: Improves Neurological Function and Enhances Cortico-Cortical Neuronal Connectivity.

In the chronic stroke model, the nh-UCBSCs or saline was administered into Sprague Dawley or SHR rats 3 months after the induction of injury by MCAO. The NSS was used in order to track the change in the behavior of these animal models at day 2, week 1, week 2, week 4 and week 8 after treatment. It was observed that after the induction of stroke, there was no significant difference in the NSS score between the group of animals treated with nh-UCBSCs and the control groups prior to treatment (figure 6: pre-transplant). However, a significant increase in the NSS score of the animals treated with nh-UCBSCs in comparison to the control groups was observed from week 2 post-treatment (figure 6: post-transplant).

In addition after direct injection of the nh-UCBSCs into the brain parenchyma, it was observed that the host neurons from the contralateral side (unaffected site) of the brain were sprouting to the site of injury. This sprouting of the nerve fibers in the brains was traced using retrograde tracing technique (figure 7: Left panel). Further greater numbers of labeled neurons were seen in the ischemic cortex of the brain (figure 7: Right panel).

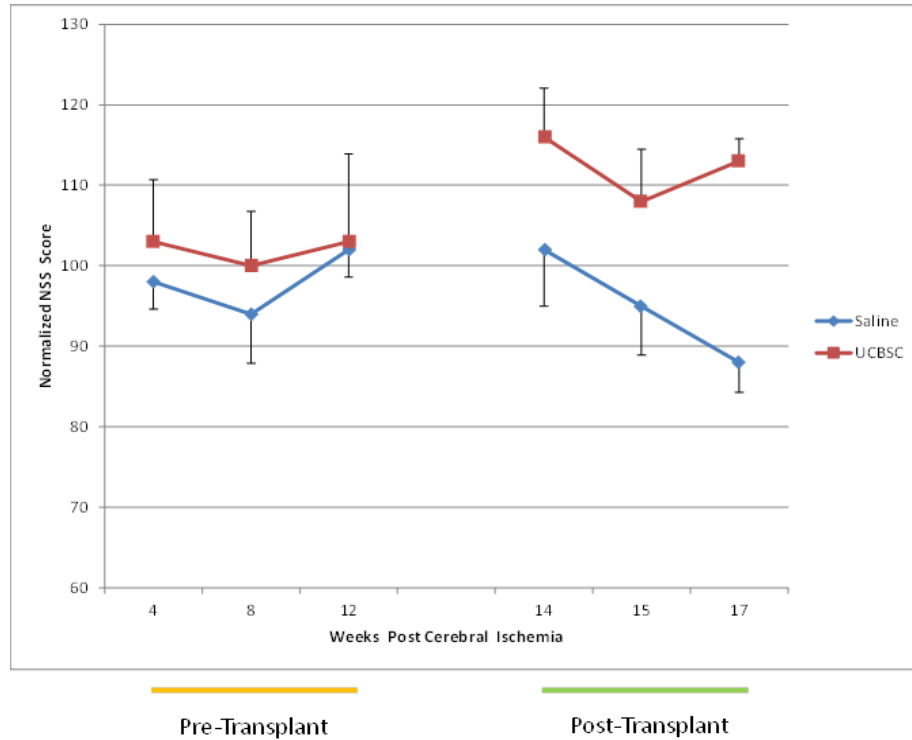


Figure 6: nh-UCBSC treatment improves neurological severity score (NSS) in rats with chronic ischemic brain injury (From C. Nan)

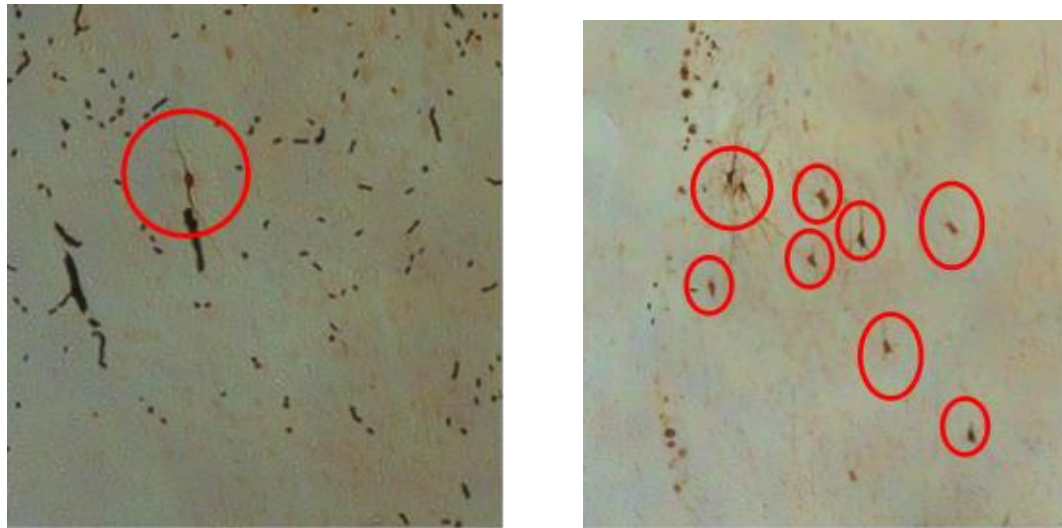


Figure 7: **Intraparenchymal transplants of nh-UCBS enhance cortico-cortical neuronal connectivity in chronic stroke.** Left panel - retrograde labeling of neuron in ischemic cortex following saline injections. Right panel – retrograde labeling of neurons in ischemic cortex following nh-UCBSC transplants. Note increase in the number of labeled neurons in the ischemic cortex following nh-UCBSC transplantation (From C. Nan).

MATERIALS AND METHODS

Isolation of a novel nh- CD34 negative human umbilical cord blood stem cells from the human umbilical cord blood:

Previous studies in the laboratory have shown effective isolation of CD34 negative non-hematopoietic stem cell population from human umbilical cord blood. The Human UCB which was obtained from the Fairview-Riverside University Hospital was processed within 4hrs after harvesting. It was diluted in PBS and supplemented with bovine serum albumin (BSA). The human UCB was then subjected to Ficoll-Hypaque density gradient centrifugation in order to separate the mononuclear cell (MNCs) population. These mononuclear cells were plated in T-25 flasks at a density of about 1 million cells in a flask and were cultured in Dulbecco's modified Eagle medium with Nutrient Mixture F-12, fetal bovine serum, growth factors like bFGF and hEGF. Antibiotics like Penicillin and streptomycin were also added to the culture media. When cells reached 60% confluency, they were split in the ratio 1:3 and were maintained under the same culture conditions.

Animals and MCAO surgery

Small animal models like rats are the most widely used animal models for stroke. The advantage of using rats is that they are relatively inexpensive to purchase and house in comparison to other small animals models ^[50]. The rat models used in the study are an average weight of about 300 grams. The permanent MCAO was performed in the SHR rats which were anesthetized with halothane and the transient MCAO was performed in Adult Sprague Dawley rats anesthetized with ketamine and xylazine intraperitoneally. A 4-0 silicon coated surgical thread was inserted through the internal carotid to the origin of the middle cerebral artery. The thread that was inserted was left in place undisturbed for 1 hour.

The modified Neurological Severity Score (mNSS) was performed 24 hours after MCAO in order to assess the neurological impairment due to the induced stroke.

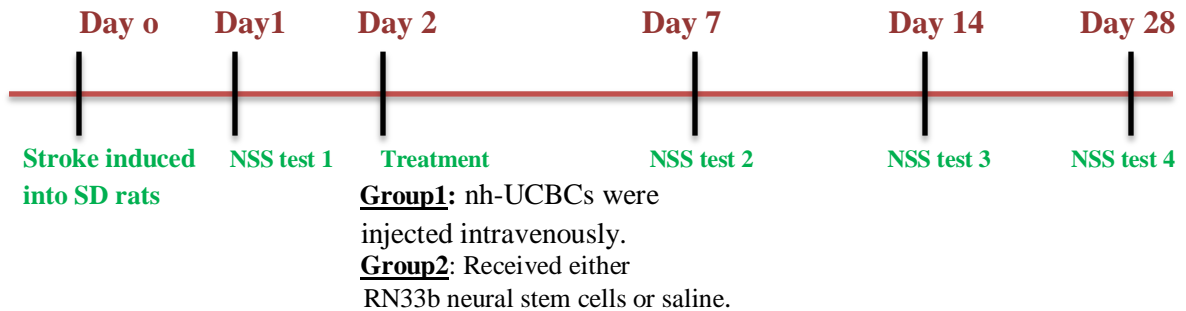
Infusion of nh-UCBCs

After the induction of stroke by MCAO, the behavior of the rats was assessed in accordance to the neurological severity score (NSS). Following this, a cell suspension of human nh-UCBCs with a final concentration of 2×10^6 cell/ml in sterile saline was injected into the saphenous vein. Animals were maintained on daily cyclosporine A administration (10mg/kg).

TYPES OF STROKE MODELS: IN ACCORDANCE TO TIME POINT OF TREATMENT

Acute Stroke Model:

a)



b)

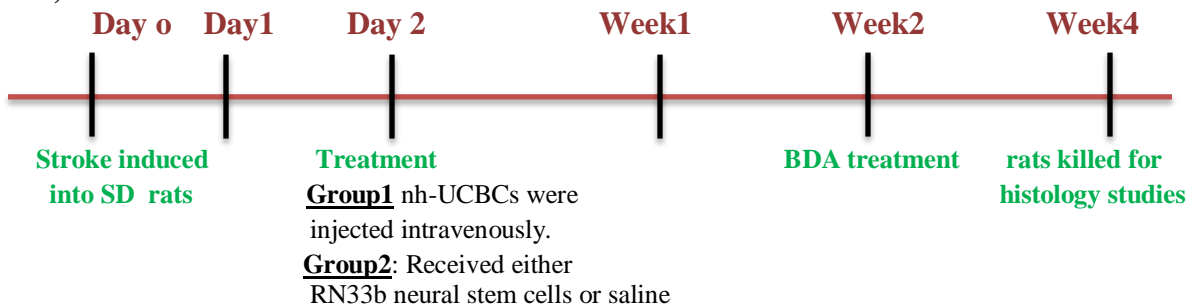


Figure 8: **Timeline for treatment with nh-UCBSc, behavior testing by mNSS and BDA treatment in an acute model for stroke.** A) In an acute model of stroke, the stroke was induced at Day 0 and nh-UCBSc cells were administered at Day 2 post-injury. Behavior testing in acute model of stroke based on modified neurological severity score (mNSS) were done on Day 0 after the induction with stroke and day7, day 14 and day 28 following the treatment with nh-UCBSc. B) BDA was administered 2 weeks post treatment with nh-UCBSc and the rats are sacrificed for histology studies after 2weeks following BDA treatment (week 4 after treatment).

In the acute stroke model, the nh-UCBCs, saline or RN33b neural were administered intravenously 48 hours after the induction of injury by MCAO. In order to study the functional behavior of the animal, functional tests based on modified neurological severity score (mNSS) was conducted on week1, week2 and week 3 after stroke induction. And for histological analysis of the brain tissues after treatment with the nh-UBSCs, the stroke was induced in the animal models at day 2, following which BDA was administered at day 14. These animal models were then sacrificed at day 28 and the brains were sectioned.

Chronic Stroke Model:

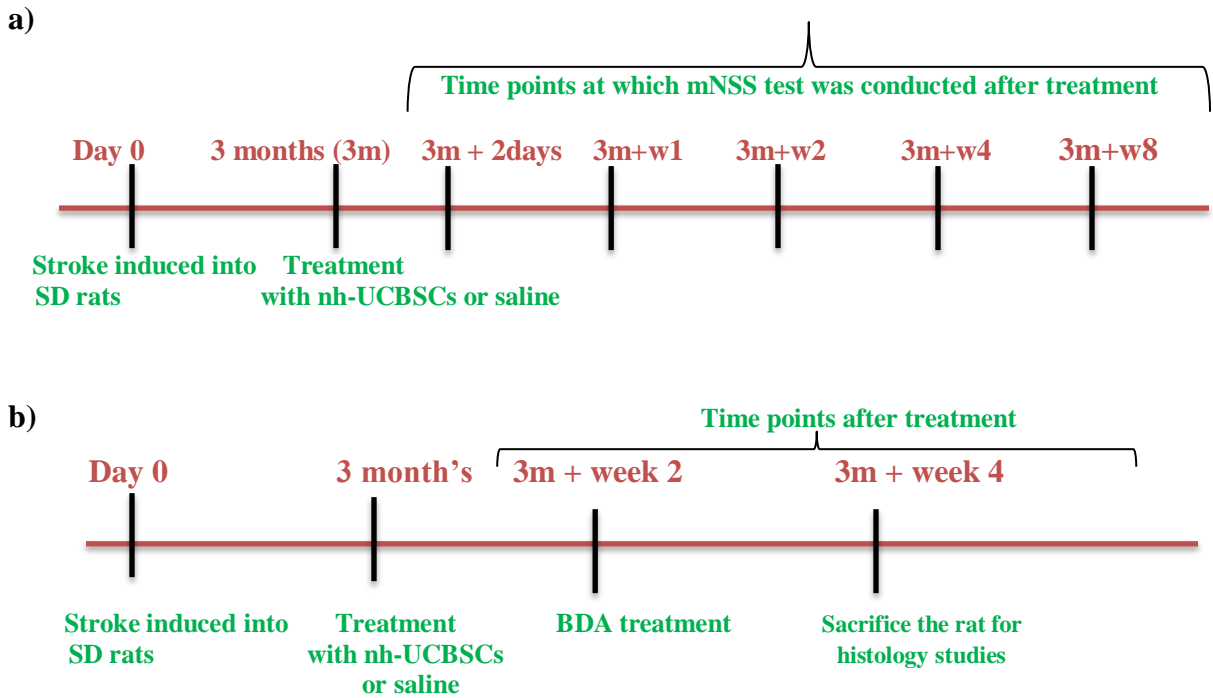


Figure 9: Timeline for treatment with nh-UCBCs, behavior testing by mNSS and BDA treatment in a chronic model for stroke. A) In a chronic model of stroke, the stroke was induced at Day0 and nh-UCBS cells are administered after 3 months post-injury. Behavior testing in a chronic model of stroke based on modified neurological severity score (mNSS) were done on Day 0 after the induction with stroke and Day 2, Day 7, Day 14 and Day 28 following the treatment with nh-UCBCs. B) BDA was administered 2 weeks post treatment with nh-UCBCs (3m+ week2) and the rats were sacrificed for histology studies after 2weeks following BDA treatment (3m+week4).

Group 1: SD rats injected with saline, (n=3)

Group 2: SD rats injected with nh-UBSCs (75000 Cells per point), (n=4)

In the chronic stroke model, the nh-UCBCs or saline was administered into Sprague Dawley or SHR rats at 3 months after the induction of injury by MCAO. The neurological severity score was used in order to track the changes in the behavior of these animal models at day 2, week 1, week 2, week 4 and week 8 Post-treatment. To study the histology of the brain tissue in a chronic model of stroke, BDA was administered 2 weeks after nh-UCBCs treatment. The animals were sacrificed 2 weeks following the BDA administration and the brains were sectioned and preserved.

Cell culture of non-hematopoietic CD34 negative Human umbilical cord blood stem cells (nh-UCBCs):

The non-hematopoietic CD34 negative Human umbilical cord blood stem cells (nh-UCBCs) were cultured on fibronectin coated plastic tissue culture plates (Sarstedt, 100*20mm) at a density of about 100, 00 cells per petriplate. The cells were provided with UCB media which consisted of 60% Dulbecco's modified Eagle medium: F12 (DMEM: F12 [low glucose] (Invitrogen) and 30% MCDB 201 medium (Sigma) supplemented with Fetal bovine serum (Becton/ Dickinson or Invitrogen), 100 unit/ml Primosin (Invitrogen), 100uM L-ascorbic acid-2-PO4 (vitamin c) (Sigma), 20nM Dexamethasone (DXM) (Sigma), 1x insulin transferring selenium (ITS) media supplement (Sigma), 1mg/ml Linoleic Acid/ BSA (Sigma), growth factors like 10ng/ml of Recombinant human epidermal growth factor EGF (Sigma) and 10ng/ml Recombinant human PDGF-BB (R & D systems). The nh-UCBCs were grown until they became 80 – 90 % confluent and formed a uniform adherent monolayer of cells. The media was changed every other day to make sure that the media in which the cells are grown is supplied with enough nutrients required for efficient growth. The nh- UCBCs were passaged when they were about 90% confluent by using TrypLE (Invitrogen) and incubating for 4 minutes at 37 °c. The TrypLE in the cell suspension was diluted by addition of UCB media and this cell suspension was centrifuged at 1000 rpm for 7 minutes. The cell pellet was resuspended in UCB media and cultured on to new fibronectin coated plastic tissue culture plate. The split ratio for every passage is 1:3 i.e., cells harvested from one confluent petriplate was split to 3 petriplates in equal amounts in

order to maintain confluency. When the nh-UCBCs were at passage 15 and were ready to be passaged further, the cells were harvested using TrypLE (Invitrogen) and the supernatant which was obtained after centrifugation of the cell suspension was saved. This supernatant is the nh-UCBS cell derived condition media.

Quantitative Real-Time Polymerase Chain Reaction Analysis:

Total RNA was isolated and purified from the high passage P (15) nh-CD34 negative UCBCs and passage P (4) fibroblast cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. The concentration of the RNA isolated was determined using a spectrophotometer (Beckman DU640). The RNA extracted from the cells is suspended in nuclease free water (Ambiotin). For a wavelength of 260nm (OD) and 1cm path length, the optical density equals 1.0 for a 40µg/ml solution of RNA. Hence, in order to determine the concentration of RNA in the sample, the following calculation was performed as:

$$\text{Total RNA concentration} = 40\mu\text{g/ml} \times A_{260} \text{ reading} \times \text{dilution factor.}$$

The cDNA was extracted from the RNA using Sensiscript Reverse Transcriptase (Qiagen) which is a standard protocol for first strand cDNA synthesis using <50 ng RNA.

Gene expression for the cytokines in the nh-UCBS cells:

- I. Quantitative real time polymerase chain reactions (q RT-PCR) with the CD34 negative nh-UCBS cells were performed using human common cytokines RT2 Profiler PCR array (96-well) format plate which profiles the expression of 84 important cytokine genes. This array includes
 - a. Interferons
 - b. Interleukins
 - c. Bone morphogenic Proteins (bmp)
 - d. Members of the TGF-B family
 - e. Platelet-derived and vascular endothelial growth factors
 - f. Tumor necrosis factor

It is used to analyze the gene expression of a focused panel of cytokines. The following q RT-PCR profile was used: 95°C for 10 min, 40 cycles: 95°C for 30 sec, 59°C

for 15 sec, 72°C for 30 sec, and 92°C for 15 sec, 55°C for 30 sec, 95°C for 30 sec and hold at 4°C.

- I. Quantitative real time polymerase chain reactions (q RT-PCR) with nh-UCBS for specific cytokines of interest. The primers tested were:

Cytokines	Roles- Pro-inflammatory/ Anti-inflammatory cytokines
IL6	Acts as both Pro and Anti-inflammatory cytokines
IL8	Growth-regulated oncogene/cytokine-induced neutrophil chemo-attractant. Major mediators of inflammatory response. Pro –inflammatory cytokine
Angiopoietin 1	Important role in vascular development. Anti-inflammatory cytokine
VEGF	Promotes vasculogenesis and angiogenesis. Anti-inflammatory cytokine
EGF	Promotes growth of epidermal and epithelial tissues. Anti-inflammatory cytokine
BDNF	Required for the survival of cortical neurons in the brain. Anti-inflammatory cytokine.

The primers mentioned above were tagged to a Taq Man probe (Lamba, 2X Taq man mix) and q RT-PCR was performed with the same q RT-PCR profile as before. In order to ensure the specificity of q RT-PCR, melt curve analyses were performed at the end of all PCRs. The gene expression levels were normalized to HPRT housekeeping gene and then analyzed using the $2^{-\Delta\Delta C_t}$ method.

Human Cytokine protein array system

In order to check the protein expression of the different cytokines in the nh- CD34 negative human UCBS cell conditioned media, a human protein cytokine kit (RayBiotech Inc.) was used. It is cytokine protein array system used to screen the expression of 88 human cytokines. The cytokine array membranes were placed in an 8 well tray and 2ml 1X blocking buffer was added to both the membranes and incubated at room temperature for 30 minutes on a shaker. After 30 minutes, the buffer was decanted and 1ml of nh-UCBS cell conditioned media was added to the membrane followed by overnight

incubation. After the incubation time, the sample was removed and washed 3 times with 2ml of wash buffer 1 at room temperature (5minutes per wash). The membranes were then washed with wash buffer II 2 times with 2ml (5 minutes per wash). Primary antibody to be added to the membrane was prepared by diluting the Biotin Conjugated anti-cytokine in blocking buffer. 1ml of the diluted biotin conjugated antibodies was added to each membrane and incubated for 1-2 hours at room temperature. Following the incubation period, the membranes were washed 3X with 2ml of Wash buffer I and 2X with 2ml of Wash buffer II. Secondary Antibody (1000 fold diluted HRP conjugated streptavidin) was prepared by adding 2 μ l HRP to 1998 μ l 1X blocking buffer. 2ml of the secondary antibody was added to each of the membranes and the tray was incubated at 4°C on a shaker to ensure uniform coverage of the antibodies. The membranes were again washed with wash buffer I and Wash Buffer II and was ready for detection. The number of biological replicates for this experiment were n=3.

Detection of the cytokine membrane:

In order to detect the signal produced by the different cytokines present in the sample, 250 μ l of 1X detection buffer C and 250 μ l of detection buffer D was added to each membrane and incubated at room temperature for 2 minutes. Following incubation, the buffer was drained and the membranes were exposed to X-ray film. The signals were detected directly from membrane using the chemi-luminescence imaging system. The time of exposure of the membrane to the X ray film depends on the intensity of the signal in the membrane.

Interpretation of the results:

The levels of the cytokine expression in the nh-UCBs cells were determined by the intensity of immunoreactivity relative to that of the standard controls, following the manufacturer's instructions. The intensities of the signal were quantified using dot blot analyzer built in the Image J software.

Pos	Pos	Pos	Pos	Neg	Neg	EN A- 78	GCS F	GM- CSF	GRO	GRO- α
I-309	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
IL-12 P40P70	IL-13	IL-15	IFN- γ	MCP -1	MCP-2	MC P-3	MCS F	MDC	MIG	MIP-1 β
MIP-1 δ	RANTES	SCF	SDF- 1	TAR C	TGF- β 1	TNF - α	TNF - β	EGF	IGF-1	Angiopoie tin
Oncosta tin M	Thrombopoi etin	VEGF	PDG F-BB	Lepti n	BDNF	BLC	Ck β B-1	Eotax in	Eotaxi n-2	Eotaxin-3
FGF-4	FGF-6	FGF-7	FGF- 9	Flt- 3liga nd	Fractalki ne	GCP -2	GDN F	HGF	IGFB P-1	IGFBP-2
IGFBP- 3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MC P-4	MIF	MIP- 3 α	NAP- 2	NT-3
NT-4	Osteoportin	Osteoproteg erin	Parc	pigf	Tgf- β 2	TGF - β 3	TIM P-1	TIMP -2	Pos	Pos

Table 1: The 88 human cytokines tested by the human cytokine protein array system

Bv2 cell culture:

Bv2 cells were a generous gift from Dr. Ling Li (College of Pharmacy, University of Minnesota). BV2 is an immortalized microglia cell line. The cells were cultured in Dulbecco's modified Eagle medium: F12 (DMEM: F12 [low glucose] (Invitrogen) with 10% heat- inactivated fetal bovine serum (FBS), 100 unit/ml primosin (Invitrogen) and streptomycin (10 μ g/mL) at 37°C. Once the cells reached 80% to 90% confluency, they were passaged in the ratio of 1:3. The cells were plated at a density of 100, 000 cells per petriplate after passaging. The cells were cultured in Bv2 media for about 5-6 hours to ensure that the cells in culture adhered to the surface of the dish and reached 40% confluency. After most of the cells were observed to have attached to the surface of the

tissue culture plate, the existing media was removed and replaced with fresh media supplemented with Lipopolysaccharide [LPS] (1:10 dilution) in order to stimulate the activation of the microglial cells. The cells were stimulated with LPS in order to recapitulate the situation of the immune cells that get activated in response to the ischemia in-vivo. These cells were then incubated for 12 hours at 37° C. Following the incubation period, the media was removed and the cells in the 3 petriplates were treated in 3 different condition media as mentioned in table (2) and were incubated again for 12 hours at 37 ° C.

Petriplate Number	Condition media	Amount added (ml)	Sample/Control
1	nh-UCBS cell conditioned media	10	Sample
2	UCB media (unconditioned media)	10	Sample
4	BV2 media	10	Control

Table 2: Culture conditions for the BV2 microglial cells following LPS stimulation

Quantitative Real-Time Polymerase Chain Reaction Analysis: Gene expression of the M1 and M2 microglial phenotype markers

The cells cultured in the three different culture conditions were harvested using TrypLE (Invitrogen). In order to stop the action of the TrypLE, the saved existing condition media was added. The cell suspensions were centrifuged at 1000 rpm for 7minutes. The total RNA from the cells cultured in the different culture conditions were extracted and purified individually using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s recommendations.

The cDNA was extracted from the RNA using Sensiscript Reverse Transcriptase(Qiagen) which is a standard protocol for first strand cDNA synthesis using <50 ng RNA.

	Microglial markers for qRT-PCR
General microglial Marker	MHC class II
M1 Phenotype marker	iNOS, CD86
M2 Phenotype marker	CD206, Arg-1, CD-163
Housekeeping genes	HPRT, β -Actinin

Table 3: Different M1 and M2 microglial phenotype markers that were checked for their expression in the BV2 cells cultured in the three different condition medias.

SYBR Green quantitative real time polymerase chain reactions (qRT-PCR) were performed in duplicates for each primer set using Eppendorf Realplex qPCR machine. To ensure the specificity of PCR, melt curve analyses were performed at the end of all PCRs. Gene expression levels were normalized to HPRT housekeeping gene and then analyzed using the $2^{-\Delta\Delta Ct}$ method. The number of biological replicates for this experiment were $n = 3$.

Tissue Culture Studies

Cell culture of mouse Neuronal stem cells (mNSCs)

mNSCs of passage P(3) were cultured in a 6 well Ultra-Low attachment plate (Sigma Aldrich Inc.) covering 2 wells at a density of 0.5×10^6 cells per well in $10 \times$ DMEM/F12 [Dulbecco's modified Eagle medium: F12] (Invitrogen) with 10% L- Glutamine. These mNSCs were maintained in an undifferentiated state by culturing them as free floating 3D clusters called neurospheres. The media in which the mNSCs were cultured was optimized with growth factors like EGF and FGF. After the formation of neurospheres was visible, the neurospheres were broken down to individual cells by constant pipetting. This is essential as it would facilitate in counting the number of cells in culture. The mNSCs obtained were plated on to a 4 well chamber slide which was pre-coated with Poly-L-ornithine for 12 hours and further coated with laminin overnight. Approximately 10,000 cells were plated on to each well of the 4 well-chamber slide and were exposed to different culture conditions (Table 4) to induce differentiation.

UCB SUP 1	UCB MED 2	FIB SUP 3	NSC MED 4
-----------------	-----------------	-----------------	-----------------

Table 4: Showing the 4 well chamber slide with the mNSCs in each chamber cultured in the following conditions.

Well 1 of chamber slide: nh-UCBS cell conditioned medium

Well 2 of chamber slide: UCB media (unconditioned media)

Well 3 of chamber slide: Fibroblast cell conditioned medium

Well 4 of chamber slide: NSC media

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The mNSCs plated which adhered to the surface of the 4 well-chamber slide were fixed by removing the different media from the wells and adding 700µl of 4% Paraformaldehyde (PFA) (Protocol) per well followed by 5-10 minutes of incubation at room temperature. After 10 minutes, the PFA was removed and the well was washed twice with PBS. Blocking buffer was prepared by adding 5% Donkey Serum (Jackson Immuno Research Laboratories) to PBS and filtering. Permeabilizing buffer [PBS-T] was prepared by adding 0.1% Tween-20 to PBS. The PBS was removed from the cells and 700µl of permeabilizing buffer was added to each well and incubated for 10 minutes at room temperature. After the incubation period, the permeabilizing buffer was removed and was blocked with blocking buffer (PBS + 5% Donkey serum) for 30 minutes. The primary antibody and secondary antibody treatments were performed according to table (5). The cells were also stained with DAPI by diluting DAPI 1:1000 in PBS.

S.No.	Primary Antibody	Company	Dilution	Secondary Antibody	Company	Dilution
1	Rabbit Anti-MAP2	Sigma	1:1000	Alexa fluoro 555 Donkey anti-rabbit IgG	Invitrogen	1:500

Table 5: Antibodies used for immunostaining and their dilutions

Neurological Severity Score

In order to determine the improvement in the behavior of the animal model affected with stroke before and after injection of the nh-CD34negative Human umbilical cord blood stem cells, the most common neurological scales known as the modified neurological severity scores (mNSS) was used. The mNSS includes many tests to check for the function of motor (muscle status and abnormal movement), sensory (Tactile, visual and proprioceptive), reflex and balance tests. In our study we used behavior tests like limb placement test, stepping test, body swing test and adhesive removal (Table 6).

Behavioral test	Function
Forelimb Placement	Assesses forelimb function and placing deficits
Stepping test	Assesses sensorimotor function, motor coordination and placing deficits during locomotion
Body swing test	Assess asymmetrical motor dysfunctions.
Adhesive removal test	Assesses tactile responses and asymmetries

Table 6: The different behavioral tests performed in the acute/ chronic stroke model based on the modified neurological severity score (mNSS).

Statistics

Statistical analyses of all the data obtained were performed by the Student t-test for comparison between two samples and to determine the statistical significance between the two groups. ANOVA one way significance test was used when there were more than two samples to determine statistical significance. Bolferoni-HOLM test was used to find the particularly significant sample, following the ANOVA test. The standard error bars in all the graphs represents the standard error means (SEM).

Results

Cell culture of CD34 negative nh-UCBCs

CD34 negative non-hematopoietic umbilical cord blood stem cells (nh-UCBCs) were grown in accordance to the procedure described in detail in the methods section of the thesis. The nh-UCBSCs have a characteristic flat, fibroblast like morphology as represented in figure 10. The cells in figure 10 were at passage 18; which is considered as high passage nh-UCBS cells.

No occurrences of teratomas were observed in the animal models after treatment with this specific nh-UCBS cell line isolated in the Department of Neurosurgery.

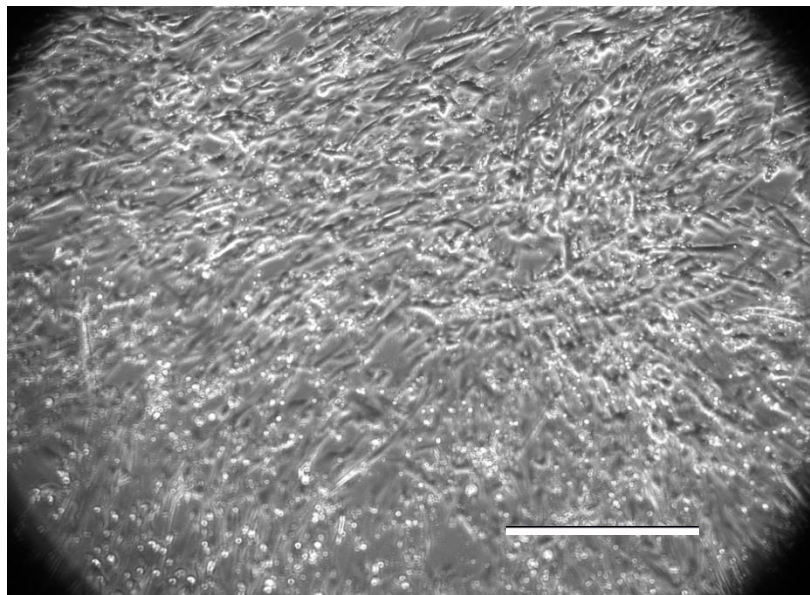


Figure 10: **Cell culture of nh-UCBCs.** The non-hematopoietic human umbilical cord blood stem cells in culture at passage P (18). 10X light microscopic image (200 μ m)

Gene expression analysis for the pro-inflammatory and anti-inflammatory cytokines present in the nh-UCBS cells.

Previous study in the lab demonstrated that injecting CD34 negative nh-UCBS cells into an acute model of stroke induced by MCAO, caused 50 % reduction in the lesion volume which suggests that the nh-UCBS cells that are being injected are modulating the immune

response in the animals with ischemic brain injury. Therefore, we examined the profile of inflammatory genes (pro and anti-inflammatory) that are present in these cells using Profiler PCR array initially followed by qRT-PCR analysis of specific cytokines.

I. Profile PCR array analysis

The use of an array enables us to test 84 different cytokines in one experiment. The expression pattern of the cytokines is summarized below.

Interleukins: The expression of anti-inflammatory cytokines like IL-10, IL-4, IL-13 and IL-8 were observed in the high passage nh-UCBS cells. The cytokine IL-6 acts both as a pro-inflammatory cytokine as well as an anti-inflammatory cytokine depending on the stimuli it is exposed to and the gene expression of IL-6 was low in the nh-UCBS cells. (Figure 11A)

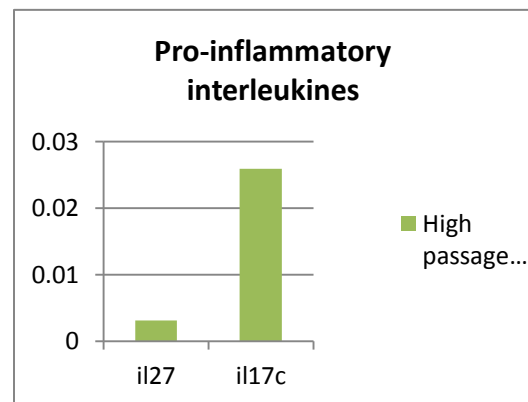
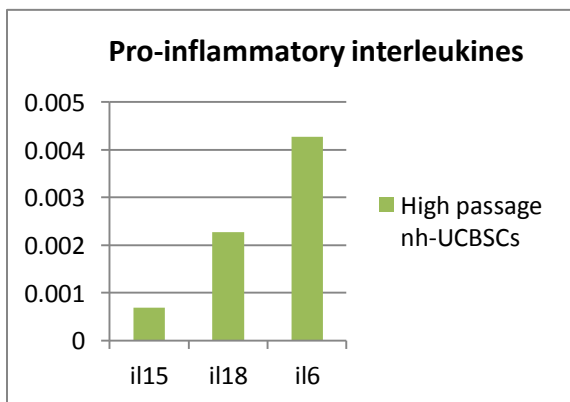
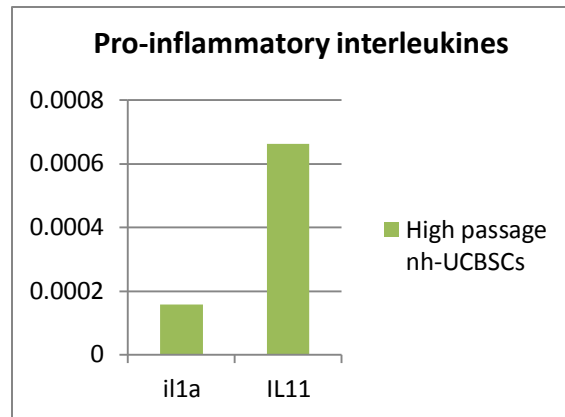
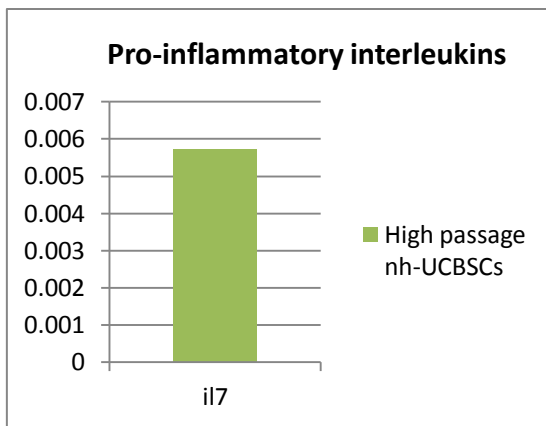
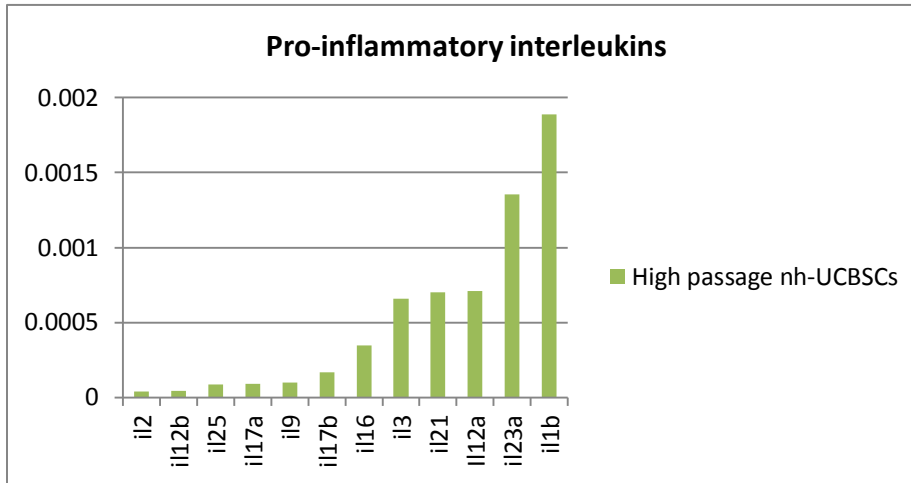
Growth factors: The gene expression of the growth factors like BMPs, CSF, FIGF, TNFSF, VEGFA, GDF and PDGF were seen in the nh-UCBS cells. It is unclear if their expression level is statistically significant due to the limitations in the experimental set-up which allows samples to be tested only once. The expression of anti-inflammatory growth factors like ANGPT1 and VEGFA responsible for contributing to the brain repair process as observed by other research groups was seen to be expressed by the nh-UCBS cells. (figure 11B).

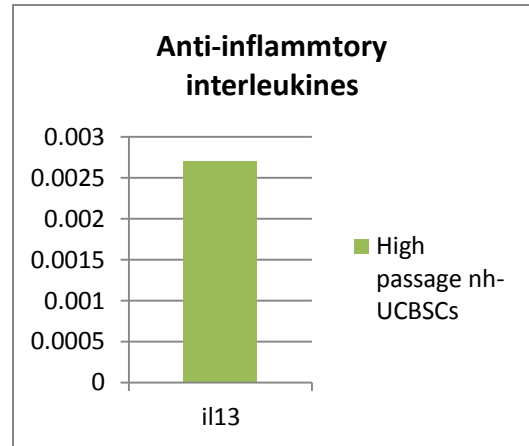
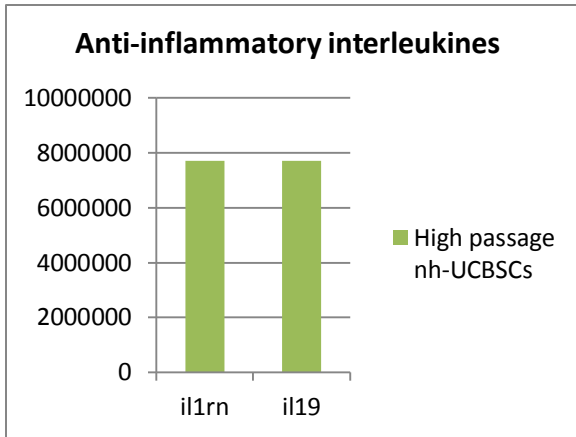
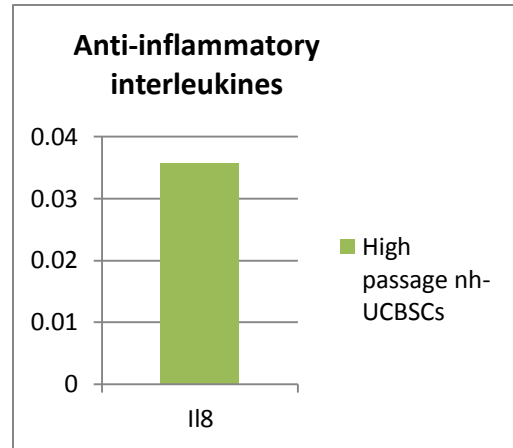
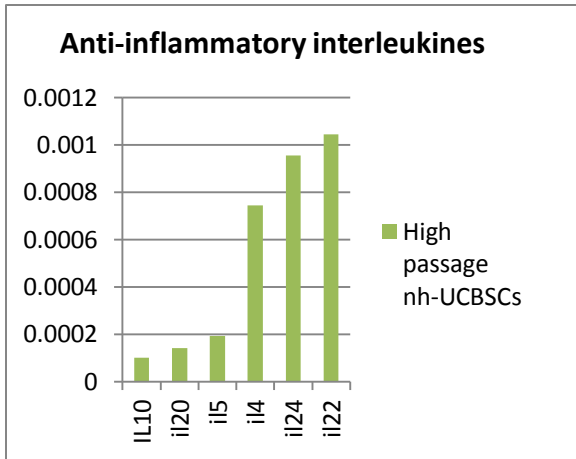
Other chemokines: In the gene expression array, other chemokines like SPP1, THPO and FASLG were tested. Of note is the expression level of secreted phosphoprotein1 (SPP1). SPP1 is a cytokine that upregulates the expression of the IFN- γ and IL-12 and is considered to be a pro-inflammatory cytokine. In the result obtained from this quantitative profile PCR array, SPP1 is not expressed in nh-UCBS cells (figure 11C).

II. qRT-PCR analysis of selected cytokines

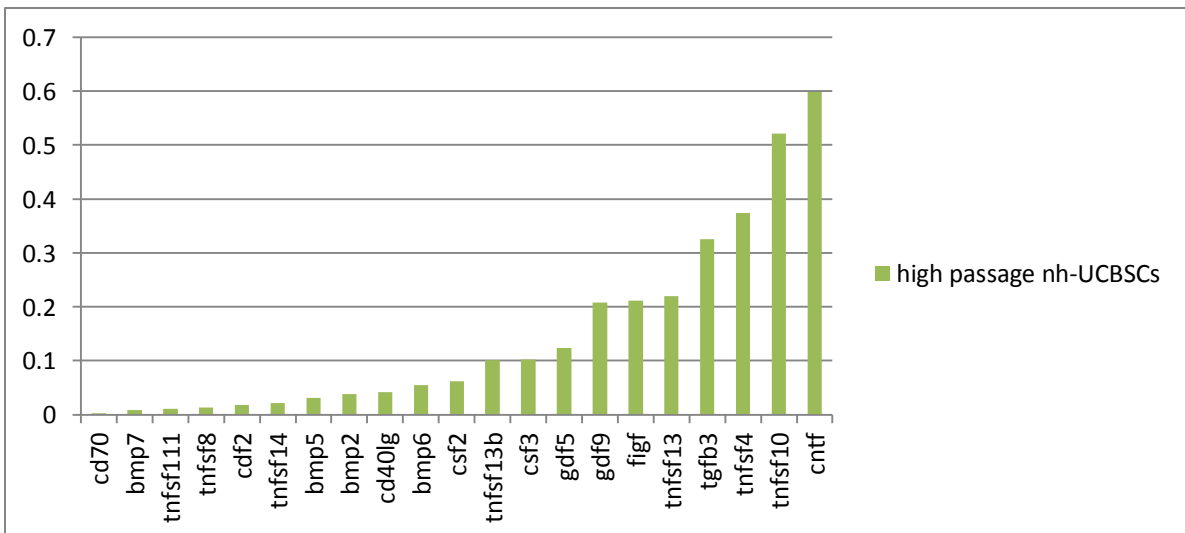
The gene expression of selected cytokines like ANGPT1, IL-8 and VEGFA was analyzed using qRT-PCR in the nh-UCBS cells and fibroblast cells. These cytokines are said to play an important role in vasculogenesis and angiogenesis. The high expression levels of the cytokines were seen in the nh-UCBS cells (figure 11D).

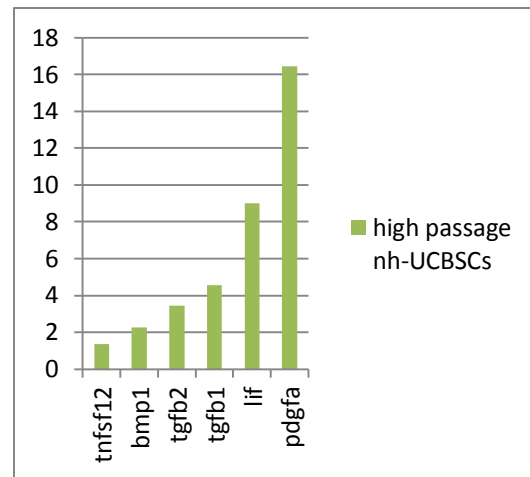
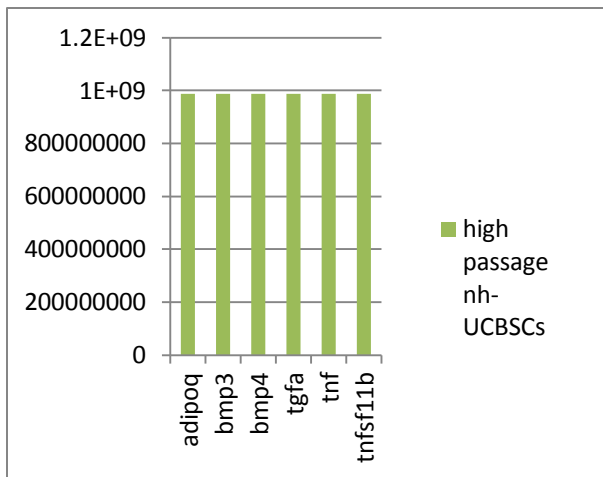
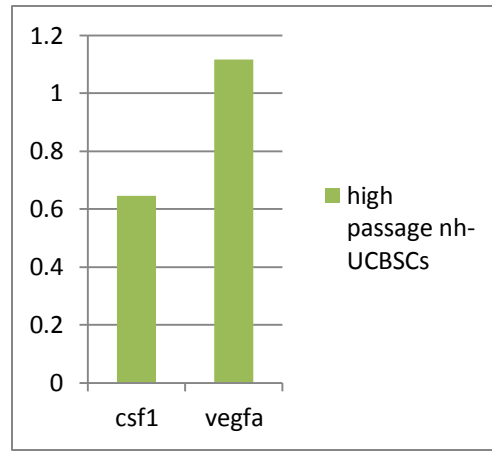
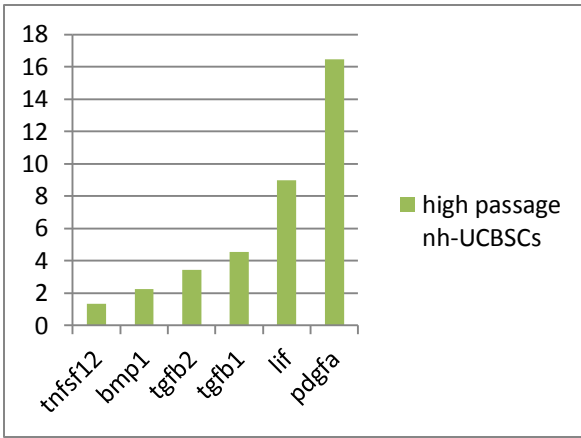
A) Interleukins:



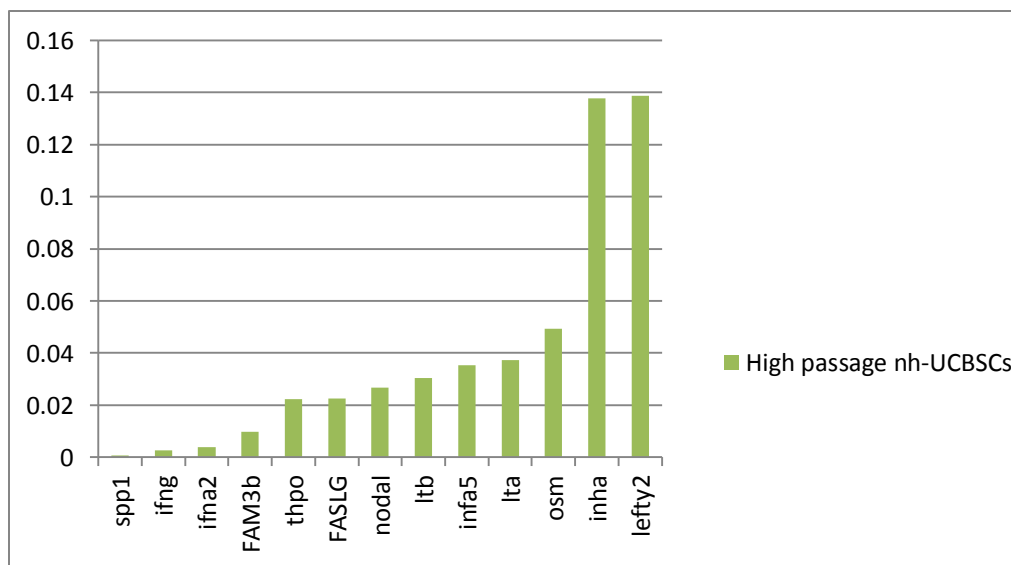


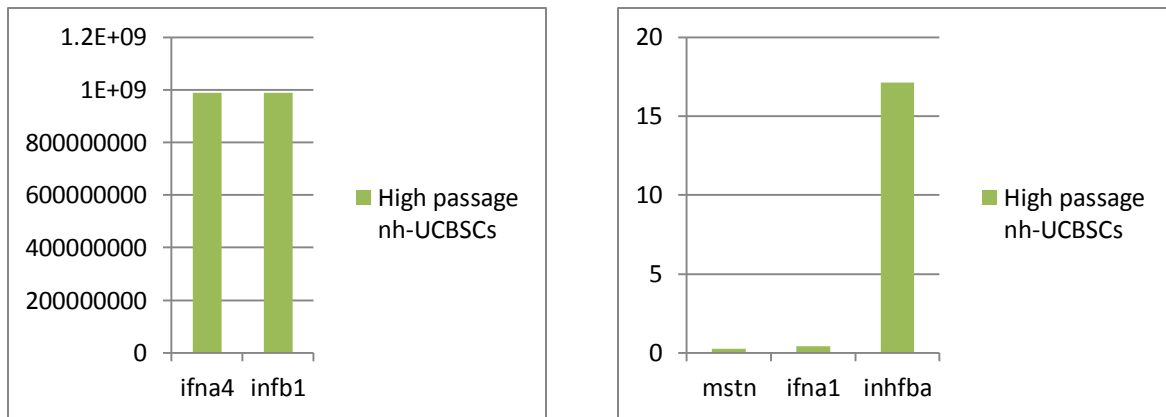
B) Growth Factors





C) Chemokines:





D) Gene expression for selected cytokines by qRT-PCR:

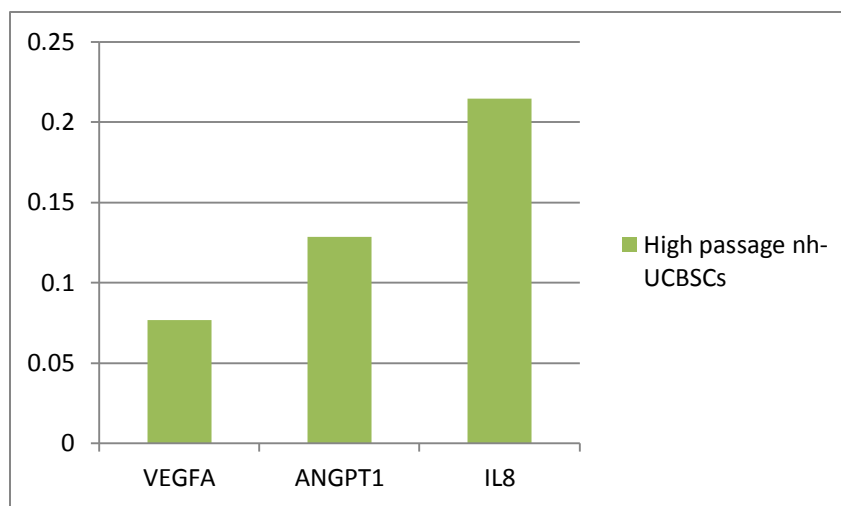


Figure11: (A-C) **Quantitative expression of the pro-inflammatory and anti-inflammatory cytokines in the nh-UCBCs (Green) by using the human common cytokines RT2 Profiler PCR array.** a) The quantitative gene expression of various Pro and anti-inflammatory interleukins in the nh-UCBCs. b) The quantitative gene expression of growth factors in nh-UCBCs c) The quantitative gene expression of chemokines in nh-UCBCs.

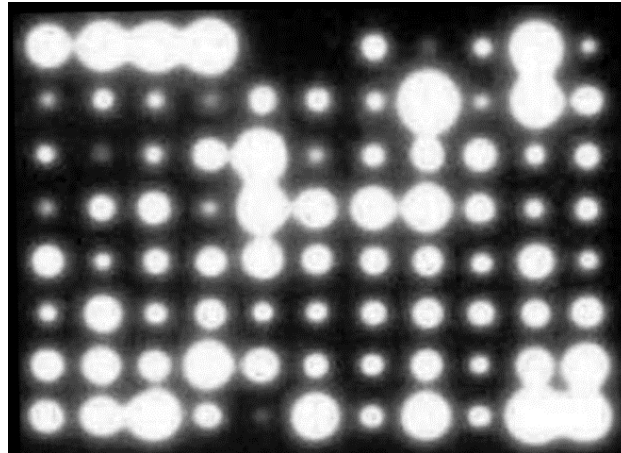
Figure (D): The quantitative gene expression of a selective pro-inflammatory and anti-inflammatory cytokines like ANGPT1, IL-8 and VEGFA in the nh-UCBCs (Green) by q RT-PCR

Protein expression for the Pro-inflammatory and Anti-inflammatory cytokines present in the nh-UCBS cell conditioned media and Fibroblast cell conditioned media.

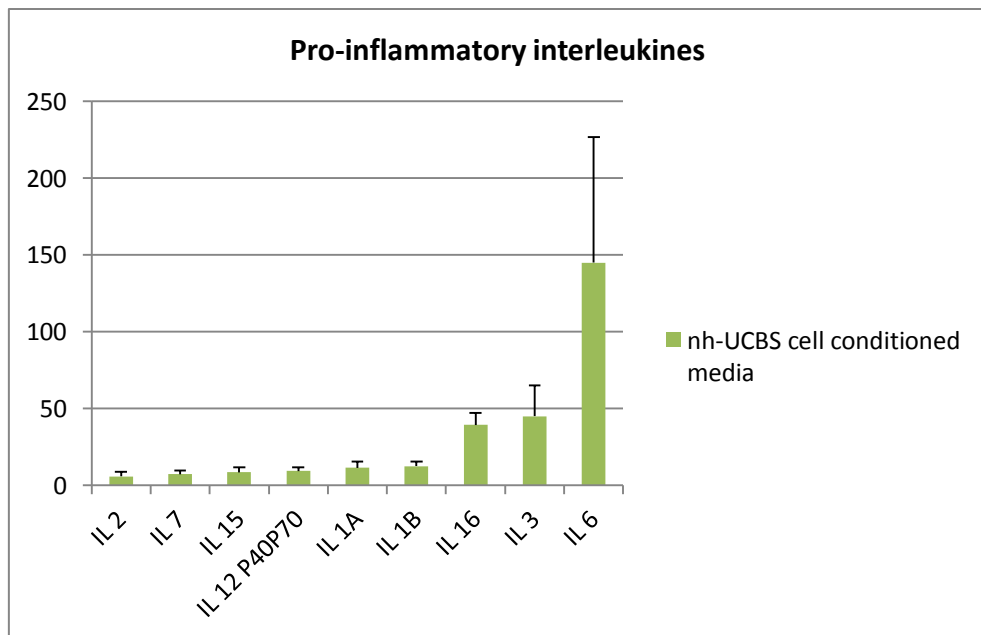
There is increasing evidence that the therapeutic effect of the mesenchymal stem cells (MSCs) isolated from the human umbilical cord blood (human UCB) helps in ameliorating the behavioral deficits induced in a stroke model due to its ability to produce cytokines and other factors that are responsible for the regulation of inflammation, angiogenesis, and also promotion of cellular growth [49]. In order to determine if the nh-UCBCs also secrete such factors that could influence the tissue environment and aid in tissue repair without directly contributing through cell engraftment or differentiation into other cell types, we checked the protein expression of the various cytokines (Interleukins, growth factors, chemokine's and MMP-inhibitors) present in the nh-UCBS cell conditioned media. We used a human cytokine protein array to test the composition of the media conditioned with nh-UCBCs. Cytokine arrays were used to determine the expression levels of 88 different cytokines (table 1) in the sample (Figure 12A). The cytokine analysis using this protein array system was done as biological replicates (n=3). In the membrane each dot represents a particular cytokine as mentioned in the materials and methods section of this thesis. The intensity of each cytokine was analyzed using dot blot analysis in image J software. The intensity of the cytokine was calculated with reference to the average intensity of the positive controls for each membrane. The negative controls are of 0% intensity in all of the membranes. The cytokine expression levels between the nh-UCBS cell conditioned media were plotted as the mean values of the intensities from all three biological replicate derived membranes. The cytokines were grouped in accordance to their sub-type. Here Figure 12b, c, d, and e represents the expression of interleukins, growth factors, chemokine's and MMP-inhibitors respectively in the sample tested. Increased expression of the growth factors like VEGF and MCSF. ANGPT1 is known to contribute to angiogenesis was observed in the nh-UCBS cell derived conditioned media. Moreover, protein expression of the anti-inflammatory cytokines like IL-4 and IL-10 was also seen in the sample tested. The protein expression of osteopontin cytokine was also high in the nh-UCBS cell condition media. Osteopontin

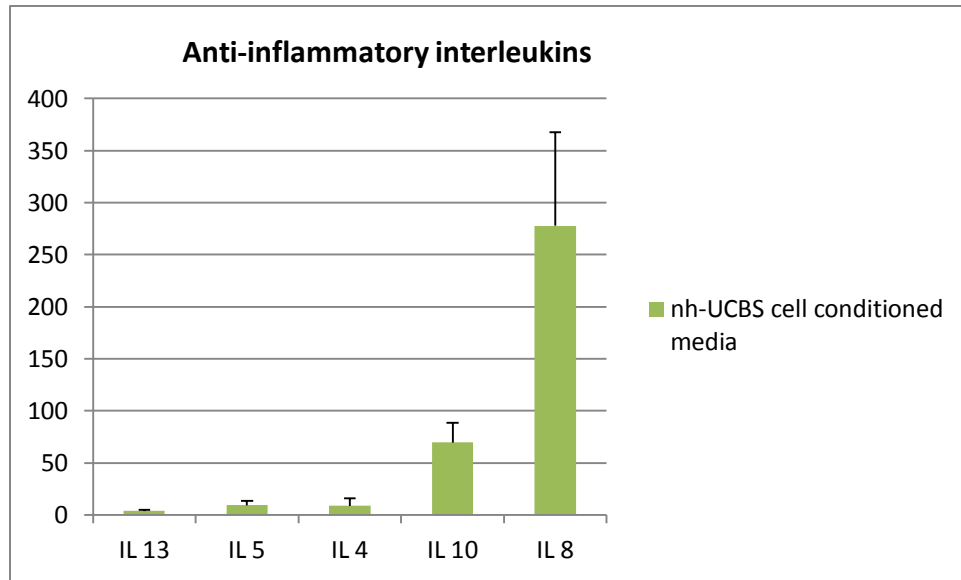
has chemotactic properties which can contribute to cell recruitment to inflammatory sites. It functions as a adhesion protein involved in cell attachment and wound healing.

A) nh-UCBS cell conditioned media incubated membrane

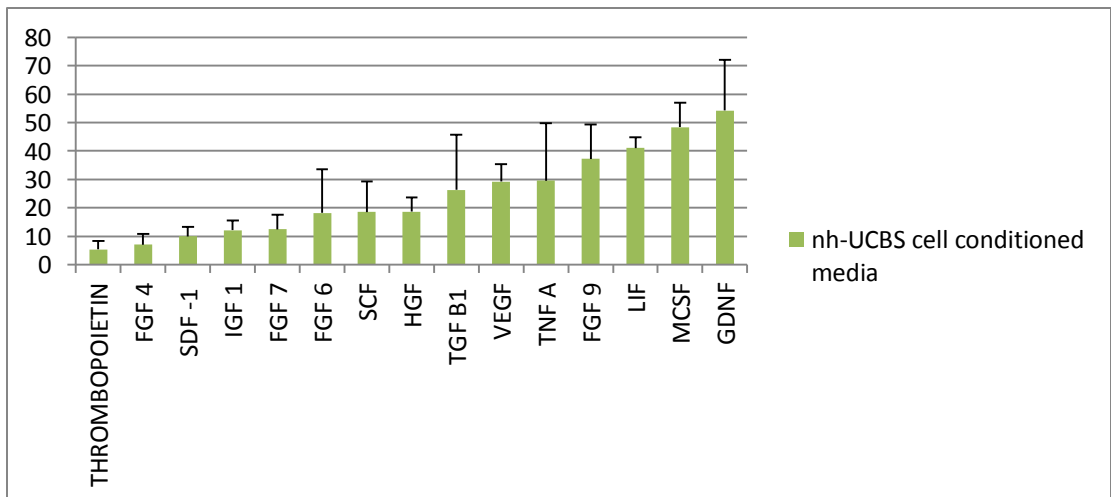
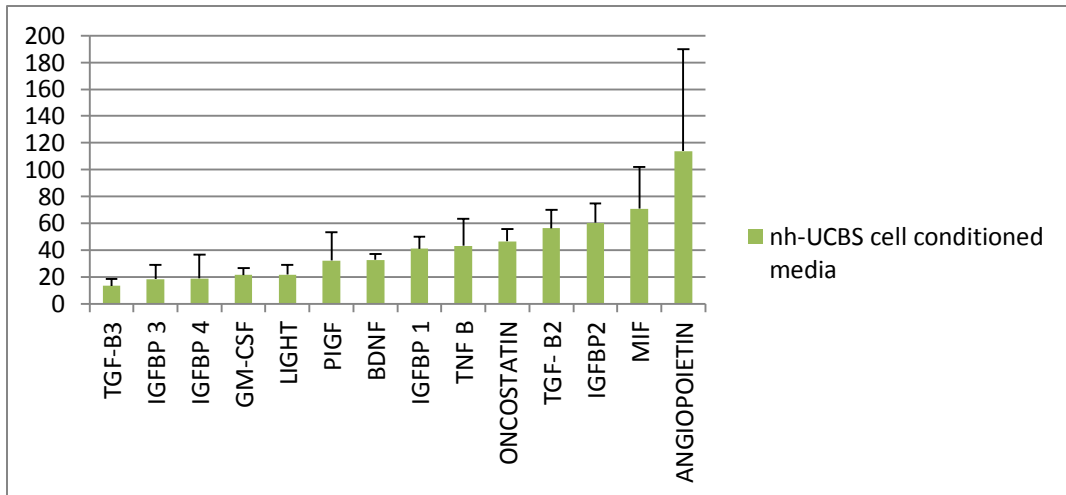


B) Interleukins

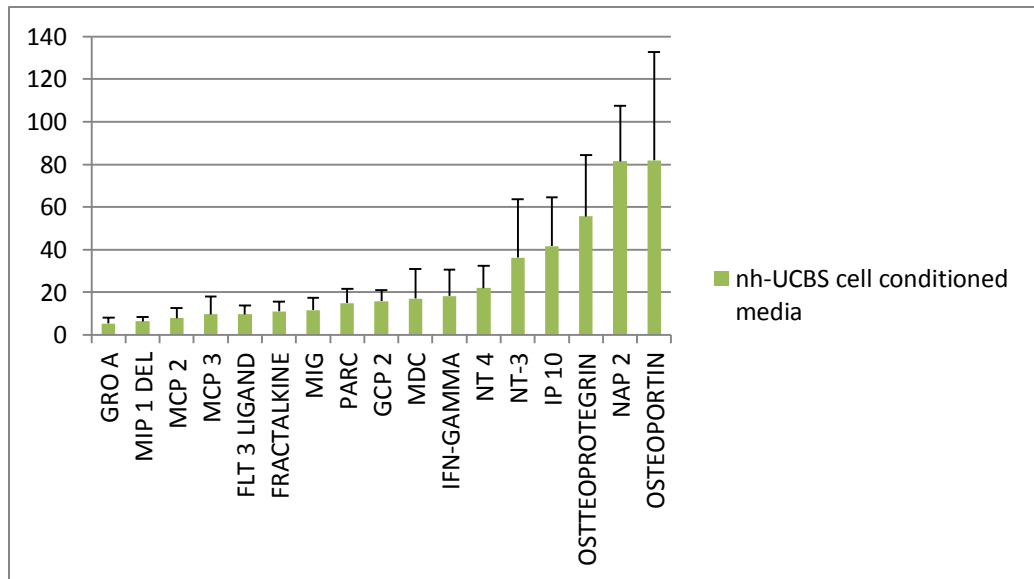
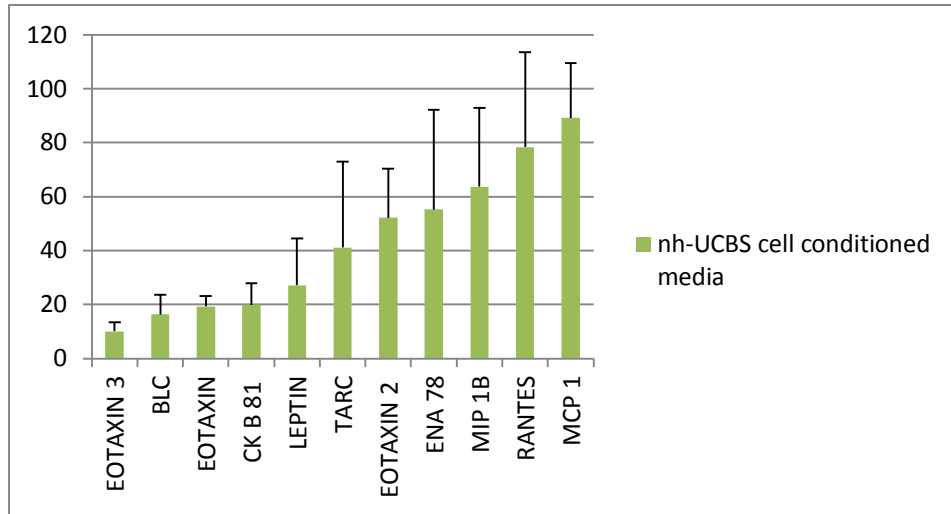




C) Growth factors:



D) Chemokines:



E) MMP inhibitors

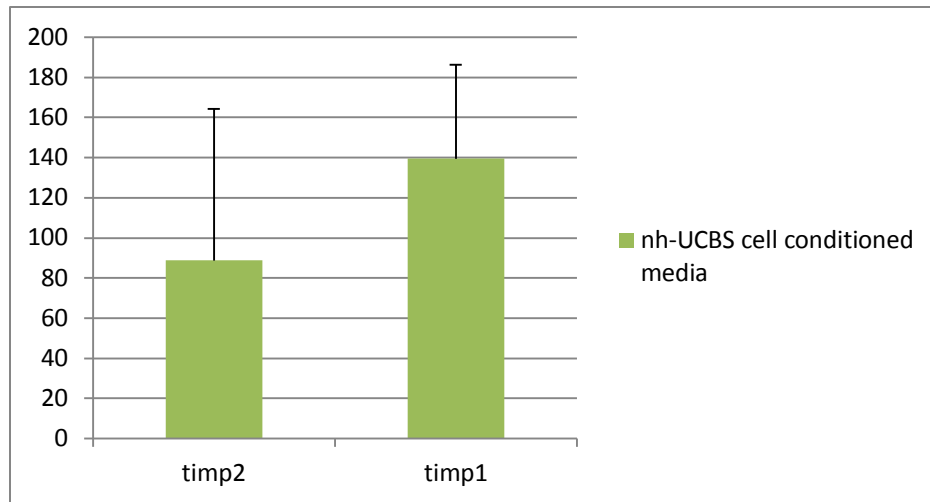


Figure 12: **Protein expression of the pro-inflammatory and anti-inflammatory cytokines present in nh-UCBS cell conditioned media (Green) by Human Cytokine protein array system.**

(A) Represents the cytokine membrane incubated with nh-UCBS cell conditioned media. The brighter the dot in the membrane, stronger is the intensity of the signal produced by the particular cytokine.

(B) Protein expression of the pro-inflammatory and anti-inflammatory interleukins present in the nh-UCBS cell conditioned media.

(C) Protein expression of the pro-inflammatory and anti-inflammatory growth factors present in the nh-UCBS cell conditioned media.

(D) Protein expression of the pro-inflammatory and anti-inflammatory chemokine's present in the nh-UCBS cell conditioned media.

(E) Protein expression of the MMP- inhibitors present in the nh-UCBS cell conditioned media.

M1 and M2 microglial phenotype

In order to examine if the nh-UCBS cells exert tissue protective benefits, by reducing the inflammatory microglial behavior, we co-cultured the BV2 cell (microglial cells) in nh-UCBS cell conditioned media. BV2 cells co-cultured in UCB media and BV2 media were used as controls in this experiment. There are two distinct phenotypes of microglial cells, viz. M1 phenotype (classical, induced by LPS/ IFN- γ) and M2 phenotype (alternative, induced by IL-4 or IL-13) ^[54]. The BV2 cells acquire classical activation pattern (M1) when activated with LPS. M1 phenotype microglia is pro-inflammatory hence promotes inflammatory response in-vitro when the BV2 cells are stimulated with LPS. The M2 phenotype microglia is anti-inflammatory and is responsible for secreting cytokines that help in wound healing and tissue repair after injury ^[53]. Hence in order to examine if the nh-UCBS cell conditioned media helps in modulating the immune response by the activation of the M2 phenotype, the BV2 cells in culture following the stimulation by LPS was treated with nh-UCBS cell conditioned media. The expression of M1 markers (iNOS, CD-86) and M2 markers (ARG-1 and CD-206) was analyzed in the cells obtained after the co-culturing of the BV2 cells in the three different culture conditions (figure 13). It was observed that Arg-1 which is a M2 phenotype marker was expressed higher in the BV2 cells co-cultured with nh-UCBS cell conditioned media when compared to the BV2 cells cultured in BV2 media (figure 13B). But the expression of Arg-1 in the BV2 cells cultured in the UCB media (unconditioned media) was similar to its expression in the cells cultured with nh-UCBS cell conditioned media (figure 13B). The expression of iNOS, a M1 phenotype marker was expressed significantly higher in the BV2 cells cultured in nh-UCBS cell condition media compared to the cells cultured in BV2 media (figure 13A), which is contrary to our hypothesis.

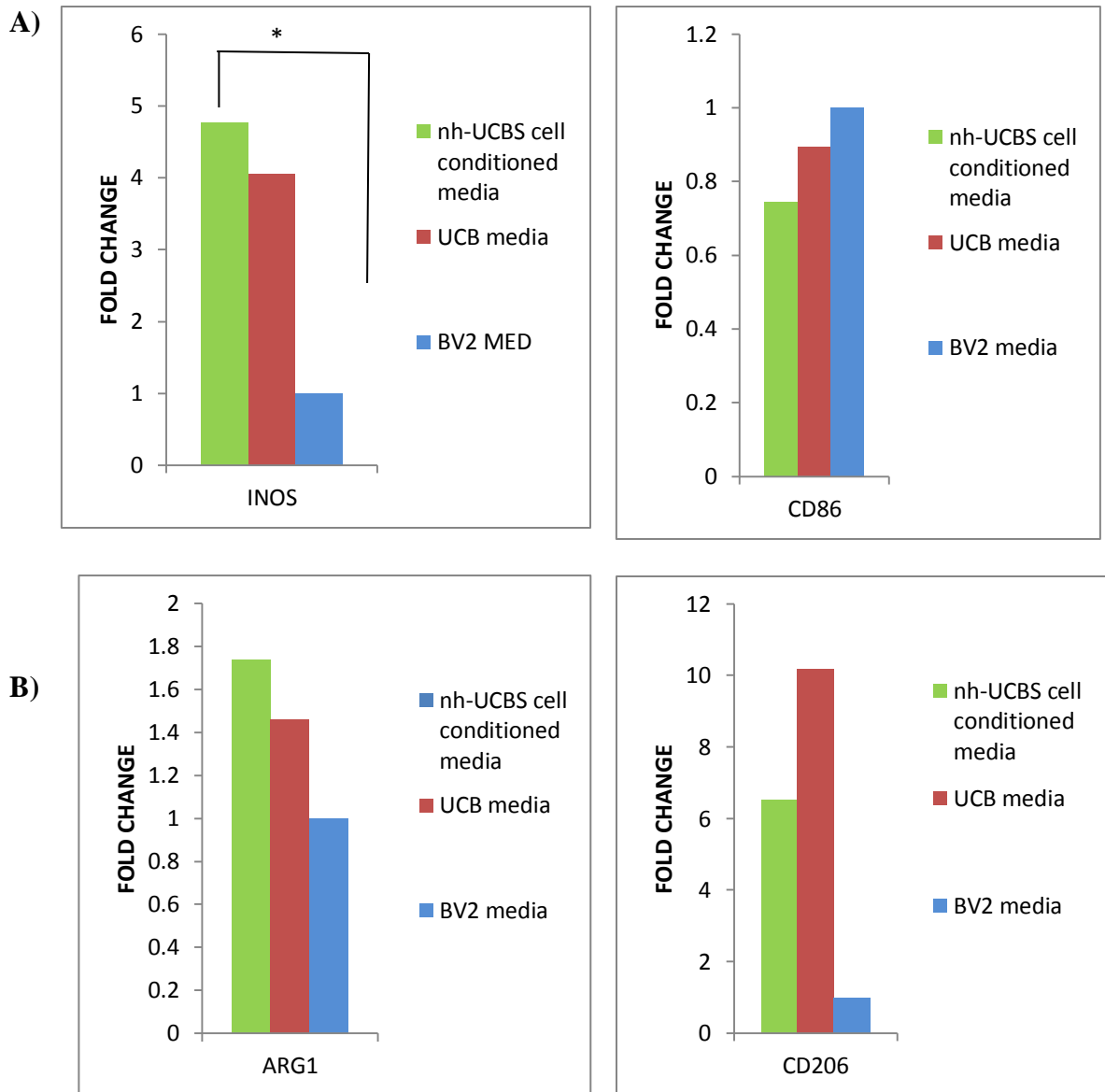


Figure 13: **Quantitative assessment of M1 (iNOS and CD-86) and M2 (Arg-1 and CD-206) microglial phenotype marker expression in the BV2 cells cultured in 3 different culture conditions: nh-UCBS cell conditioned media (Green), UCB media (unconditioned media) (Red) and BV2 media (Blue) by qRT-PCR**

A) The quantitative expression of the M1 markers: iNOS and CD-86 in the cells cultured in the 3 different culture conditions by qRT-PCR.

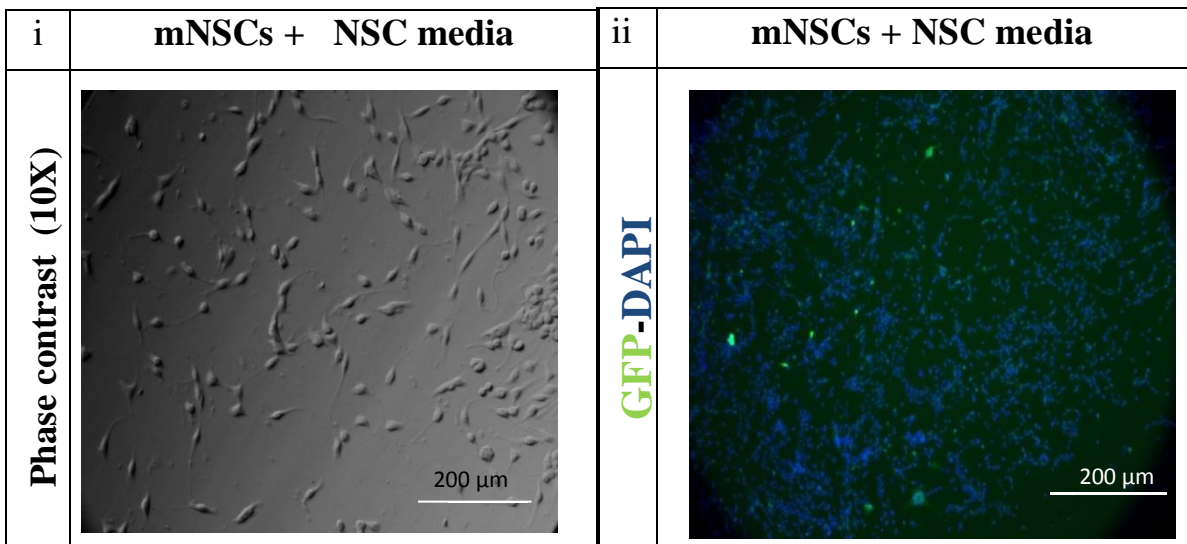
B) The quantitative expression of the M2 marker: Arg1 and CD-206 in the cells cultured in 3 different culture conditions by qRT-PCR.

[Number of biological replicates=3. Asterisks denote statistical significance (atleast $P < 0.05$; ANNOVA one way significance test and Bolferoni HOLM test)]

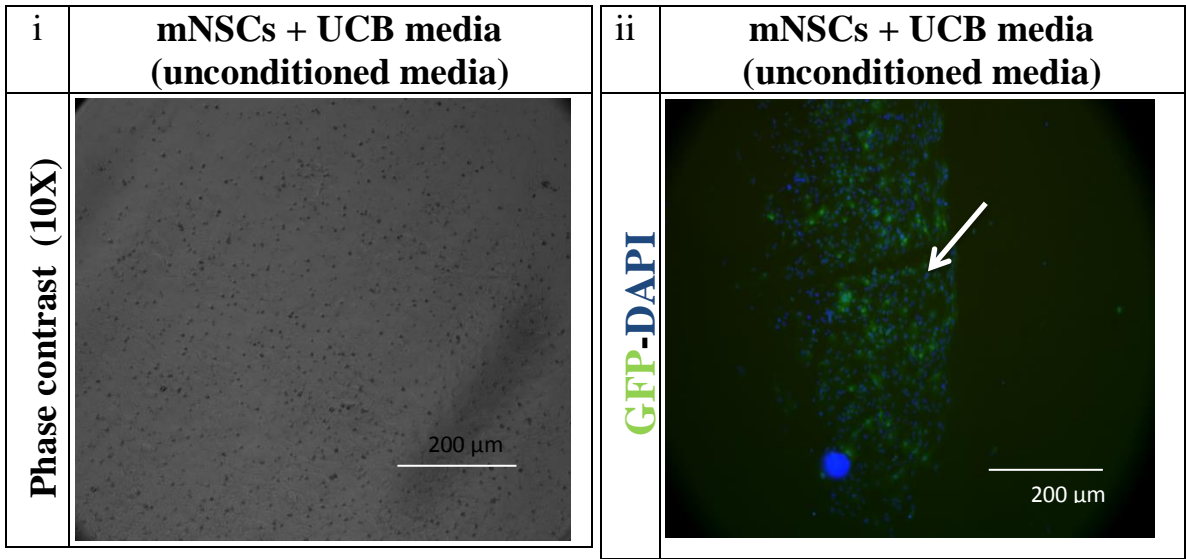
Trophic effects of nh-UCBSC on neural stem cells

In this experiment, we examined whether the neural stem cells grown in nh-UCBS cell conditioned media has the ability to differentiate into neurons with longer dendrites in comparison to the mNSCs grown in other culture conditions like UCB media (unconditioned media), fibroblast cell conditioned media and neural stem cell (NSC) media. It was observed that the mNSCs cultured in nh-UCBS cell conditioned media formed a greater number of colonies of neural stem cells, in comparison to the mNSCs cultured in the other condition media (data not shown). Moreover the size of each colony was bigger when mNSCs were cultured in nh-UCBS cell conditioned media (figure 14D). This indicates that the nh-UCBS cells are secreting certain growth factors that are helping in this robust proliferation of neuronal stem cells. The number of colonies formed by the mNSCs cultured in fibroblast condition media was greater than the number of colonies formed in UCB media, but less than the number of colonies formed in nh-UCBS cell conditioned media. Though it was not possible to visualize the increase in the length of the dendrites when the NSCs were cultured in the nh-UCBS cell conditioned media, the significant increase in the number of colonies and increased size of each colony when the mNSCs were cultured in nh-UCBS cell conditioned media compared to other culture conditions, shows that these nh-UCBS cells are responsible for secreting certain factors that contribute to neuroplasticity

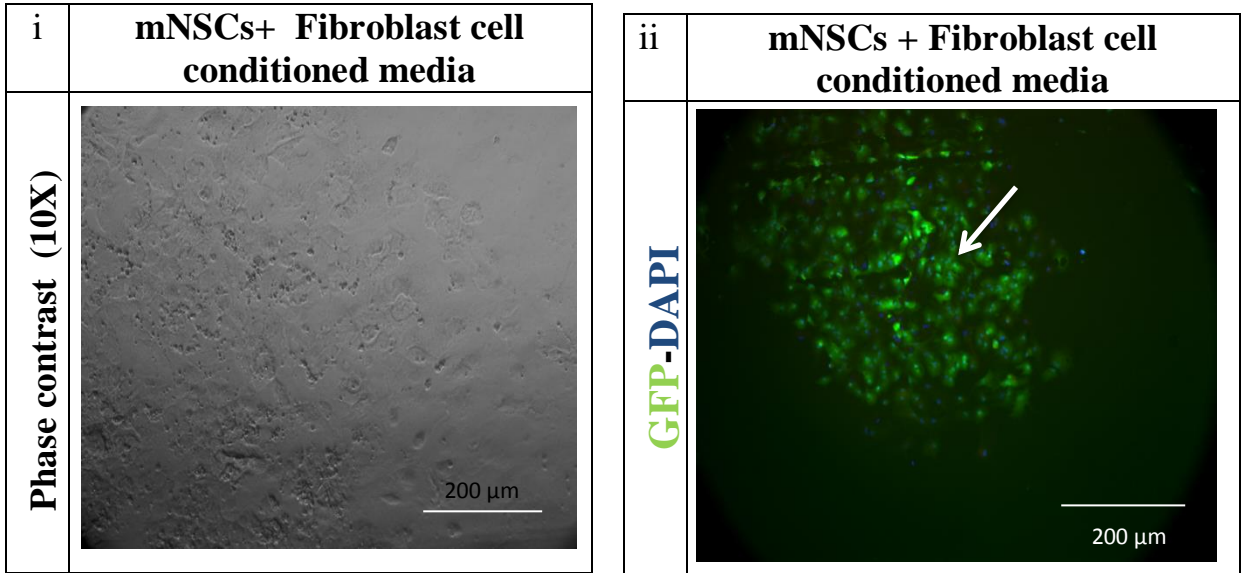
A)



B)



C)



D)

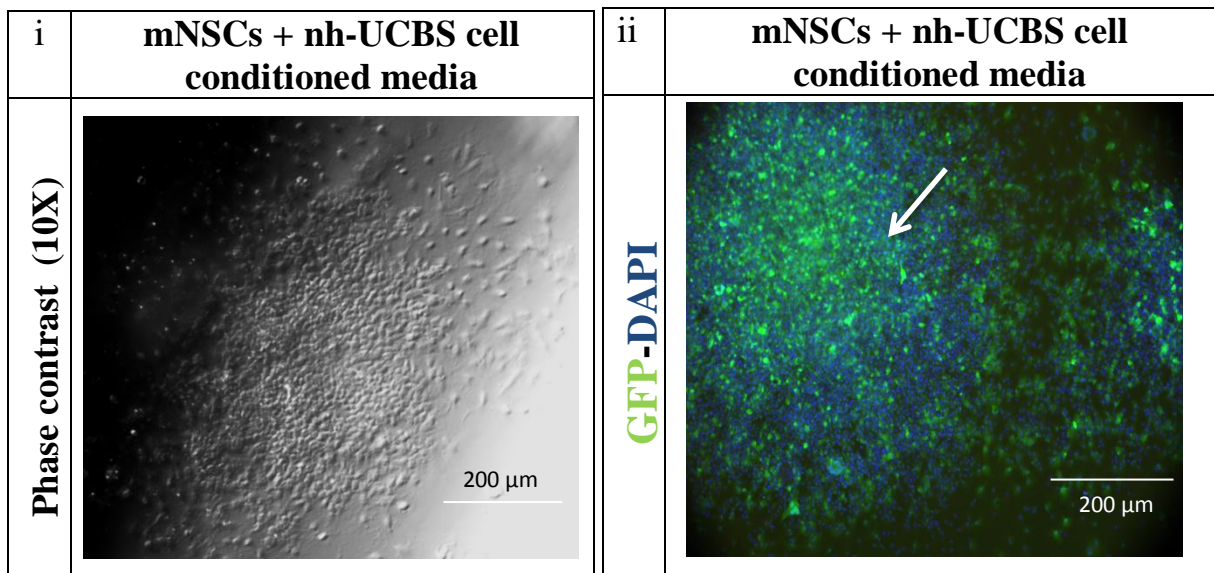


Figure 14: **Immunocytochemistry of the mNSC in the 3 different culture conditions in the 4 well chamber slide**

(A) (i) Phase contrast image of the mNSCs cultured in Neural stem media (ii) Staining for the mNSCs in NSC media. Merged image of DAPI (blue) and GFP-positive neural stem cells (GREEN).

(B) (i) Phase contrast image of the mNSCs cultured in UCB media (unconditioned media) (ii) Staining for the mNSCs in UCB media. Merged image of DAPI (blue) and GFP-positive neural stem cells (GREEN). Apoptosis of mNSCs was observed when cultured in UCB media. The numbers of GFP positive mNSCs were very few. The number of colonies formed was very scanty with not many cells in every colony. Hence the size of the colony was very small.

(C) (i) Phase contrast image of the mNSCs cultured in fibroblast cell conditioned media (ii) Staining for the mNSCs in fibroblast cell conditioned media. Merged image of DAPI (blue) and GFP-positive neural stem cells (GREEN). The morphology of the neural stem cells mNSC seems changed.

(D) (i) Phase contrast image of the mNSCs cultured in nh-UCBS cell conditioned media (ii) Staining for the mNSCs in nh-UCBS cell conditioned media. Merged image of DAPI (blue) and GFP-positive neural stem cells (GREEN). The neuronal stem cells cultured in the nh-UCBS cell conditioned media formed many colonies (n=20). The size of every colony was larger when compared to the colonies in other culture conditions i.e. there were more number of cells forming a colony when mNSCs were cultured in nh-UCBS cell conditioned media.

[Arrows indicate colony formation by mNSC in B, C and D]

DISCUSSION

It is known from previous work in the laboratory that a novel population of CD34 negative non-hematopoietic stem cells can be isolated from the human umbilical cord blood. Those cells when injected systemically into a rat 48 hours after the induction by stroke or direct injection of these stem cells into the brain parenchyma at 3 months after ischemic stroke, helped in the alleviation of the stroke induced deficits, improving the behavior of the animal model seen by performing various functional tests. The human cells injected into the animal model were not present in the brain 2-3 days after administration, hence it was hypothesized that the recovery process in the animal model is mediated by a mechanism other than cell-replacement. The focus of this thesis was to explore the other mechanism(s) of action that was responsible for the improved recovery in animal model induced with stroke.

Ischemic brain stroke triggers the complicated inflammatory cascade in the brain that eventually causes pronounced cell death adjacent to the obstructed vasculature. In the first part of this study we postulated that the nh-UCBS cells that are injected into the stroke induced animal model helps in preventing the apoptotic cascade and also modulates the inflammatory response to the ischemic injury thereby improving the functional behavior of the animal. Hence the initial focus of the study was to analyze the expression level of the various pro- and anti-inflammatory cytokines in the non – hematopoietic umbilical cord blood stem cells. This was done using a quantitative RT2 PCR profile array in which the 96 well plate had a variety of built in primers for the cytokines of interest. We observed that the gene expression level of the variety of interleukins, growth factors, MMP inhibitors and other chemokine's was in the nh-UCBS cells. Of specific interest, high expression of growth factors like ANGPT1 and VEGF responsible for angiogenesis and vasculogenesis was observed in the nh-UCBS cells. This preliminary observation needs to be repeated with more replicates to generate significant results. Another interesting observation was the expression of the chemokine secreted phosphor protein1 (SPP1) which is a pro-inflammatory cytokine that promotes the migration of inflammatory cells to the wound site and also acts an adhesive protein to retain the cells at the site. SPP1 was not seen to be expressed in the nh-UCBS cells.

Further studies need to be performed to understand the significance of this observation. Next, in order to confirm the gene expression level of the cytokines, we checked the secretion of the pro-inflammatory and anti-inflammatory level of cytokines in the nh-UCBS cell conditioned media. Protein level of a variety of interleukins, growth factors, chemokine's and MMP inhibitors were observed in the nh-UCBS cell conditioned media, The protein expression of ANGPT1 was significantly high in the nh-UCBS cell conditioned media, which correlated with the gene expression of the ANGPT1 cytokine in the nh-UCBS cells.

We next hypothesized that nh-UCBS cells can modulate the neuroinflammatory immune response by activating microglial cells. To test this hypothesis, we studied the influence of the nh-UCBS cell conditioned media on the BV2 microglial cell line. Microglial BV2 cells were co-cultured with the nh-UCBS cell conditioned media after the stimulation of the BV2 cells by LPS. The LPS is responsible for activating the inflammatory response from these immune cells in an *in-vitro* environment. We postulated that the nh-UCBS cell conditioned media will help in the transition of the M1 phenotype of macrophage to the M2 phenotype which is the responsible phenotype for wound healing and tissue repair. Though in our observations, we noticed that the nh-UCBS cell conditioned media did not significantly reduce the gene expression of the M1 phenotype macrophages, as seen by the expression of the M1 markers (iNOS and CD-86) in the Bv2 cells cultured in three different culture conditions, we did observe a significant increase in the expression of M2 marker (Arg-1) in the BV2 cells co-cultured in the nh-UCBS cell conditioned media compared to the BV2 cell condition media which states that the nh-UCBS cells are activating the transition from the M1 to M2 phenotype. However, the unconditioned culture media used to grow nh-UCBS cells also had a positive effect on the generation of the M2 phenotype which is a confounding factor in this interpretation.

The second part of the study was to understand the mechanism of non-neural stem cell based approach for mediating recovery from stroke which is neuroplasticity. We expected to see the increase in the dendritic length of the neurons cultured in the nh-UCBS cell conditioned media compared to the neuronal stem cells

cultured in other control culture condition media. But we observed that the nh-UCBS cell condition media helped in the increase of the number and size of the colonies formed by neural stem cells. We believe that this might be due to the various growth factors secreted by the nh-UCBS cells that help in the robust proliferation of the nh-UCBS cells. But the exact growth factor which is responsible for this is yet to be known.

Overall we had expected to see the expression of anti-inflammatory cytokine like IL-10 to be high in the nh-UCBS cell and in the nh-UCBS cell conditioned media. It is known that treatments that enhance IL-10 expression around the infarct site, reduces the infarct volume ^[48] ^[52] and similar observation of reduced infarct size was noticed after treatment with nh-UCBS cells in a rat model of stroke. Cytokine like IL-10 also may directly bind to NF- κ B, the key transcriptional regulator of genes thereby inhibiting its activity and thereby modulating the release of the pro-inflammatory cytokines such as TNF- α and IL-1 β in the stroke induced model. We observed that the gene expression of the most studied anti-inflammatory cytokines like IL-4 and IL-10 with regard to contributing to the recovery process after ischemic brain injury was high in the nh-UCBS cells. We also believe that the nh-UCBS cells release a number of neuroprotective growth factors that is responsible for the mechanism of neuroplasticity and neurorepair which ultimately improves the neurobehavior of the animal model induced with stroke.

It is however unclear whether the nh-UCBS cells that are injected into the animal model is reducing the inflammation by releasing cytokines that help in lessening cell death within the infarcted region, if its due to the activation of the macrophage cells in the brain and peripheral organs of the body which changes from M1 to M2 phenotype thus helping in the clearance of cellular debris or whether it is a combination of the two events. But it is certain that the nh-UCBS cell treatment alters the cellular and molecular program initiated by cerebral ischemia.

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