

# Differentiating neurons derived from human umbilical cord blood stem cells work as a test system for developmental neurotoxicity

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**Abstract** Differentiating neuronal cells derived from human umbilical cord blood stem cells have been used as an *in vitro* tool for the assessment of developmental neurotoxicity of monocrotophos (MCP), an organophosphate pesticide. The differentiating cells were exposed to MCP during the different stages of maturation, viz., days 2, 4, and 8, and changes in the makers of cell proliferation, neuronal differentiation, neuronal injuries, and receptors were studied. We found significant upregulation in the different MAPKs, apoptosis, and neurogenesis markers and downregulation in the cell proliferation markers during neuronal differentiation. We further identified significant upregulation in the expression of different MAPKs and proteins involved in oxidative stress, apoptosis, and calpain pathways in the mid-differentiating cells exposed to MCP. The upregulated levels of these proteins seem to be the main cause of alteration during the differentiation process towards apoptosis as a fine-tune of pro-apoptotic and anti-apoptotic proteins are desirable for the process of differentiation without apoptosis. The decreased acetylcholinesterase activity, dopaminergic, and cholinergic receptors and increased acetylcholine levels in the differentiating neuronal cells indicate the vulnerability of these cells towards MCP-induced neurotoxicity. Our data confirms that differentiating neuronal cells derived from human umbilical cord stem cells could be used as a powerful tool to assess the developmental neurotoxicity in human beings.

**Keywords** Umbilical cord blood stem cells · Developmental neurotoxicity · Apoptosis · Alternative testing strategies · Monocrotophos · Organophosphates

## Introduction

Most children with autism or other developmental disabilities have severe language-related problems or disorders [1, 2]. Organophosphorus pesticides could be detected in the amniotic fluid and are able to cross the placenta [3, 4]. Prenatal exposure of organophosphorus pesticides has already been related with different disabilities, including reduced head size [5], low body weight [6], and reflex problems [7, 8] in neonates and low IQ in preschool children [9–11]. Although several reports have been published on the molecular mechanism of these pesticides, very little is known about the developmental neurotoxic potential of these pesticides during embryo/brain development [12–17]. The majority of chemicals have never been tested for their developmental neurotoxic (DNT) effects, so we cannot ignore the possible involvement of these chemicals behind such type of disabilities. The evidence for developmental neurotoxicity arising from organophosphate exposures is not ambiguous, because a plethora of literature is available on animal studies and residual effects in humans following acute intoxication [16, 18–20]. Developing brain seems to be more vulnerable to these kinds of pesticides as compared to the adult brain [16, 21, 22] due to the low amount of oxygen, high mitosis, and less-developed blood–brain barrier. Therefore, developmental neurotoxicity assessment is a serious concern for environmental chemicals, drugs, and new chemical entities. Animal-based DNT models have shown their own limitations by deviating from the rule of 3R (reduction, refinements, and reproducibility), and furthermore, mechanistic studies on these models also largely suffer to correlate data with human beings

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[23]. The use of cell cultures has proven to be a powerful approach to elucidate the mechanisms involved in the developmental neurotoxicity [24, 25], but, to date, most of the mechanistic *in vitro* studies are limited to use non-stem, transformed cell lines and quite often, the extrapolation of such *in vitro* data to the *in vivo* situations [26]. Stem cells provide a new tool for the better understanding of the mechanisms involved in developmental toxicity/neurotoxicity as well as overcomes the other problems of *in vitro* data studies by using transformed cell lines [26, 27]. This approach can work more appropriately for DNT, since the unique property of unlimited proliferation and pluripotency of stem cells makes them a tremendous resource for virgin human biological material and mimicking the same conditions as those during brain development. Thus, we hypothesized to exploit the plasticity and pluripotency potential of human cord blood stem cells to develop a homogenous, highly predictive, and innovative *in vitro* tool to understand the mechanisms involved in human specific neural developmental, injuries, and repairs in addition to identifying the stage-specific biomarkers of organophosphate exposure [28].

Attempts were made to study mechanisms involved in neuronal development as well as developmental neurotoxicity by using different growth factors and to study the differentiation factors mediating alterations in the expressions and activity of early, midterm, and mature neural markers throughout the differentiation in cultured neuronal cells, derived from human umbilical cord blood stem cells (hUCBSCs). The influence of monocrotophos (MCP), known as developmental neurotoxic organophosphate pesticide, was studied on the alterations in the expression marker genes involved in the neuronal development, repair, and injuries on differentiating neuronal cells derived from the umbilical cord blood stem cells. Data indicate the potential application of differentiating neural cells derived from hUCBSCs to understand the mechanisms involved in chemical-induced developmental neurotoxicity in human beings. Data indicate the possible application of differentiating nervous cells, derived from hUCBSCs as homogenous, high-throughput *in vitro* tool to understand the mechanisms involved in chemical-/drug-induced developmental neurotoxicity in human beings.

## Experimental Procedures

### Reagents and Consumables

All the chemicals, reagents, and kits used for this study were purchased from Stem Cell Technologies, Vancouver, BC, Canada, and Sigma St. Louis, USA, unless otherwise stated. All cytokines and growth factors such as the recombinant human nerve growth factor (rhNGF), basic fibroblast growth factor, thrombopoietin, stem cell factor, fetal liver tyrosine

kinase-3 ligand [FLT-3 ligand] were purchased from Pepro-Tech (Rocky Hill, NY). Culture wares and plastic wares were procured from Nunc, Denmark, and Corning Incorporated, NY. Autoclave Milli-Q water was used in all the experiments. MCP [dimethyl (*E*)-1-methyl-2-methyl carbanoyl vinyl phosphate C7H14NO5-P; Catalog No. PS-609; purity 99.5 %)] was purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

### Ethical Clearance for Collection and Transportation of Human Tissues

The entire study was carried out by following the protocols and procedures approved by Institutional Human Ethical Committees of Indian Institute of Toxicology Research (IITR), Lucknow, India, and King George's Medical University, Lucknow, India. The informed consent of parents was obtained prior to collecting blood from umbilical cord.

### Isolation, Characterization, Bulk Production, and Neuronal Differentiation of Umbilical Cord Blood Stem Cells

Umbilical cord blood stem cells (hUCBSCs) were isolated, characterized, and cultured for the bulk production and differentiated into the neuronal cells as described earlier by us [13, 15].

### Transcriptional Changes in Markers Associated with the Stemness, Proliferation, Neuronal Differentiation, and Cell Injuries

Expression analysis was done for genes responsible for stemness, proliferation, neuronal differentiation, and cell injuries at transcriptional level using TaqMan-based quantitative real-time PCR. TaqMan low-density array of 96 genes in 384-well plate formats was designed and procured from Applied Biosystems, USA (Electronic supplementary material (ESM) Figure S1). Relative quantification of fold changes in mRNA expression during the different maturity of neuronal cells was detected on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA) and analyzed by SDS2.3 software for relative quantification (Applied Biosystems, USA). In brief, RNA was isolated from neuronal cells all through the differentiation using GeneElute mammalian total RNA Miniprep Kit (Catalog No#RTN-70, Sigma, USA). The purity and yield of RNA were assessed by Nanodrop ND-1000 Spectrophotometer V3.3 (Nanodrop Technologies Inc., Wilmington, DE, USA), and quality was assessed by running RNA onto 2 % denaturing agarose gel. Total RNA (2  $\mu$ g) was reverse-transcribed into cDNA by SuperScript III first-strand cDNA synthesis Kit (Catalog No#18080-051, Invitrogen Life Science, USA) and treated with RNase-free DNase I to remove any potential DNA contamination. cDNA

was further pre-amplified by primer pool provided by Applied Biosystems with a customized TaqMan low-density array as describe by the manufacturer's protocol. Real-time reactions were carried out in triplicate, and GAPDH was used as internal control to normalize the data. Differentiation-induced alterations in mRNA expression of different genes are expressed in relative quantity compared with unexposed control groups at respective days.

#### Translational Changes in Markers Associated with the Stemness, Proliferation, Neuronal Differentiation, and Cell Injuries

Expression analysis was done for the markers associated stemness, proliferation, and neuronal differentiation at protein level using Western blot analysis. At various maturities (days 0, 2, 4, and 8), cells were pelleted and lysed using CelLytic™ Cell Lysis Reagent (Catalog No# C2978, Sigma, USA) in the presence of 1× protein inhibitor cocktail (Catalog No# P8340, Sigma, USA), 2 mM PMSF, and 1 mM sodium orthovanadate. After protein estimation by BCA protein assay (Catalog No# G1002, Lamda Biotech, Inc., St. Louise, MO, USA), equal amounts (35 µg/well) of proteins were loaded in 10–12 % Tricine–SDS gel [14] and blotted on polyvinylidene fluoride membrane using a wet transfer system. After blocking for 2 h at 37 °C, the membranes were incubated overnight at 4 °C with specific anti-protein primary antibodies specific for stemness markers, viz., CD34<sup>+</sup>, CD133, C-MYC, OCT4, SOX2, NANOG, SSEA3, and SSEA4 and neuronal markers, viz., NF-M, NF-H, NF-L, Nenn, PSD95, Synaptophysin, Nestin, BDNF, NGF (1:1,000), and actin beta (1:3,000) in blocking buffer (pH 7.5). The membranes were then incubated for 2 h at room temperature with secondary anti-primary immunoglobulin G (IgG)-conjugated with horseradish peroxidase (Calbiochem, USA). The blots were developed using by luminal (Cat. No. 34080, Thermo Scientific, USA), and densitometry for protein specific bands was done in Gel Documentation System (Alpha Innotech, USA) with the help of AlphaEase™ FC StandAlone V. 4.0.0 software. Actin-β was used as internal control to normalize the protein data. Differentiation-induced alterations are expressed in fold change comparing the data with respective unexposed controls.

#### Immunocytochemical Localization of Protein Involved in Neurogenesis

Immunocytochemical localization of marker proteins of neurons in differentiating neuronal cells at days 0, 2, 4, and 8 was conducted following the protocol described earlier by us [15]. Briefly, hUCBSCs ( $1 \times 10^4$  cells/well) were seeded in a laminin (25 g/ml)-coated eight-well chamber slide (Labtek, Campbell) and incubated for 24 h to adhere onto the surface. The

adhered HSCs were induced to differentiate into neuronal subtypes up to day 8. At various maturities (days 0, 2, 4, and 8), the cells were fixed in 4 % paraformaldehyde for 20 min. The cells were then washed with phosphate-buffered saline (PBS) twice and incubated for 15 min in PBS containing 0.02 % TritonX 100 and 0.1 % BSA to block the nonspecific binding site. Cells were then washed with PBS and incubated at room temperature for 2 h with primary antibodies against specific proteins, viz., GAP43 (1:500), MBP (1:500), NCAM-L1 (1:200), and Neuritin (1:500) (all antibodies were from Chemicon International). Following incubation with primary antibodies overnight, cells were washed three times with PBS for 5 min each to remove the unbound antibodies. Then, fluorescein isothiocyanate (FITC/rhodamine)-conjugated secondary antibodies were added to each well and kept on a rocker shaker in dark for 2 h at room temperature. Cells were then washed with PBS three times for 5 min each. Thereafter, the cells were fixed with anti-fade Vectashield and visualized under an upright fluorescence microscope (Nikon Eclipse 80i equipped with Nikon DS-Ri1 12.7-megapixel camera, Japan) using specific filters for FITC and rhodamine.

#### Identification of Non-cytotoxic Doses of Monocrotophos (MCP)

Stock solution of MCP was made up in neurobasal medium, and fresh working concentrations were made by diluting the stock solution in the same medium. Prior to start experiments, viability was assessed, and batches showing more than 95 % cell viability were only used for the study. The non-cytotoxic doses of MCP were identified by exposing the stem cells derived differentiating neuronal cells (days 2, 4, and 8) to MCP ( $10^{-8}$  M– $10^{-2}$  M) for 12–96 h by using MTT tetrazolium bromide salt and lactate dehydrogenase assay kit.

#### MCP-Induced Alterations in the Expression and Activity of Markers Involved in Neuronal Development, Injury, and Repair

On the completion of expression studies in stem cells derived differentiating neuronal cells and identification of non-cytotoxic does of MCP, cells were subjected to study the responsiveness these cells against the exposure of MCP. Investigations were further carried out to study MCP-induced alterations in the expression and activity of markers involved in neuronal development, injury, and repair with special emphasis on oxidative stress, apoptosis, and MAPK-induced pathways. The cells at various maturities were exposed to MCP ( $10^{-5}$  M, 3 h) for mRNA expression and ( $10^{-5}$  M, 6 h) for protein expression. Then, cells were processed for expression studies (mRNA and protein) with the help of quantitative real-time PCR and Western blot analysis as described above.

Plate scheme of the genes studied with the relevant information are provided in ESM Figure S1. Western blot analyses were done only those proteins which were altered on transcriptional levels.

#### FACS Analysis

Apoptosis was detected by deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays using APO-BrdU TUNEL Assay Kit with Alexa Fluor 488 anti-BrdU (Molecular Probes, Invitrogen Detection Technologies, USA, Catalogue No. A23210) by flow cytometer (BD-LSRII) and analyzed by Cell Quest 3.3 software. Debris was excluded by forward and sideways light-scattering.

#### Acetylcholinesterase Activity and Levels of Acetylcholine

Effects of MCP on acetylcholinesterase activity and on acetylcholine levels were studied in culture differentiating neuronal cells derived from hUCBSCs at only cells during day 4 of the differentiation. Studies were undertaken to investigate the effect of MCP at concentration  $10^{-5}$  M for 6 h and cells pretreated with some inhibitors, viz., SB203580, a p38 inhibitor; zDEVD-fmk, an apoptosis inhibitor; and SP600125, a JNK inhibitor with concentration of 10  $\mu$ g/ml for 1 h followed by MCP  $10^{-5}$  for 6 h. Acetylcholinesterase enzyme activity and acetylcholine levels were estimated by the help of fluorescence-based Amplex red acetylcholine/acetylcholinesterase assay kit (catalog no. A12217; Molecular Probes; Invitrogen Detection Technologies, USA). Following the different exposures, cells were harvested by centrifugation at  $200\times g$  for 10 min at 4 °C. Cells were sonicated in 40 mM Tris buffer (pH 8.0) and centrifuged at  $12,000\times g$  for 10 min at 4 °C. Supernatants were then processed as per the manufacturer's protocol. Fluorescence was measured at the excitation wavelength 530 nm and the emission wavelength 590 nm by using the Synergy HT multiwell plate reader (Synergy HT, USA).

#### Dopamine and Cholinergic (Muscarinic) Receptors

Effects of MCP on dopamine (DA-D2) and cholinergic (muscarinic) receptors were studied in cultured differentiating neuronal cells derived from hUCBSCs at only cells during day 4 of the differentiation, since this stage of differentiation was found to be more vulnerable to MCP. Studies were undertaken to investigate effect of MCP at concentration  $10^{-5}$  M for 6 h following the standard protocols [29]. After 4 days of differentiation, neuronal cells were also pretreated with some inhibitors like SB203580, a p38 inhibitor; zDEVD-fmk, an apoptosis inhibitor; and SP600125, a JNK inhibitor with concentration of 10  $\mu$ g/ml for 1 h followed by MCP  $10^{-5}$  for 6 h to check the effect of these proteins in neuronal cells injuries. Specific

radio-ligands, viz., 3H-Spiperone (15 Ci/mmol,  $1\times 10^{-9}$  M), 3H-QNB (42 Ci/mmol,  $1\times 10^{-9}$  M) and specific competitor, viz., haloperidol ( $1\times 10^{-6}$  M) and atropine sulfate ( $1\times 10^{-6}$  M) was used in the assay of DA-D2 and cholinergic receptors, respectively.

#### Membrane Preparation

Cells of both treated and control groups were harvested and centrifuged at 1,000 rpm for 10 min at 4 °C. The cell pellet was subsequently suspended in 5 mM Tris-HCl buffer (pH 7.4) and sonicated. The sonicated cell suspension was then centrifuged at 20,000 rpm for 20 min at 4 °C, and the membrane pellets were suspended in 40 mM Tris-HCl buffer (pH 7.4) and were stored at -80 °C until assay. Protein estimation of each sample was done BCA Protein Assay (Catalog No# G1002, Lamda Biotech, Inc., St. Louise, MO, USA).

#### Neurotransmitter Receptor Binding Assay

Radioligand receptor binding assay was carried out using Robotic Liquid Handling System (Multi Probe II EX, Packard, USA). Multiwell plates (Millipore MSFBN6B, 96-well) were arranged on the deck and mapped with the WINPREP software. Sequential addition of the assay components was also programmed. The incubation mixture in a final volume of 250  $\mu$ l contained 40 mM Tris buffer (pH 7.4), cell membrane, and radioligand specific for each receptor. Binding assays were carried out in triplicate in each case, and specific competitors were run to titrate the assay system. The reaction mixture in the plate was incubated for 15 min at 37 °C followed by filtration of the contents over the vacuum manifold. Plates were washed with buffer twice, filtered over vacuum, and dried. Scintillation cocktail 70  $\mu$ l (Microscint O, Packard, USA) was added to each well, and plates were left overnight. Counting as counts per minute was carried out using plate counter (Top Count-NXT, Packard, USA). The binding in the absence of MCP or competitor is the total binding. Finally, percentage change in the specific binding following exposure of MCP to 4 days differentiated neuronal cells derived from UCBSCs cells was calculated.

#### Immunocytochemical Localization of MCP-Induced Alterations in the Activity of Choline Acetylcholinesterase (ChAT) and Tyrosine Hydroxylase (TH)

Immunocytochemical localization of ChAT and TH was done in differentiating cells (days 0, 2, 4, and 8) following the exposure to MCP ( $10^{-5}$  M) for 6 h. Unexposed cells processed under identical experimental conditions were used as control. The procedure followed was identical to that described for immunocytochemical localization of neuronal marker



proteins. The quantification of MCP-induced alterations in the expression of ChAT and TH activity was done using the Image Analysis Software Leica-QWin 500. Data were calculated as mean $\pm$ SE of at least 20 microscopic fields.

### Statistical Analysis

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data are expressed as mean $\pm$ SE. Statistical analysis was performed using one-way analysis of variance and post hoc Turkey's test to compare control and treated groups. A *P* value $<$ 0.05 was considered to be statistically significant.

## Results

### Isolation, Purification, Culture, and Differentiation of Hematopoietic Stem Cells

Stem cells were isolated from 93 human umbilical cord blood samples using robotic automated RoboSep, MACS system by segregating those based on CD markers (CD34<sup>+</sup>, CD133<sup>+</sup>, CD38<sup>-</sup>, CD45RA<sup>-</sup>). After this process, 80–85 % purity of stem cells with more than 95 % viability could be achieved. Purified stem cells were cultured and characterized as discussed earlier by us (Kashyap et al. 2012). hUCBSCs were induced to differentiate into neuronal subtypes and stage-specific differentiation (Fig. 1) as described earlier [15].

### Expression Studies at Transcriptional Level

Morphological changes (Fig. 1) observed were accompanied by detectable upregulated expression in transcripts of various neuronal marker genes. Quantitative RT-PCR analysis revealed that the mRNA expression of markers associated with stemness, viz., CD133, MYC, NANOG, SHH, Klf4, SOX2, POU5F1, and FRAP1 were found to be affected significantly (*p* $<$ 0.001) with the progress of differentiation. The quantitative reduction trend in the expression of stemness markers was most severe for CD34, i.e., 2.66 $\pm$ 0.13-fold on day 4 as compared with 9.6 $\pm$ 1.8-fold on day 2 and even undetectable by day 8 of differentiation. A similar trend was also recorded for CD133 (0.32 $\pm$ 0.08-fold, 0.13 $\pm$ 0.02-fold), Klf4 (0.77 $\pm$ 0.12-fold, 0.53 $\pm$ 0.10-fold), POU5F1 (0.51 $\pm$ 0.08-fold, 0.15 $\pm$ 0.03-fold), and SOX2 (1.47 $\pm$ 0.13-fold, 1.25 $\pm$ 0.11-fold) at days 4 and 8 of differentiation, respectively. The expression of SHH could be detected only at day 2 of differentiation (0.31 $\pm$ 0.01-fold), when compared with undifferentiated cells at day 0. In case of NANOG, a biphasic response was observed. At days 2 and 4, expression was increased (2.57 $\pm$ 0.19-fold and 4.09 $\pm$ 0.24-fold, respectively) and thereafter at day 8 reduced to 2.93 $\pm$ 0.01-fold of control. However, all the values were

significantly higher than the undifferentiated cells of day 0. MYC was also found downregulated on days 4 and 8 of differentiation. The expression of cell survival target FRAP1 (mTOR) was found to be increased at all points of differentiation, i.e., day 2 (1.21 $\pm$ 0.15-fold), day 4 (1.71 $\pm$ 0.23-fold), and day 8 (2.6 $\pm$ 0.39-fold) of differentiation (Fig. 2a).

Generally, the expressions of mRNA levels of genes involved in the differentiation were found to increase with the increase in differentiation, except in case of NGF, CHRM2, and NR4A2. The expression reached the highest levels, i.e., 609.96 $\pm$ 12.0-, 7.13 $\pm$ 0.79-, 20.59 $\pm$ 2.7-, 18.45 $\pm$ 1.4-, 4.38 $\pm$ 0.21-, and 7.86 $\pm$ 0.68-fold of undifferentiated control cells at day 8 for NFM, MBP, NFH, NCM, BDNF, and STAT4, respectively. Markers associated with early differentiation, viz., nestin (NES), and proliferation, i.e., WNT1 showed significantly higher expressions at day 2 (51.19 $\pm$ 4.5- and 13.47 $\pm$ 0.87-fold, respectively), and thereafter, a reduction in subsequent differentiation, i.e., day 4 (24.30 $\pm$ 2.3- and 0.002-fold), and day 8 (8.14 $\pm$ 1.1- and 0.001-fold). However, the overall expression of nestin was higher than the undifferentiated cells. Though, the expression of NGF was increased significantly (56.07 $\pm$ 0.52 and 62.15 $\pm$ 0.42) by days 2 and 4, respectively, but it reduces by day 8 (25.15 $\pm$ 0.12 fold) when compared with undifferentiated control cells. Except the expression of gene for caspase-3 and P<sup>53</sup>, all the genes associated with apoptosis were found to be upregulated all through the differentiation. On the other hand, various studied MAP kinases were also shown to be associated significantly (*p* $<$ 0.001) with the differentiation process, except MAP kinase 8 (Fig. 2b–d).

### Expression Studies at Translational and Cellular Levels

Results show linearity in the expression pattern of markers studied at transcriptional and translational levels all through the differentiation of cells. Data of protein expression of few representative markers studied by using Western blot analysis and immunocytochemical analysis are summarized in Figs. 3 and 4. Significant alterations in stemness markers, viz., CD34 (0.21 $\pm$ 0.01), CD133 (0.05 $\pm$ 0.01), C-MYC (0.31 $\pm$ 0.01), OCT4 (0.34 $\pm$ 0.04), SOX2 (0.11 $\pm$ 0.02), Nanog (0.27 $\pm$ 0.03), SHH (0.06 $\pm$ 0.01), and CD90 (0.35 $\pm$ 0.05) were recorded at day 8 of differentiation, respectively. Early differentiation marker Nestin shows initial increase, i.e., 1.21 $\pm$ 0.08 folds at day 2 of differentiation and brought down to 0.73 $\pm$ 0.09- and 0.41 $\pm$ 0.04-fold of control at days 4 and 8 of differentiation, respectively. The maximum protein expression for mature neuronal markers, viz., NF-M (1.87 $\pm$ 0.07-folds of control), NF-H (2.84 $\pm$ 1.2-folds of control), NFL (1.49 $\pm$ 0.08), Nenn (3.17 $\pm$ 0.49), PSD95 (3.31 $\pm$ 0.96), Synaptophysin (3.93 $\pm$ 1.2), NGF (1.55 $\pm$ 0.4), and BDNF (2.16 $\pm$ 1.4-folds of control), were observed at day 8 of differentiation. Most of the neuronal marker proteins were expressed in cells at day 8 of

differentiation, and in further differentiation, the increase in the magnitude of expression of these proteins was non-significant. The result of Western blots was also found comparable with immunocytochemical localization for the significant functional expressions of marker proteins of neuronal structure (Fig. 4).

#### Identification of Non-cytotoxic Doses of Monocrotaphos (MCP)

Non-cytotoxic doses of MCP were identified in neuronal cells at various time points of maturity (0, 2, 4, and 8 days) by using standard endpoints such as MTT and LDH assays [15].

#### Transcriptional Changes in Marker Genes in Differentiating Cells Exposed to MCP

The results of MCP-induced ( $10^{-5}$  M for 3 h) changes to the expression of markers involved in stemness, neuronal differentiation, apoptosis, oxidative stress, metabolism, MAP kinases, and transcription factors in differentiating cells are summarized in Fig. 5a–f. MCP exposure significantly ( $p < 0.001$ ) reduces the expression of genes involved in stemness all through the differentiation. The cells were found to be more vulnerable to day 4 of differentiation as indicated by maximum reduction in the expression of CD34 ( $0.019 \pm 0.00$ -fold), SOX2 ( $0.586 \pm 0.03$ -fold), SHH (negligible), POU5F1 ( $0.329 \pm 0.01$ -fold), and FRAP1 ( $0.332 \pm 0.01$ -fold) when compared with unexposed cells of the respective day. In totality, the expression level of the mRNA of these genes was significantly lower than unexposed cells of the respective days, except in case of a non-significant increase of POU5F1 at day 2 of differentiation (Fig. 5a).

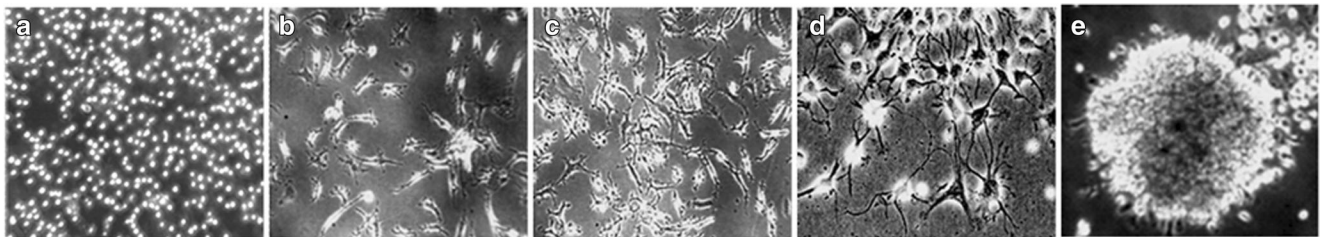
In general, all the neuronal markers have shown significant responses against the exposure of MCP. Cells at day 4 of differentiation have shown maximum downward alterations in the expression of mRNA of most of the neuronal genes studied except NCAM ( $2.81 \pm 0.34$  maximum at day 4), NGF ( $0.49 \pm 0.01$  and maximum at day 8), and MAP2 (negligible at day 4). Cells at day 2 of differentiation were also found to be

vulnerable to MCP exposure as evidence by CHRM2 ( $0.52 \pm 0.02$ ), NGF ( $0.10 \pm 0.001$ ), and NFM ( $0.27 \pm 0.01$ ). Significant downregulation was observed in the expression of early neuronal markers such as NES ( $0.71 \pm 0.02$ ;  $0.370.71 \pm 0.01$ -fold of unexposed control) at days 4 and 8 of differentiation and CHRM2 ( $0.520 \pm 0.02$ ;  $0.270 \pm 0.001$ ;  $0.040 \pm 0.00$ ) at days 2, 4, and 8 of differentiation (Fig. 5b).

MCP-induced alterations in the mRNA expression of apoptosis marker genes are presented in Fig. 5c. Similar to the expression pattern of neuronal markers, cells at day 4 of differentiation were found to be most vulnerable to MCP exposure. At day 4, significant ( $p < 0.001$ ) upregulation in the expression of pro-apoptotic genes, i.e., Bax ( $2.04 \pm 0.17$ -fold), Bad ( $2.11 \pm 0.16$ -fold), Caspase-9 ( $1.340 \pm 0.08$ -fold), Caspase-3 ( $1.44 \pm 0.12$ -fold), was observed at  $10^{-5}$  M concentration after 3 h, whereas downregulation was observed for anti-apoptotic genes such as Bcl<sub>2</sub> ( $0.63 \pm 0.04$ -fold). CAPN1 ( $0.66 \pm 0.03$ -fold), CAPN2 ( $0.26 \pm 0.01$ -fold), and TP53 ( $0.16 \pm 0.16$ -fold) were also found significantly downregulated at day 4 of differentiation. On the other hand, same MCP exposure could not pose any significant alterations in the mRNA expression of Bad, Bax, Caspase-8, Caspase-9, Caspase-3, TP53, and CALP2, in cells differentiated for the days 2 and 8.

MCP-induced alterations in the mRNA expression of MAP kinases and immediate early response genes, viz., C-fos and C-jun are presented in Fig. 5d. Data show the significant downregulation of MAP3K5 ( $0.29 \pm 0.001$ -fold), MAPK1 ( $0.66 \pm 0.02$ -fold), and MAPK11 ( $0.547 \pm 0.02$ -fold), in cells differentiated for 4 days. However, the levels of mRNA for MAPK1 ( $1.91 \pm 0.09$ -fold) and MAPK10 ( $4.74 \pm 0.52$ -fold) were found upregulated significantly ( $p < 0.001$ ) at day 2 and 4 of differentiation, respectively. Cells have also shown significant ( $p < 0.001$ ) upregulation of mRNA for C-FOS ( $2.13 \pm 0.31$ -fold) whereas downregulation in the expression of C-JUN ( $0.59 \pm 0.03$ -fold) at differentiated day 4 when compared with unexposed control cells of the same day of differentiation.

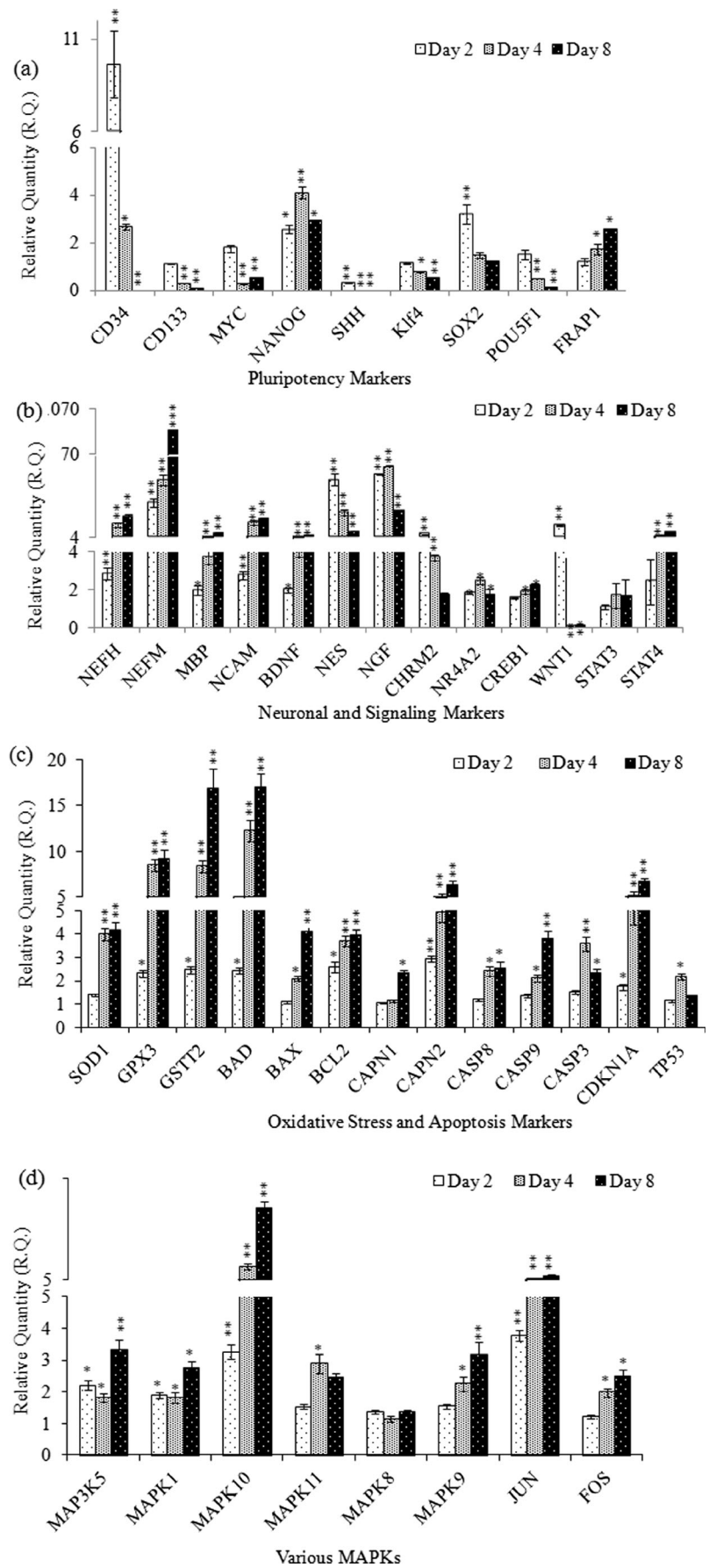
MCP-induced alterations in the mRNA expression of genes involved in oxidative stress and metabolism are presented in Fig. 5e. MCP-induced responses were the most significant



**Fig. 1** Human cord blood stem cells were cultured in myelocult medium for proliferation and differentiated into neuronal lineage using neurobasal medium containing different growth factors. Images were taken at  $\times 200$  magnification using Leica phase contrast microscope with Leica IM50 software (Leica Microsystems). **a** Purified population of umbilical cord

blood stem cell (UCBSCs) in culture at day 0. **b** Early-differentiating neuronal cells from UCBSCs at day 2. **c** Mid-neuronal differentiation at day 4. **d** Well-differentiated neuronal cells at day 8. **e** Floating colony in proliferation medium containing different growth factors

**Fig. 2** Growth factors induced alterations in the transcriptional expression of markers involved in stemness markers (a), neuronal markers (b), apoptosis marker (c), and different MAP kinases (d) using TaqMan low-density array (TLDA) PCR analysis at days 2, 4, and 8). \* $p < 0.05$ , \*\* $p < 0.001$



( $p < 0.001$ ) for CYP2B6 (5.60±0.86-fold) and CYP2E1 (4.82±0.72-fold) in cells differentiated for 8 days. However, the expression of these cytochrome P450s got downregulated significantly at days 2 and 4 of differentiation. MCP exposure also poses significant reduction in the mRNA expression of GPX3 (0.28±0.06-fold), CAT (0.55±0.01-fold); CYP2E1 (0.14±0.00-fold) and GSR (0.24±0.001-fold) at day 4 of differentiation. MCP-induced changes were non-significant for the expression of GSTP1 and GSTT2, all through the differentiation.

Similar to the other markers, the mRNA expression of transcription factors, viz., PTEN (0.62±0.06), DLG4 (0.2±0.01), EGR1 (0.4±0.02), and COX10 (0.56±0.03) were affected maximum in cells at day 4 of differentiation. Interestingly, all the transcription factors studied were showing up-regulation in the expression levels of mRNA by day 2 of differentiation, but thereafter, there was a downregulation, except in case of NFKB1 (1.2±0.09) at day 4 of differentiation (Fig. 5f).

#### Translational Changes in Marker Proteins in Differentiating Cells Exposed to MCP

The Western blot analysis of MCP-induced ( $10^{-5}$  M for 6 h) changes to the expression of markers involved in neuronal differentiation, apoptosis, and oxidative stress in differentiating cells are summarized in Fig. 6. In a general trend, the expression level of marker proteins, viz., Bad, Bax, Bcl2, activated Caspase-9 and Caspase-3, pP38, and pJNK1/2 were increased till day 8 of differentiation. The 4 days differentiated

cells were showing maximum vulnerability to the MCP exposure (Fig. 6a–c).

#### Induction of Apoptosis

The observations of TUNEL assay show that MCP ( $10^{-5}$  M) exposure for 6 h induces significant apoptosis (23.72±5.8 %) in 4 days differentiated cells, when compared with similarly differentiated unexposed control cells, i.e., 6.26±1.8 %. Similarly, MCP exposure induces apoptosis (10.76±1.5 % and 10.95±2.8 %) in cells at 2 and 8 days of differentiation, which was statistically significant ( $p < 0.001$ ) in respect to the corresponding unexposed control cells, i.e., 2.46±2.52 % (2 days) and 6.95±1.8 % (8 days) (Fig. 7a–f).

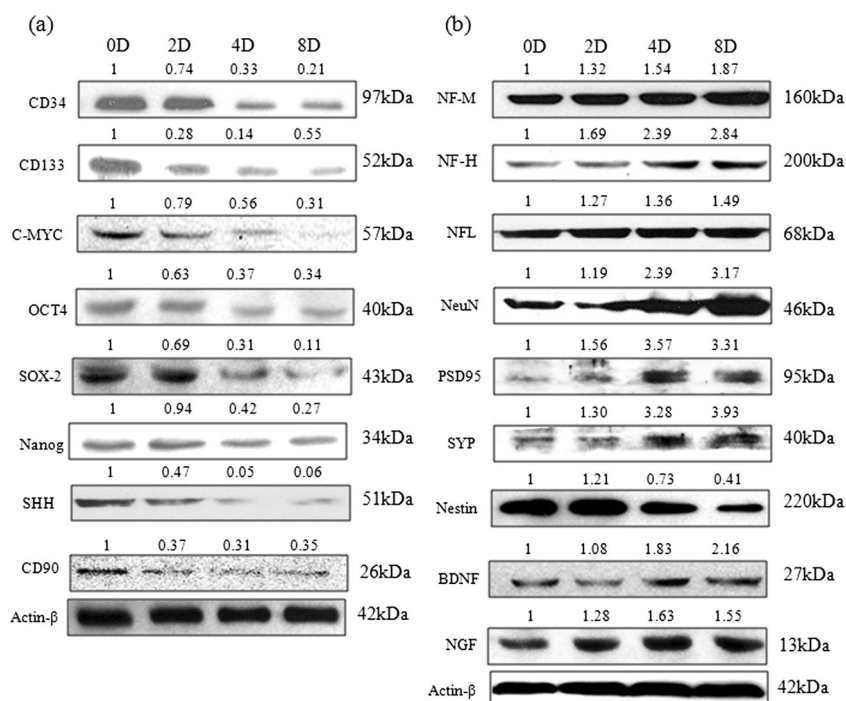
#### Acetylcholinesterase activity and acetyl choline levels

The acetylcholinesterase activity was found to be significantly decreased while the levels of acetylcholine were found to be significantly upregulated in the MCP-exposed differentiating cells of day 4 (Fig. 8a and b). The different MAPK inhibitors, viz., SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and apoptotic inhibitor zDEVD-fmk, could check the altered levels of these neuronal enzymes and chemical in MCP-exposed differentiating cells of day 4 (Fig. 8).

#### Dopaminergic and Cholinergic Receptor Levels

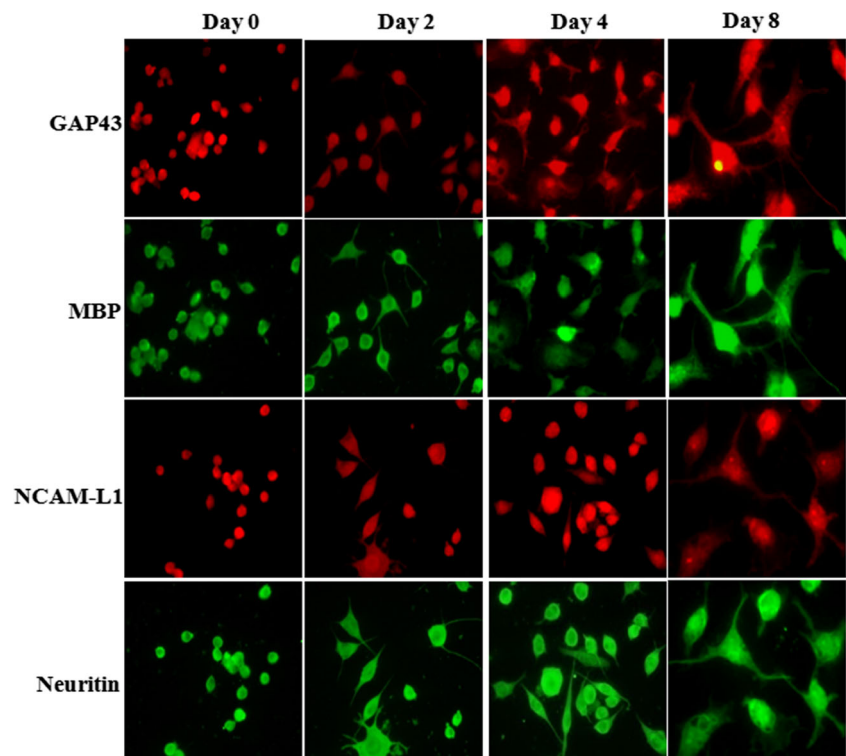
Result highlights from the experiments carried out to study the involvement of different neurotransmitter receptors

**Fig. 3** Translational analysis of growth factors induced alterations in the expression of markers involved in stemness (a) and neuronal differentiation (b) (Western blot analysis at days 0, 2, 4, and 8 day of maturities)





**Fig. 4** Representative microphotographs of immunocytochemical localization of neuronal marker proteins in stem cell-derived neuronal cells at various maturity, i.e., days 0, 2, 4, and 8 (0D, 2D, 4D, and 8D, respectively). Images were captured at  $\times 400$  magnification using Nikon Eclipse 80i upright fluorescence microscope equipped with Nikon DS-Ri1 12.7 megapixel camera, Japan. The specific filters used were for FITC and rhodamine



(cholinergic and dopaminergic) in the neurotoxicity of MCP-exposed mid-day 4 differentiating neuronal cells are represented in Fig. 8c and d. A significant decrease in specific binding of 3H-Spiperone and 3H-QNB was observed after the exposure of MCP ( $10^{-5}$  M for 6 h) in differentiating cells of day 4 while different MAPK inhibitors, viz., SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), and apoptotic inhibitor zDEVD-fmk, could check the reduction of these neuronal receptors in MCP exposed differentiating cells of day 4.

Levels of Choline Acetyltransferase (ChAT) and Tyrosine Hydroxylase (TH)

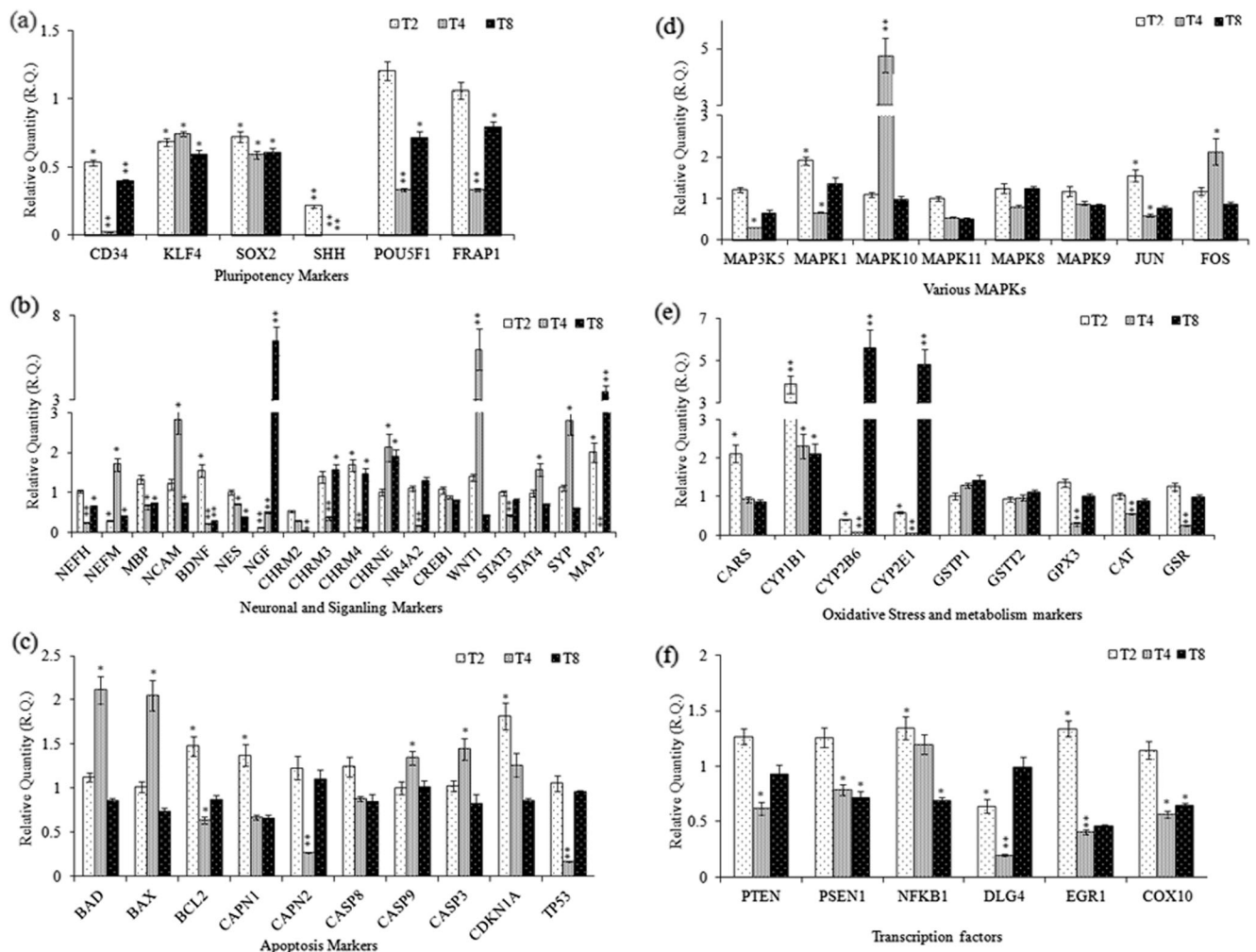
The results of immunocytochemistry confirm that the culture has the functional cholinergic and dopaminergic neuronal cells, which responded to MCP exposure. The response to MCP exposure was statistically highly significant in the cells differentiated for days 0, 2, and 4. However, the expression of both the enzymes was found to be reduced in cells differentiated for day 8, and response against MCP also could not achieve significant levels (Fig. 9).

## Discussion

The present investigations were aimed to study the neuronal differentiation of hUCBSCs and their applicability to use as an in vitro tool for assessing chemical-induced developmental

neurotoxicity. Human umbilical cord blood was selected to obtain the stem cells, as it has been reported as one of the richest sources of stem and progenitor cells [30–32] and also has comparatively less ethical concerns, as placenta is a waste material after the birth of child [13]. The various cytokines and growth factors such as TPO, FLT-3 ligand, SCF, FGF-basic, etc., are known to play their key role in early hematopoiesis, proliferation of hematopoietic progenitors [33–36], suppression of apoptosis in  $CD34^{+}CD38^{-}$  cells in culture [37], short-term expansion and regulation of surface antigens [38], and improving the homing capacity of umbilical cord blood cells [39].

The cord blood-derived  $CD34^{+}Thy1^{+}$  hematopoietic stem cells have a high commitment towards neuronal lineage, similar to fetus-derived neural stem cells [31, 40, 41]. Using a cocktail of various growth factors such as recombinant rhNGF in serum-free neurobasal medium, we found that  $CD34^{+}Thy1^{+}$  hematopoietic stem cells can be induced to undergo morphological and phenotypic changes consistent with developing neuronal cells. NGF is a potent neurotrophic factor that regulates development, maintenance, and function of the nervous system [42]. NGF works through its receptor TrkA (tropomyosin-related kinase) and plays a crucial role in the neuronal differentiation via activating different MAPKs [43, 44]. These differentiated cells showed a typical neuron-like morphology and expressed various cell type-specific markers for neurons. The data from quantitative real time-PCR and Western blot analysis revealed significant expression of various neuronal markers, viz., NEFH, NEFM, MBP, NCAM,



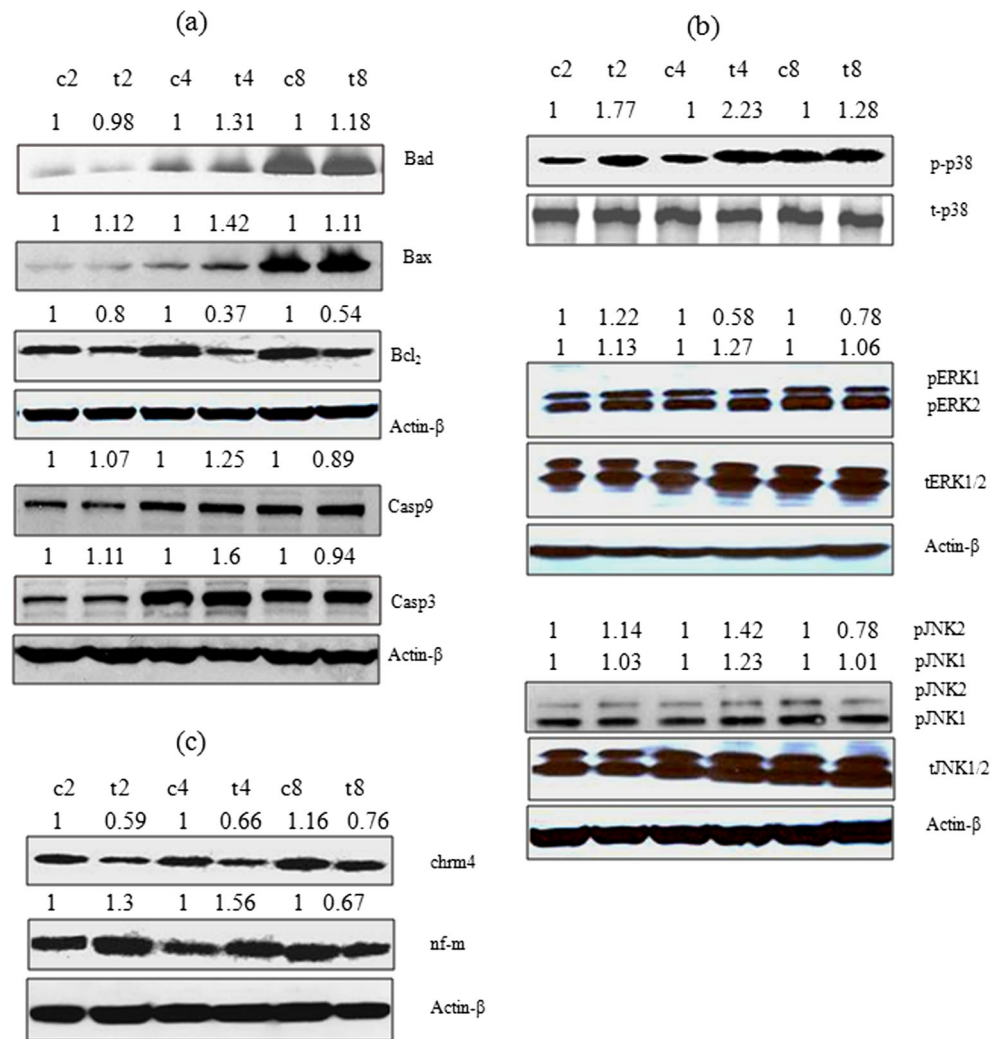
**Fig. 5** MCP-induced alterations in the levels of expression of mRNA of genes involved in pluripotency (a), neuronal differentiation (b), apoptosis (c), various MAPKs (d), oxidative stress and metabolism (e), and transcription factors during different maturation stages of differentiating

neuronal cells derived from human umbilical cord blood stem cells. (T2: 2 days differentiated cells, T4: 4 days differentiated cells, T8: 8 days differentiated cells). \* $p < 0.05$ , \*\* $p < 0.001$

BDNF, etc., which were found elevated after the onset of differentiation from days 2 to 8. The expression of NGF was also recorded increasing during differentiation and reduced at day 8, showing the complete maturity and thus the decrease in the endogenous expression. The expression of WNT was completely shut down during the days 4 and 8 which were showing the hinderance of cell division during differentiation and further confirms the process of normal differentiation. Nestin is a well-known marker of neural precursor cells, which was found to be upregulated at day 2, which concludes that the differentiation started from this time point. The elevated expressions of these neuronal markers are consistent with the observations of Schez Ramos et al. [45] and Zigova et al. [46]. The reduction of early markers of neuronal differentiation during the increased differentiation process further confirms a normal differentiation process. Different MAPKs such as Mapk 1/9/10/11, MAP3Ks, ASK1, CREB, as well as apoptotic markers, viz., BAX, Bcl2, Casp-8, Casp-9, BAD,

Calp 1, and Calp 2, were increased during increased differentiation, showing their direct involvements in the process of differentiation. Oxidative markers, viz., GPX3, SOD1, SOD3, and GSTT2, also have shown their involvement in the maintenance of homeostasis in differentiating neuronal cells, derived from cord blood stem cells. MAPKs are serine/threonine-specific protein kinases that stimulate under the influence of various extracellular stimuli such as xenobiotics, mitogens, stresses, and pro-inflammatory cytokines and regulates various cellular multiple signaling pathways involved throughout the process of mitosis, differentiation, proliferation, and cell survival/cell death [47]. Different types of MAPKs have shown their role in the variety of cells differentiation, including neuronal cells [48, 49] [1, 2], osteogenic differentiation [50, 51], and angiogenesis [52]. Zegui and colleagues [53] have shown that extracellular signal regulated kinase (ERK)1/2 phosphorylation is an early signaling event required for the neuronal differentiation. They also showed a dramatic

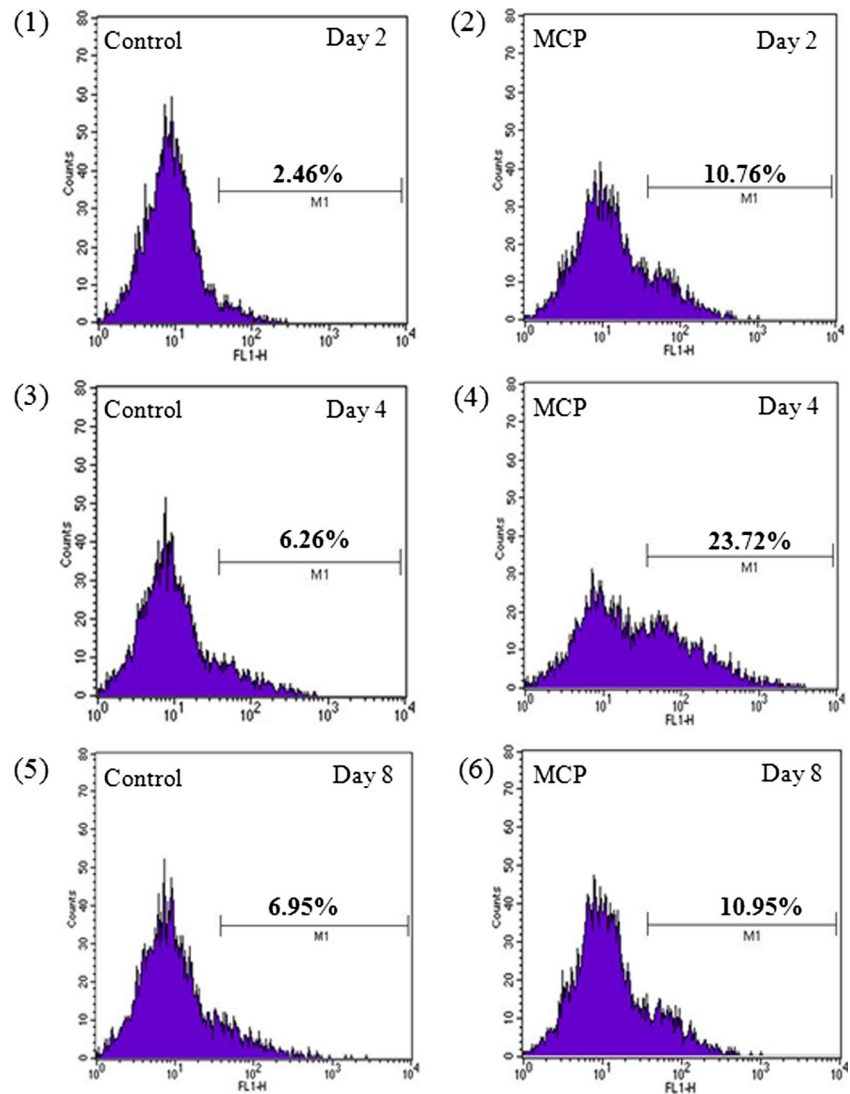
**Fig. 6** MCP-induced alterations in the levels of expression of proteins involved in apoptosis (a), various MAPKs (b), and neuronal markers during different maturation stages of differentiating neuronal cells derived from human umbilical cord blood stem cells (c). Lane 1 (c2): unexposed cells at day 2 of differentiation; lane 2 (t2): cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 2 of differentiation; lane 3 (c4): unexposed cells at day 4 of differentiation; lane 4 (t4): cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 2 of differentiation; lane 5 (c8): unexposed cells at day 8 of differentiation; lane 6 (t8): cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 2 of differentiation, \* $p < 0.05$ , \*\* $p < 0.001$



increase in ERK1/2 phosphorylation (p-ERK 1/2) 1–5 days after differentiation, which was attenuated in the presence of the p-ERK 1/2-specific inhibitor U0126 confirmed by significant reduction in the number of NeuN- positive cells and the expression of associated cytoskeletal proteins [53]. Many studies have proven non-apoptotic role for pro-apoptotic genes in the regulation of gene expression at hindbrain boundaries. The ectopic expression of hindbrain boundary markers is also dependent on TP53 activity and its downstream apoptotic effectors. The pro-apoptotic Bcl-2, puma, and bax are required for hindbrain boundary marker expression, and gain of function of the Bcl2–caspase pathway leads to ectopic boundary marker expression [54]. In many instances, the kinases affected by caspase activity also mediate signals that provoke the phenotypic changes associated with cellular differentiation/transformation. Enzymes such as MEK kinase 1 (MEKK1), ste20-like kinase (SLK), and the p21-activated kinases have important roles in cellular remodeling and are known to be cleavage-activated by caspase-3. In the cellular context investigated, these kinases were reported to promote apoptosis. Nonetheless, it is conceivable that caspase-

directed activation of these kinases may also have non-apoptotic outcomes when activated under a different circumstance [55]. Caspase-8 is critical for embryonic development as mice deficient in this caspase die in utero with accumulated defects within the heart and neural tube and show deficiencies in hemopoietic progenitor cell production [56–58]. Calpains are also necessary for differentiation process during development because they are involved in the breakage of Myc proteins, which are deeply involved in multiple biological processes, including cell proliferation, growth, metabolism, apoptosis, differentiation, and tumorigenesis. Myc protein have been found to be capable of both inhibiting and facilitating differentiation depending on the biological context. Recently, people have identified a new mode of Myc regulation in differentiating muscle cells in which c-Myc protein is proteolytically cleaved by calcium-dependent calpains in the cytoplasm. This cleavage serves two purposes. First, it inactivates the transcriptional function of Myc by removing its C terminus, a region responsible for the interaction of Myc with Max and DNA. Second, it alters cytoskeletal architecture and accelerates muscle differentiation through the activity of the

**Fig. 7** Detection of apoptosis in cultured differentiating neuronal cells derived from human umbilical cord blood stem cells exposed to MCP ( $10^{-5}$  M) for 6 h. Apoptosis was detected by terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) assays using APOBrdU TUNEL Assay Kit with Alexa-Fluor-488 anti-BrdU (Molecular Probes, Invitrogen Detection Technologies; Catalog No. A23210) by a flow cytometer (BD-LSRII) and analyzed by Cell Quest 3.3 software. (1) Unexposed control population of early differentiation neuronal cells of day 2 derived from human umbilical cord blood stem cells. (2) neuronal cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 2 of differentiation. (3) Unexposed control population of mid-differentiation neuronal cells of day 4 derived from human umbilical cord blood stem cells. (4) neuronal cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 4 of differentiation. (5) Unexposed control population of differentiation neuronal cells of day 8 derived from human umbilical cord blood stem cells. (6) neuronal cells exposed to MCP ( $10^{-5}$  M) for 6 h at day 8 of differentiation. Graphical representation of mean  $\pm$  SE values of triplicate experiments carried out for detection of apoptosis induction



remaining N-terminal cleavage product (termed Myc-nick) [59]. In differentiating ES cells, there was increased nuclear translocation of STAT3 and decreased protein expression levels of GDNF, BDNF, and NGF [53]. We also found the reduced levels of NGF in fully mature neuronal cells, but the levels of BDNF were increased during differentiation. The different origin of stem cells cannot be denied behind these unparallel results. The gradual STAT4 with neuronal differentiation shows the stronger role to this transcription factor in the differentiating neurons derived from UCBCs.

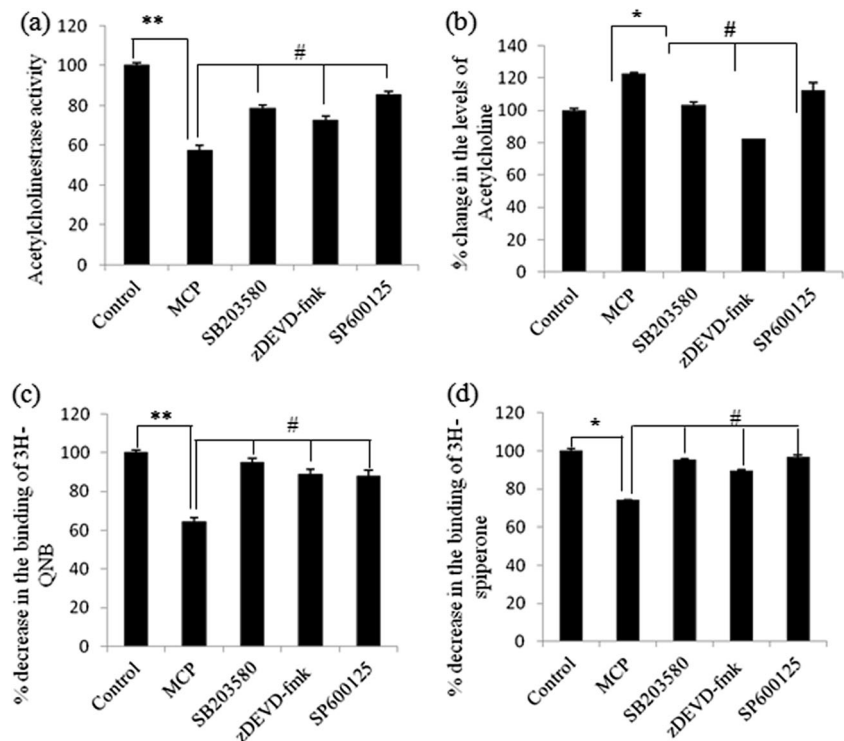
The mRNA expressions of cholinergic receptors like CHRM2/CHRM3 (muscarinic type of receptor) and NR4A2 (dopaminergic precursor), as well as protein expressions of acetyl cholinesterase (AChE) were found expressed after the onset of differentiation from days 2 to 8, which confirmed that the neurons differentiated from stem cells may be of mixed cholinergic and dopaminergic types. At the same time, the expression of artemin

(ARTN) was found elevated during differentiation, which has been shown to support the survival, neurite outgrowth, cell migration, and differentiation of peripheral neurons [60, 61]. A significant expression of dopamine DA-D2 and cholinergic receptor also confirm mixed cholinergic and dopaminergic neuronal population.

Notably, the expression of CD34, CD133, Nanog, and MYC, cell type markers for hematopoietic stem cells, were found decreasing all through the differentiation of stem cells into neuronal cells, and by day 8, the markers of stemness were almost diminished, which again strongly support normal neuronal differentiation. The expressions of few of the stemness markers were found increased at day 2 of differentiation. This might be due to immediate homeostatic response of cells against anti-proliferative activity due to growth factors inducing neuronal differentiation in the cells against their wish. To find out the suitability of these differentiating neuronal cells derived from UCBCs as a tool for developmental



**Fig. 8** Activity of acetylcholinesterase enzyme (a) and levels of acetylcholine (b) in monocrotophos exposed to 4 days differentiated neuronal cells derived from human umbilical cord blood cells were assessed by using fluorescence-based Amplex red acetylcholine/acetylcholinesterase assay kit (catalog no. A12217; Molecular Probes; Invitrogen Detection Technologies, USA). The levels of different neuronal receptors, viz., cholinergic (c) and dopaminergic (d) were calculated by radioligand receptor binding assay using Robotic Liquid Handling System (Multi Probe II EX, Packard, USA) in multiwell plates with the help of WINPREP software



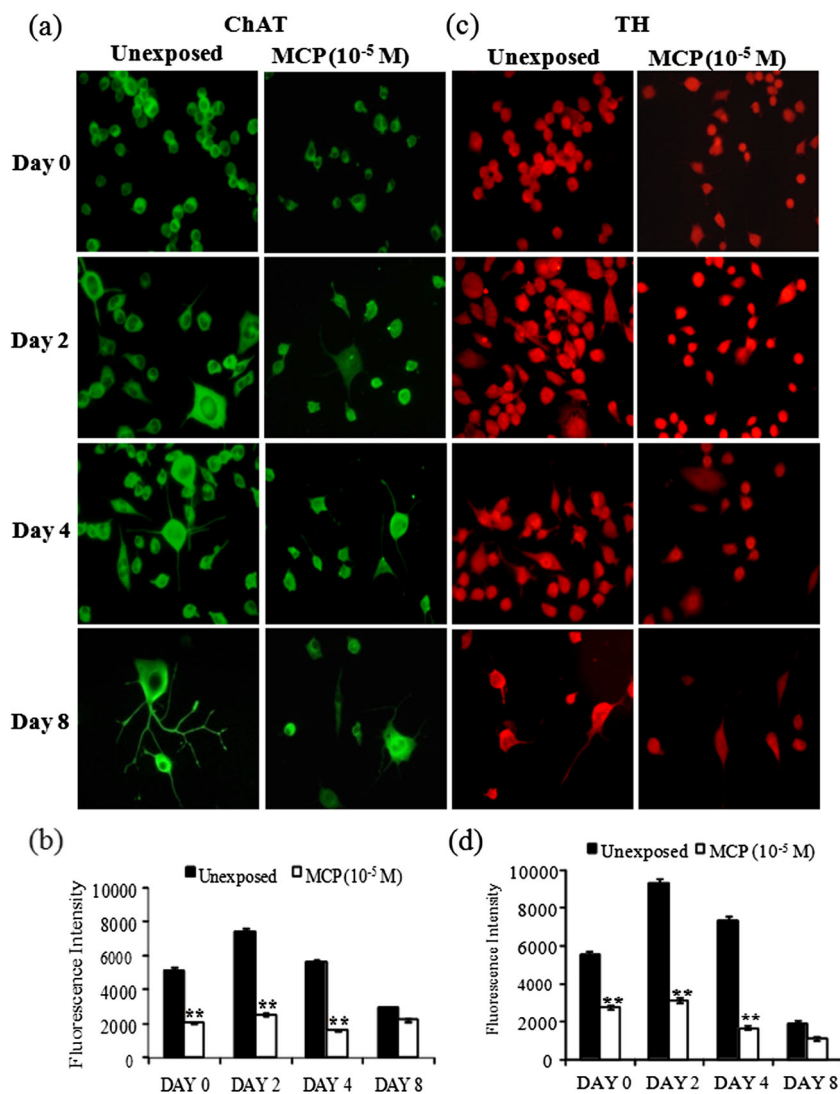
neurotoxicity, we exposed these differentiating cells to known neurotoxicant-monocrotophos.

Higher concentrations of MCP have already been reported to be cytotoxic in PC12 cells [12, 14, 62], Caco-2 cells [62], and primary cultures of peripheral blood lymphocytes and hemocytes [63–65]. Consistent with these reports, higher concentrations of MCP have been found to be cytotoxic in stem cells derived from differentiated and undifferentiated neuronal cells at various points of maturation [15]. These higher concentrations of MCP caused physiological stress and led to the cell death at 24 h onwards in human stem cell-derived neuronal cells. MCP at lower concentrations does not cause physiological stress, and the effects were non-significant until the end of incubation. It has been suggested that these kinds of observation could be obtained due to mitochondria-mediated apoptosis, as reported in PC12 cells. Some workers have suggested that MCP exerts cytotoxicity by inducing signaling pathway in the production of cytokines linked with cellular injury and apoptosis [66]. Possible involvement of oxidative stress either by generating reactive oxygen and nitrogen species or by the stress-activated transcription factors such as NF kappa B, AP-1, and P53 cannot be ignored [12, 14].

In order to find out the responsiveness of the markers involved in the neuronal differentiation, damage, and the repair processes, these differentiating cells were exposed to

MCP at various points of maturity. Although the cells were showing significant responsiveness against MCP all through the differentiation, the mid- and early differentiating cells at days 4 and 2 of differentiations were found to be more vulnerable than well-differentiated mature cells to the toxic insult of MCP. In our investigations, we observed the downregulation in the markers of neuronal pluripotency, viz., CD34, POU5F1, FRAP1, SOX2, KLF4, and differentiation, viz., NFH, MBP, BDNF, CHARM3 and 4, NR4A2, STATE3, and MAP2 in mid-differentiating (day 4) cells exposed to MCP. Previously, we have reported that MCP interacts with the different residues in the kinase domain of AKT1 proteins, which are generally occupied by the ribose of ATP and hence cease phosphorylation of AKT1, which supposed to necessary for AKT1 activation and cell proliferation by inactivating GSK3 protein [13]. Reduced levels of FRAP (mTOR) in MCP-exposed mid-differentiating cells also confirm hindrance in AKT phosphorylation, as mTOR works as PDK2 and phosphorylates it at serine 473 residue on kinase domain. Kirby et al. [67] also reported that that SB216763, a glycogen synthase kinase-3 (GSK3) inhibitor, can maintain pluripotency in mouse and human ESCs via Canonical Wnt/ $\beta$ -catenin signalling. They further demonstrated that SB216763-treated mouse embryonic stem cells (mESCs) can be maintained in a pluripotent state with indistinguishable morphology in the absence of exogenous leukemia inhibitory factor when cultured on mouse embryonic fibroblasts and also having the expression of pluripotent-specific genes Oct4, Sox2, and Nanog. Elaboration with these data embryoid bodies

**Fig. 9** Representative microphotographs of immunocytochemical localization of neuronal markers choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) in stem cell-derived neuronal cells at various maturity, i.e., days 0, 2, 4, and 8 (0D, 2D, 4D, and 8D, respectively) in MCP exposed and unexposed control cells. Images were captured at  $\times 400$  magnification using Nikon Eclipse 80i upright fluorescence microscope equipped with Nikon DS-Ri1 12.7-megapixel camera, Japan. The quantification of ChAT and TH expression was done using the Image Analysis software Leica Q-Win 500. Data were calculated as mean of 6 SE of at least 20 fields from three independent experiments. **a** Localization of choline acetyltransferase (ChAT); **b** graph representing change in the expressions of choline acetyltransferase (ChAT) in MCP exposed and unexposed cells during different maturity; **c** localization of tyrosine hydroxylase (TH); **d** graph representing change in the expressions of tyrosine hydroxylase (TH) in MCP exposed and unexposed cells during different maturity



generated from these mESCs having early-stage markers for all three germ layers and also found to efficiently differentiate into cardiac-like cells and MAP2-immunoreactive neurons [67]. In accordance with this study, we also concluded that, in the current study, MCP works as an AKT1 inhibitor, and MCP-exposed differentiating cell populations could not inhibit GSK3 protein, resulting in the loss of markers involved in the process of pluripotency via hindering  $\beta$ -catenin. Several reports have also demonstrated the involvements of  $\beta$ -catenin as an important determinant of self-renewal and pluripotency [68–71]. Zimmer et al. [72] have also demonstrated suitability of in vitro models to estimate the alteration in the differentiation markers and neuronal functions such as catecholaminergic transmission in neuronal differentiating mESC exposed to subtle doses of methyl mercury (MeHg). Exposure to MeHg in differentiating cells downregulates several neuronal transcripts, including dopamine receptors and endogenous neurotrophic factors, which are required for the maturation of neurons. Our data elucidated

the downregulated various neuronal markers which was further confirmed by receptor binding assay for dopaminergic and cholinergic receptors as well as via estimating neurotransmission (AChE/ACh) enzyme within the cells of mid-differentiation (day 4) exposed to MCP. We also observed significant upregulation in apoptosis markers, viz., BAD, BAX, CASP 9 and 3, and in various MAPKs, viz., MAPK10 and FOS while significant downregulation was observed in BCL2, TP53, CALP2, JUN, MAPK6, MAP3K5, and MAPK1 in mid-differentiating (day 4) cells exposed to MCP. Role of different pro-apoptotic, anti-apoptotic, and caspase proteins are well defined in the different cells, including neuronal cells [12–14]. The high vulnerability of mid-differentiating cells towards apoptosis in comparison to mature neuronal cells reflects the developmental neurotoxic behavior of monocrotophos. Data of genes involved in the apoptosis during differentiation and MCP-exposure indicate that the upregulated levels of caspase-3 in the day 4 differentiated cells may be one of the reasons to switch its role from

differentiation to apoptosis after the MCP-exposure in comparison to unexposed differentiating cells of day 4 which could also be confirmed by assessing higher TUNEL-positive cells. The downregulated levels of markers associated with oxidative stress and xenobiotics metabolism enzymes, viz., GPX, CAT, GSR, CYP2B6, CYP2E1, as well as transcription factor involved in neurogenesis, viz., DLG4, EGR, and COX10 in mid-differentiating cells exposed to MCP also confirms our hypothesis that these cells are under much more pressure of apoptosis in comparison to exposed population of differentiating cells of other maturity and is well correlated to the data of markers associated with apoptosis. The increased levels of pro-apoptotic genes, viz., BAD, Bax, Caspases, Calpain, and reduced levels of xeno-metabolizing P450s, viz., 2B6, 2E1, and anti-oxidative markers, viz., catalase, GSR, GPX3, as well as anti-apoptotic Bcl2 protein into MCP-exposed day 4 differentiated seems to be the main cause of neurotoxicity on this stage. The downregulated levels of CALP2, JUN, MAPK6, MAP3K5, and MAPK1 may have loss of differentiation due to increased apoptosis in the mid-differentiating cells of day 4. Similar kind of alteration patterns are also there in stem cell-derived neuronal cells, as well as in developing rat brain [73–75] exposed to lithium-pilocarpine, 2.5 h hypoxia (92 % nitrogen and 8 % oxygen), and pilocarpine hydrochloride, respectively. In the parallel studies, Slotkin and colleagues have also reported the similar findings in PC12 cells exposed to organophosphorus/organochlorine pesticides [19, 20, 24].

Increased levels of acetylcholine, reduced activity of acetylcholinesterase enzyme, as well as reduced neuronal cholinergic and dopaminergic receptors confirms monocrotophos-induced neurotoxicity in stem cell-derived mid-differentiating neuronal cells. The specific inhibitors of different MAPKs (SB203580 and SP600125) and apoptosis (zDEVD-fmk) also confirm the involvement of different MAPKs and apoptosis in the process of monocrotophos-induced developmental neurotoxicity. We have already reported the involvements of reactive oxygen species, oxidative stress, and caspases in the MCP-induced neurotoxicity in PC12 cells, as well as human umbilical cord blood stem cells [12–14]. Significant AChE inhibition (50–82 %) in the rat brain regions are already reported within 2.5 h after exposure of monocrotophos in Wistar rats [76]. Inhibition of acetylcholinesterase is one of the positive interferences of neurotoxicity at low doses and could lead to developmental abnormalities, including reduced birth weight, length, and head circumference, as well as reflex abnormalities and affected mental development in neonates [7, 77–79]. The loss of neuronal receptors has also been reported as one of the main reasons for developmental neurotoxicity. The previous studies have already demonstrated the role of defective dopamine signaling in the impaired spatial learning and memory in children and adults with neurofibromatosis type 1 and Parkinsonism [80]. The current study also

reflects poor neuronal transmission, as cholinergic receptors are also necessary for transmission of information from one neuron to another and finally to lead to information on the form of action.

## Conclusion

A remarkable observation of the present study is that chemical-induced neuronal cells derived from human umbilical cord stem cells could be used as a powerful tool to assess the developmental neurotoxicity in human beings with more precision in comparison to other tumor cells of humans or rodents.

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