Summary
Organic chemicals and pollutants are found increasing in our environment (water, air, soil and food) persistently over the past decades. Many of them are well known to be neurotoxic in human brain. Humans are being exposed to these potentially toxicants throughout their entire life, beginning with starting of life as an embryo. Brain is the most important and complex organ of the body as it controls behavior and several important physiological functions. It is separated from rest of the body by blood brain barrier, which prevents entry of blood borne chemicals into the brain and also restricts exit of chemicals from the brain. However, several of the chemicals, being lipophilic readily cross the blood brain barrier, are biotransformed in the brain and produce toxicity. Brain cells usually have no or low regeneration power as they remain mostly in post-mitotic phase of cell cycle. In addition, the content of lipid is high which make brain cells more susceptible to toxicant induced cell death. It is well documented that the developing brain in fetuses and children is more vulnerable to toxic agents than the adult brain even at low exposure level that are usually not harmful to mature brain. In part, this may be due to no or low metabolic functionality in differentiating cells and the fact that the adult brain is well protected by the blood brain barrier and developing blood-brain barrier is not efficient enough to restrict the entry of xenobiotics from maternal environment.

Cytochrome P450s (CYPs) are heme-thiolate containing proteins involved in metabolism of various xenobiotic chemicals as well as endogenous chemicals like steroids, fatty acids, hormones, vitamins, cholesterol and neurotransmitters. CYPs play key role in metabolism, bioactivation, toxication, detoxification and elimination of various environmental chemicals and pollutants which come in contact by invading our ecosystem. The expression, inducibility and activity of several members of CYPs family (CYP1A1, 1A2, 1B1, 2B6, 2D6, 2E1 and 3A4), involved in metabolism, toxicity and detoxification have been documented in mature brain following the experimental exposure of environmental chemicals and drugs. However, the expression and inducibility of CYPs during neurogenesis is not known. The use of pure population of cultured cells of neuronal origin has been suggested as a powerful tool to study and elucidate such mechanism based studies. But in vitro studies with developmental stages have been hampered since most of the studies were conducted with mature adult cells only and non availability of developing brain tissues due to ethical dubious. But, till date most of the mechanistic in vitro studies are limited on transformed proliferating normal,
immortalized, or tumor cells. Quite often, the extrapolation of such *in vitro* data to the *in vivo* situation has been found less perfect. Stem cells isolated from human umbilical cord blood have been used as the primary source material, since stem cells are known to have comparable properties to primary human cells, i.e., unlimited proliferation ability, plasticity to generate other cell types and a more readily available source of human cells. Stem cell technology provides a new tool for better understanding the toxicity mechanisms involved and how to potentially predict and avoid toxicity in humans. This approach can work more appropriately for developmental neurotoxicity, since the unique property of unlimited proliferation and pluripotency of stem cells make them tremendous resource for virgin human biological material. Thus, we hypothesized the applicability of plasticity and pluripotency potential of human cord blood stem cells to convert them into neuronal subtypes and develop homogenous, highly predictive, and innovative *in vitro* tool to understand the mechanisms involved in developmental stage specific xenobiotic metabolism and to identify stage specific biomarkers of exposure and effect for developing human brain.

Umbilical cord blood is one of the richest sources of stem cells. Unlike, embryonic stem cell, use of cord blood stem cells is non-controversial as it is generally discarded material after the birth of child. In addition, the non-invasive collection make it comparatively wonderful tool for regenerative medicine. Umbilical cord blood stem cells have been thought to possess higher proliferating capacity due to longer telomeres than other somatic stem. Moreover, umbilical cord blood can be cryopreserved for longer period that may be used in transplantations. Transplantations of umbilical cord blood have lower risk of graft-versus-host rejection in compare to bone marrow due to MHC-II compatibility demonstrating superiority of umbilical cord blood in clinical applications.

*First Objective: To convert the human umbilical cord blood stem cells into neuronal subtypes*

First objective of the present study was to develop/standardize the protocols for isolation, purification, characterization and bulk production of hematopoietic stem cells from human umbilical cord blood. Stem cells were isolated from 125 samples using robotic, automated and magnetic cell separator i.e. RoboSep™ (Stem Cell Technologies, Vancouver, BC, Canada), MACS system. Hematopoietic stem cells were isolated by segregating them on the basis of cell surface CD markers (CD34⁺ and CD38⁻) using bispecific antibodies against cell surface at one end and secondary antibody conjugated
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with iron core dextran coated nanoparticles at another end. After this process, these purified population of cells were characterized by immunophenotyping of CD markers (CD34, CD38 and CD45) using FACS and colony forming unit assay. Following immunophenotyping, 85-90% purity of stem cells with more than 95% viability could be achieved. Hematopoietic stem cells also showed clonogenic potential towards various lineages viz., erythroid, granulocytes and monocytes. Purified stem cells were cultured in selective serum containing myelocult culture medium supplemented with hydrocortisone, cytokines and growth factors cocktails for more than four months up to passage 15-20. At each passage, the purity of hematopoietic stem cells was ascertained for the expression of stem cell markers. By selective proliferation, the number of CD34+ cells could be increased up to 94.9%, 97.5% and 99.8% at III, IV and V passages respectively. This much of purity of stem cells was maintained till last passage i.e., 15-20. Our findings suggest the extensive self renewal and undifferentiated proliferation capacity of cultured hematopoietic stem cells for such a prolonged period of time.

The cord blood derived hematopoietic stem cells were found to have a high commitment towards neuronal lineage similar to fetus-derived neural stem cells. Using a cocktail of various growth factors, rhNGF and retinoic acid in serum free neurobasal medium, these hematopoietic stem cells can be induced to undergo morphological and physiological changes consistent with developing neuronal cells. These differentiated cells showed a typical neuron-like morphology and expressed various cell type specific markers for neurons. The quantitative Real Time-PCR, immunocytochemical localization and western blot data revealed that the significant mRNA transcripts and antigens of various neuronal markers like GAP43, NCAM, NF-M, NF-H, PSD95, TUJ-1 and SYP etc. were found elevated after the onset of differentiation at day 2 and the expression of these markers became more significant at day 8. The transcript and protein of neuron associated to growth factors, NGF and BDNF were also recorded increasing from day 2 to day 8. The expression of CREB was up regulated from day 2 to day 8. CREB is neuron associated transcription factor, which promote neuronal differentiation by activating upstream regulators like MAP Kinases. The expression (mRNA and protein) of acetyl cholinesterase (ACHE) was found expressed after the onset of differentiation from day 2 to day 8, which confirms the neurons differentiation from stem cells to be cholinergic type. Differentiating cells were also showing significant elevated expression of artemin (ARTN) which is known to support the survival, neurite outgrowth, cell...
migration and differentiation of peripheral neurons. Thus, it was inferred that differentiating cells are cholinergic type and peripheral neurons. The expression of nestin, a neural precursor marker, was found maximum at day 2 of differentiation, which reduces in subsequent differentiations i.e., day 4 and 8. In developmental neurogenesis, all the neuronal markers were found to be expressed at an early stage of development and the maximum expression of these markers were recorded at day 8 of differentiation. After neuronal induction, TUJ-1, NF-M and SYP were expressed along the cell body and neurite-like processes, in a similar fashion to developing neurons. Notably, the expression of CD34, CD133, Nanog, MYC and SHH, cell type markers for hematopoietic stem cells, were found decreasing all through the differentiation of stem cells into neuronal cells. The markers of stemness were almost diminished by day 8 of differentiation, which strongly support neuronal differentiation. In general, early differentiating stages (day 1, 2, 4) were more responsive to the growth/ differentiation factors than well differentiated mature neuronal cells (day 8 and 12).

**Second Objective: To study the expression and inducibility of xenobiotic metabolizing cytochrome P450s all through the differentiation following the exposure of known inducers of P450s**

In this objective, these differentiating human neuronal cells were then subjected to study the constitutive and inducible expression (mRNA and protein) and activity of selected xenobiotic metabolizing CYPs (1A1, 2B6, 2E1 and 3A4), receptor regulators (AHR, CAR and PXR), and phase II enzyme GSTP1-1 following the exposure of known inhibitor (cimetidine) and inducer of CYPs (rifampin). The CYPs inhibitory mechanism of cimetidine is primarily based on the binding of it with the heme group of CYPs, thus inhibits the expression at protein level. Rifampin, well known antibiotic, is used in the present study as it is known to induce the expression of all the genes of xenobiotic metabolism. The constitutive expression and substrate specific catalytic activity of CYP1A1 and 3A4 were found increasing gradually till day 8 of differentiation, while CYP2B6, CYP2E1, CAR and PXR showed highest expression and activity at day 4. There was no significant increase in the expression of AHR and GSTP1-1 during differentiation. After profiling constitutive expression, inducible expression of xenobiotic metabolizing enzymes, regulators and phase II enzyme GSTP1-1 were studied at all the stages of neuronal maturities following the experimental exposure of cimetidine (universal inhibitor of CYPs), and rifampin (universal inducer of CYPs).
induction of xenobiotic metabolizing genes, the non-cytotoxic doses of cimetidine, rifampin and monocrotophos (MCP; a widely used developmental neurotoxic organophosphate pesticide) were identified at different stages of maturity (day 0, 2, 4 and 8) using standard endpoints of cytotoxicity viz., tetrazolium bromide MTT assay and trypan blue dye exclusion assay. MTT assay was found to be most sensitive followed by trypan blue dye exclusion assays. MTT assays represent the mitochondrial catabolism. Thus, it may be suggested that cimetidine, rifampin and MCP or their metabolites may interact either directly to the cellular proteins or oxidative stress signaling pathways. Such differences in sensitivity due to organelle specificity of chemicals have already been reported for different toxicants, even in the same cell system. In general, there was no significant variation in the sensitivity of cells against the exposure of cimetidine, rifampin and MCP at day 0, 2, 4 and 8 of differentiation. Human stem cells derived neuronal cells at various maturity i.e., day 0, 2, 4 and 8 were then subjected to exposure of 250 μM cimetidine, and 250 μM rifampin for 3, 6 and 12 h for mRNA, protein expression and enzymatic activity studies respectively. Following the exposure, differentiating neuronal cells were investigated to measure the exposures induced alterations in the expression and activity of xenobiotic metabolizing CYPs, regulators and GSTP1-1 at various points of maturity. Data obtained were found in consistent manner having same trend at transcriptional (Real Time PCR analysis), translational (Western Blot and immunocytochemical localization analysis) and substrate specific enzymatic functionality level. Significant up-regulation in the expression of CYPs (1A1, 2B6, 2E1 and 3A4), their receptors (AHR, CAR and PXR) and phase II metabolizing enzyme GSTP1-1 under the influence of rifampin confirms the responsiveness of differentiating neuronal cells to xenobiotics. Down-regulation of all the CYPs under the influence of inhibitor (cimetidine) further confirmed the responsiveness of these differentiating neuronal cells. In general, the magnitude of expression of CYPs studied was highest at day 2 of differentiation followed by 0, 4 and 8 days differentiated cells respectively.

Third Objective: To study the responsiveness of differentiating stem cells against known developmental neurotoxicant pesticide- monocrotophos

The xenobiotic metabolizing capability of differentiating neuronal cells derived from human hematopoietic stem cells at various maturity i.e., day 0, 2, 4 and 8 were further validated against the exposure of known developmental neurotoxic organophosphate
pesticide monocrotophos (MCP). The differentiating cells were subjected to exposure of MCP (10^{-5}M), cimetidine (250 \mu M) for 1 h followed by MCP (10^{-5}M), and rifampin (250 \mu M) plus MCP (10^{-5}M) concurrently for 3, 6 and 12 h for mRNA, protein expression and enzymatic activity studies respectively. After the completion of exposure periods, cells were processed for profiling of exposures induced alterations in the expression (mRNA and protein) and activity of xenobiotic metabolizing CYPs (1A1, 2B6, 2E1 and 3A4), regulators (AHR, CAR and PXR) and GSTP1-1 at various points of maturity. MCP induced significantly the expression of all the genes of xenobiotic metabolism. Pre-exposure of cimetidine could not directly influence the CYPs induction in MCP exposed cells. The overall magnitude of induction of CYPs and regulators by MCP was higher than the levels induced by rifampin, in general, co-exposure of rifampin and MCP showed statistically highly significant additive effect in the induction of mRNA/ protein expression of all the genes studied. In addition, the magnitude of MCP induced catalytic activity (EROD, PROD, NDMA-d and EMD) was greater than the induction levels following the exposure of rifampin. The cells receiving co-exposure of rifampin and MCP for 12 h showed additive effect in the induction of catalytic activity of all the CYPs. The cells showed maximum expression and induction in the activity of CYP3A4 all through differentiation i.e., 0, 2, 4, 8 days. In general, the magnitude of expression of CYPs studied was highest at day 2 of differentiation followed by 0, 4 and 8 days differentiated neuronal cells respectively. Such stage specific vulnerability of the cells may be due to the complexity in tightly controlled mechanism of cell differentiation/ maturation and higher intracellular metabolic activities in early stages of differentiation.

To the knowledge, this is the first report showing the expression and inducibility of CYPs (1A1, 2B6, 2E1, and 3A4), their regulators (AHR, CAR, and PXR), and phase II enzyme GSTP1-1; in developing neuronal cells derived from human hematopoietic stem cells. We found the increase in the expression and activity of CYPs with the progress of neuronal cell differentiation, which suggests the possible association of CYPs activity in the neuronal development of developing brain. These differentiating cells responded significantly to rifampin (known CYP inducer), cimetidine (known CYP inhibitor) and monocrotophos (MCP). The inducible expression of CYPs also confirms the metabolizing capabilities of human cord blood stem cell-derived neuronal cells for a wide range of xenobiotics, including pesticides, organic solvents, polycyclic aromatic hydrocarbons, drugs, etc. Thus, our data identifies the CYP mediated xenobiotic
metabolic capabilities of human umbilical cord blood stem cell derived neuronal cells at various stages of maturity.

Based on the findings obtained from human stem cells derived neuronal cells, the final inferences drawn are as under:

- Our findings demonstrate the undifferentiated proliferation and possibility of functional neuronal differentiation of hematopoietic stem cells derived from human umbilical cord blood under the influence of specific culture conditions.

- Significant expression of early neuronal markers could be detected by day 2 of differentiation. Cells got morphological and physiological maturation by day 8 of differentiation.

- Early differentiating stages were found to be more vulnerable to xenobiotics than well differentiated mature cells.

- This *in vitro* system could be utilized to address cellular and molecular events inducing and controlling the differentiation.

- This study provides the evidences for stage specific xenobiotic metabolism capability of developing neurons which could be homogenous tool to identify stage specific biomarkers of exposure and effect.

- The human stem cell based *in vitro* system could be utilized to predict human specific developmental neurotoxicity and pre-screening tool to assess neurotoxicity/developmental neurotoxicity potential of environmental chemicals/drugs.