**Integrative genomic and proteomic profiling of human neuroblastoma SH-SY5Y cells
reveals signatures of endosulfan exposure**

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Abstract:

Endosulfan, an organochlorine pesticide, is known to induce multiple disorders/abnormalities including neuro-degenerative disorders in many animal species. However, the molecular mechanism of endosulfan induced neuronal alterations is still not well understood. In the present study, the effects of sub-lethal concentration of endosulfan (3 μ M) on human neuroblastoma cells (SH-SY5Y) was investigated using genomic and proteomic approaches. Microarray and 2D-PAGE followed by MALDI-TOF-MS analysis revealed differential expression of 831 transcripts and 16 proteins in exposed cells. A gene ontology enrichment analysis revealed that the differentially expressed genes and proteins were involved in variety of cellular events such as neuronal developmental pathway, immune response, cell differentiation, apoptosis, transmission of nerve impulse, axonogenesis etc. The present study attempted to explore the possible molecular mechanism of endosulfan induced neuronal alterations in SH-SY5Y cells using an integrated genomic and proteomic approach. Based on the gene and protein profile possible mechanisms underlying endosulfan neurotoxicity were predicted.

Keywords: Endosulfan; neuroblastoma cells (SH-SY5Y); genomics; proteomics.

Abbreviations: API5, Apoptosis inhibitor 5; AIFM2, Apoptosis-inducing factor mitochondrion-associated 2; HTT, Huntingtin; FOS, FBJ murine osteosarcoma viral oncogene homolog; FADD, Fas (TNFRSF6)-associated via death domain; EGR1, Early growth response 1; ACIN1, Apoptotic chromatin condensation inducer 1; XRCC5, X-ray repair complementing defective repair in chinese hamster cells 5; G-CSF, Granulocyte-colony stimulating factor; ZNF244, Zinc finger protein 244

1. Introduction

Organochlorine pesticides are being extensively used since 1950 in agriculture and other sectors (Peyre et al., 2012). Most of them have been totally banned or restricted in many countries including India because of their persistence and toxicity. One such chlorinated cyclodiene insecticide is endosulfan, which has been widely used on crops like soya, cotton, tea, coffee, cashew and vegetables in many countries (Homme et al., 2015). Total consumption of the endosulfan in India was reported 15,537 metric tonnes during the period of 2005-2010 (Gandhi et al., 2015). Endosulfan has been classified as persistent organic pollutant owing to its persistence and toxic nature (UN, 2011). Following properties of endosulfan signify its persistence, toxicity and long range transport in the environment; a high octanol-water partition coefficient (K_{ow}) value 3.55-3.62, allows endosulfan to bioaccumulate/bioconcentrate in different organs of the biological systems. It exerts toxic effect through various routes of exposure such as body contact, inhalation and ingestion. Being vaporized at lower temperature and being persistent in the environment for long period of time, endosulfan can be transported over long distance. Endosulfan has been banned in many countries, but it's still used in some countries including India at a large scale (Songa et al., 2012).

Epidemiological and toxicological data revealed hepatotoxicity (Paula et al., 1995), neurotoxicity (Silva and Gammon, 2009), and teratogenicity (Gupta et al., 1978) along with endocrine- (Bisson and Hontela, 2002), immuno- (Banerjee and Hussain, 1987) and reproductive dysfunctions (Silva and Gammon, 2009) upon chronic and acute exposure to endosulfan. Endosulfan exerts genotoxic effects in mammalian germ cells (Pandey et al., 1990), bacterial cells (Chaudhuri et al., 1999), human lymphocytes (Kumar et al., 2011), and hepatocellular

carcinoma (HepG2) cells (Lu et al., 2000). In addition, exposure to endosulfan has been reported to induce oxidative stress (Shao et al., 2012) and apoptosis (Rastogi et al., 2014).

Since endosulfan can cross blood brain barrier, neuro-degenerative properties of endosulfan constitute the principle end point of concern. Work presented in several articles has demonstrated that exposure to endosulfan results in variety of behavioral deficits and neurological disorders comprising of epilepsy, convulsion, hyperactivity, behavioral impairment and paralysis (Usha and Harikishan, 2004; Paul and Balasubramaniam, 1997). Recently, elevated level of brain dopamine and serotonin has been reported in amphibians due to exposure to endosulfan (Homme et al., 2015).

Although the above mentioned studies demonstrate neuro-degenerative properties of endosulfan, the mechanism underlying these alterations is poorly understood at molecular level. The present study attempted to explore the possible molecular mechanism of endosulfan induced neuronal alterations in a dopaminergic human neuroblastoma cell line (SH-SY5Y) using an integrated genomic and proteomic approach. Genomic and proteomic signatures identified in this study can be further explored for the development of specific neurodegenerative biomarkers of endosulfan exposure.

2. Materials and methods

2.1. Chemicals

Minimum essential medium (MEM), F-12 nutrient medium, fetal bovine serum (FBS), streptomycin, penicillin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen (Life Technology, Spain). Dimethyl sulfoxide (DMSO) and endosulfan [mixture of endosulfan α and β isomers (7:3)] were procured from Sigma Aldrich

(USA). The stock solution of endosulfan was prepared in DMSO. A final concentration of DMSO (1% v/v) was used in the cell culture studies.

2.2. Cell culture

SH-SY5Y cell line was procured from American Type Culture Collection. The cells were grown in Eagle's MEM/Ham's F-12 (1:1), supplemented with 10 % of FBS, 10 U/ml of streptomycin, 10 mg/ml of penicillin, 1 mM sodium pyruvate, and 1 mM non-essential amino acid. Cells were maintained in the humidified incubator at 37°C and 5% CO₂.

2.3. Cell viability assay

Cell viability of SH-SY5Y cells upon exposure to endosulfan was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate (2×10^4 cells/well in 200 µl of medium). The plate was incubated at 37°C and 5% CO₂ for 24 h to allow attachment of the cells to plate surface. After the incubation cells were treated with various concentrations (2.5, 5, 10, 20, 30, 40, 50, 100 µM) of endosulfan for 24 h. 1% (v/v) of DMSO served as vehicle control. The cells were further processed for MTT assay as described earlier (Gandhi et al., 2015). The assay was performed in triplicate, and the results were expressed as percent of viability compared to the control cells. Lethal concentration-50 (LC₅₀) of endosulfan was estimated from dose response curve.

2.4. Microarray preparation and processing

Cells were exposed to sublethal concentration (3 µM) of endosulfan for 24 h. Total cellular RNA was isolated using Rneasy mini kit (Qiagen, UK) according to the manufacturer's protocol. RNA

was quantified with NanoDrop 1000 spectrophotometer and its integrity was verified using Bioanalyzer 2100 (Agilent, USA). Affymetrix Primeview GeneChips (Human), containing 49395 probes corresponding to 23000 human genes, were used to identify changes in gene expression pattern in response to endosulfan exposure. Fifty nanogram of total RNA was converted into Cy3 labeled cRNA using Quick Amp kit (Agilent Technologies, CA) as described in the manufacturer's protocol. The A260/280 nm ratio and quantity of the cRNAs were determined using Agilent Bioanalyzer. Hybridization of each sample was carried out at 48°C for 16 h. Hybridized microarray slides were washed according to manufacturer's instruction, and scanned using microarray scanner (Agilent, USA). The experiment was performed in triplicate.

2.5. Microarray data analysis

Microarray data were first normalized with Gene spring Gx12.5 software (Agilent Technologies, CA). Genes with signal intensity < 20.0 percentile were removed from analysis to eliminate background noise followed by pre-processing, normalization and quality control analysis. Differential gene expression between control and exposed samples, was defined based on three statistical parameters: a) fold change of ≥ 1.5 or ≤ -1.5 (exposed vs. control); b) a false discovery rate corrected q-value < 0.05; and c) $p \leq 0.05$ [analysis of variance (ANOVA)]. Gene microarray data have been submitted to National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (accession no. GSE65140). Gene functional analysis and clustering was performed using, Database for Annotation, Visualization, and Integrated Discovery (DAVID).

2.6. Quantitative polymerase chain reaction (qPCR)

We validated microarray results with qPCR using the $\Delta\Delta C_t$ method. Total RNA was isolated following the same procedure used in microarray experiment. RNA was converted to cDNA using cDNA reverse transcription kit (Applied Biosystem, USA). qPCR was carried out using SYBR green master mix (Applied Biosystems, USA) on ABI 7200 thermal cycler. PCR primers used for the validation of the selected genes are given in Table 1. RNA polymerase II (RP II) was used as the housekeeping gene [3]. After initial denaturation at 95°C for 10 min, reactions were cycled 40 times using the following parameters: 95°C for 15 s, primer annealing at 57-58°C for 10 s, and primer extension at 72°C for 30 s. The C_t value of each target gene was normalized with C_t value of housekeeping gene (RP II). The experimental design included three biological replicates and two technical replicates within each biological replicate. Relative fold change between control and exposed samples was calculated using $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) and statistical significance was determined using t-test at $p < 0.05$.

2.7. Two-dimensional electrophoresis (2DE)

The cells were exposed to sublethal concentration (3 μ M) of endosulfan for 24 h and 1% DMSO as control. After 24 h exposure, cells were harvested for protein extraction (Dhondge et al., 2012). Isoelectric focusing (IEF) was performed on immobilized pH gradient strips (17cm, pH 4-7) followed by electrophoresis on 12% SDS-PAGE gel. Image analysis and densitometry were performed as described (Dhondge et al., 2012). The experiment was performed in triplicate and the significantly ($p < 0.05$) differentially expressed protein spots were excised by robotic spot cutter (Bio-Rad, USA).

2.8. Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS)

Excised gel pieces were destained followed by tryptic digestion using In-gel tryptic digestion kit as per manufacturer's protocol (Thermo Scientific, USA). The digested peptides were extracted, dried completely by lyophilization and suspended in Tris-acetate buffer. The digested peptide sample was mixed with matrix solution containing 5 mg/ml α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% (v/v) TFA, and 2% (w/v) ammonium citrate (1:1) and spotted onto the MALDI plate and air dried (Bruker Daltonics, USA). The samples were analyzed by Ultraflex III MALDI-TOF-MS (Bruker Daltonics, USA) instrument and peptide mass fingerprint was acquired using Flex analysis software (Bruker Daltonics, USA). The obtained masses were analyzed using Mascot search (Matrix Science, UK) conducted against NCBI protein database to identify the respective proteins. Biological function of the identified proteins was determined using UniProt database.

3. Results

3.1. Cytotoxicity

Colorimetric MTT assay, measures the reduction MTT by mitochondrial succinate dehydrogenase enzyme. Since the MTT is reduced by metabolically active cells only, the level of activity is a measure of the viability of the cells. In the present study endosulfan was found to exert concentration-dependent increase in cytotoxicity ($p < 0.01$) in SH-SY5Y cells with LC_{50} value of 15.19 μ M (Fig. 1). Vehicle control (1 % DMSO) did not show any effect on cell viability when compared to control cells ($p < 0.01$). Based on these results, we choose sub-lethal

concentration of endosulfan (3 μ M) for further studies. Gene/protein expression changes were then characterized at sub-lethal concentration of endosulfan.

3.2. Effect of endosulfan on multiple cellular pathways

Microarray data analysis revealed (Fig. S1) differential expression of genes in endosulfan exposed cells. Out of the 831 differentially expressed genes, 137 genes showed up-regulation, whereas 694 genes exhibited down-regulation in exposed cells. Up-regulated (Fig. 2A) and down-regulated (Fig. 2B) genes were assigned functional categories based on gene ontology using DAVID database.

The up-regulated genes were found to be involved in variety of biological processes such as signal transduction (21%), regulation of metabolic process (13%), homeostatic process and transcription (8%), regulation of catalytic activity (8%), chromatin modification (8%), cell proliferation and differentiation (8%), cellular component biogenesis (8%), apoptosis (6%) and cell cycle process (2%). On the other hand large number of genes involved in RNA transport (26%), mitosis (13%) axonogenesis (11%), negative regulation of apoptosis (5%), histone acetylation (3%), immune response (2%) etc., were found to be down-regulated (Fig. 2B).

2D electrophoresis and MALDI-TOF-MS analysis revealed the differential expressions of 16 proteins in SH-SY5Y cells after 24 h exposure to endosulfan as compared to control (Fig. 4). Table 2 entails the list of 16 differentially expressed proteins identified with their fold change and biological functions. Out of the sixteen proteins, ten proteins were found to be down-regulated whereas the remaining proteins showed up-regulation. Biological functions of the identified proteins were analyzed using UniProt database. The differentially expressed proteins were found to be involved in various biological networks such as neuronal development, immune

response, transcription, cell adhesion, cytoskeleton organization, cell differentiation, calcium homeostasis and lipid metabolic process (Table 2).

3.3. *Effect of endosulfan on expression of genes and proteins involved in neuronal pathway*

Further analysis was carried out using UNIPROT and NEXTPROT databases to investigate the effect of endosulfan on expression of genes/proteins involved in neuronal pathway. Many differentially expressed genes (AIFM2, HTT, API5, FOS, FADD, XRCC5, and EGR1) and proteins (SDK2, G-protein regulated inducer of neurite out growth-1, Granulocyte colony-stimulating factor, polycystic kidney disease protein 1-like 1) were found to be involved in different functional networks of neuronal pathway. Genes identified through UNIPROT and NEXTPROT databases were further validated using quantitative RT-PCR. Results obtained by quantitative RT-PCR were in agreement with the results obtained by microarray analysis (Fig. 3). Table 3 represents the list of validated genes, their biological functions and fold change expression in microarray and qRT-PCR analyses ($p < 0.05$).

API5 expression was down-regulated compare to control. API5 is known to have neuro-protective role that is important for neuronal survival and synaptic plasticity during neuro-regenerative processes in the brain. Other genes [AIFM2 ($\uparrow$) and HTT ($\downarrow$)] and proteins [SDK2 ($\downarrow$) and G-protein regulated inducer of neurite out growth-1 ($\uparrow$)], which are essential for neuronal development (neurogenesis, neuron projection etc) were found to be differentially expressed.

Endosulfan exposure was also found to alter the expression of genes and proteins involved in the process of neurodegeneration and apoptosis. Anti-apoptotic genes such as API5 and HTT were found to be down-regulated, whereas pro-apoptotic genes (AIFM2, FOS, and FADD) were found to be up-regulated upon exposure to endosulfan. XRCC5, a gene involved in double strand

DNA repair, was found to be down-regulated, whereas EGR1 expression was up-regulated in exposed cells. Granulocyte colony-stimulating factor, a key protein associated with negative regulation of neuron death, was found to be down-regulated. In addition, polycystic kidney disease protein 1-like 1, protein involved in neuropeptide signaling pathway was found to be up-regulated.

4. Discussion

The present study elaborates the role of various genes and proteins along with their mode of action in response to endosulfan exposure on neuroblastoma cells. Endosulfan exerts neurotoxicity mainly through two modes. One is attributed to the chlorinated part which alters the electrophysiological and enzymatic properties of nerve cell membranes that in turn causes change in the kinetics of Na⁺ and K⁺ ion flow through the membrane. Additionally calcium transport of Ca²⁺-ATPase activity can be hindered along with disturbances in phosphokinase activities (Wayland and Laws, 1991). Second mode of action is due to the cyclodiene part which hampers the action of the neurotransmitter gamma-aminobutyric acid (GABA), which is responsible for uptake of chloride ions by neurons. This results in only partial repolarization of the neuron that leaves it in uncontrolled excitation state (Klaassen and Watkins, 1999). The molecular insights behind these disturbances in endosulfan exposed neuronal cells are still under-explored.

Limited reports are available on the neuronal alterations induced by endosulfan exposure. These investigations were mostly focused on neurotransmitters and a neurological disorder comprises of epilepsy, convulsion, hyperactivity and paralysis (Wilson et al., 2014; Usha and

Harikrishnan, 2004) and did not address the underlying molecular mechanism. Besides neurotransmitter signaling, neurological alterations also involve other functions such as synaptic plasticity, neuronal development, apoptosis, and neuro-peptide signaling. The present study reports different genes and proteins involved in endosulfan induced molecular alterations in SH-SY5Y neuroblastoma cells using the combination of two different omic approaches (genomics and proteomics).

Results obtained in our study entail that endosulfan exposure can alter expression of key genes and proteins involved in multiple biological pathways. Majority of the genes and proteins were found to be involved in regulation of neuronal developmental pathway, cell adhesion, apoptosis, signal transduction, regulation of transmission of nerve impulse, etc (Fig. 2, table 2). Certain genes and proteins were involved in neuronal developmental and immune response pathways. Wilson et al. have already reported damage in nigrostriatal dopamine system (Wilson et al., 2014) while Aggarwal et al. (Aggarwal et al., 2008) reported perturbation in immune responses due to endosulfan exposure. Similarly endosulfan altered cell adhesion, cytoskeleton organization, cell differentiation, and cell cycle processes in the present study. In agreement with this earlier studies have shown that exposure to endosulfan can alter phenotype of liver cells by inducing cell detachment and disruption of the actin cytoskeleton (Peyre et al., 2012). Kang et al demonstrated that endosulfan can inhibit neural stem cells differentiation and proliferation and also interfere with the gap junctional intercellular communications which plays a crucial role in the maintenance of cellular homeostasis (Kang et al., 2001). Exposure to endosulfan has also been reported to induce apoptotic cell death in SH-SY5Y cell line (Jia and Misra, 2007). Further, Song et al. reported that endosulfan exposure leads to alterations in ARE-mediated transcription

in HepG2 cell line (Song et al., 2012). In agreement with this, genes and proteins related to apoptosis and transcription was found to be differentially expressed due to endosulfan exposure.

API5 is an anti-apoptotic gene; and its down-regulation may lead to apoptosis (Ramachandran et al., 2012). API5 negatively regulates ACIN1 expression. Down regulation of API5 causes ACIN1 over-expression, which after cleavage by caspase-3 enhances the apoptotic chromatin condensation (Sahara et al., 1999). Down-regulation of API5 gene in current study indicates that endosulfan can lead to neuronal cell death whereas; up-regulation of AIFM2 has been reported to induce caspase independent neuronal cell death (Sean et al., 2002). It has been shown to cause high molecular weight DNA fragmentation and chromatin condensation (Sean et al., 2002). Up-regulation of AIFM2 and down-regulation of API5 in our study indicated that endosulfan can induce apoptotic cell death or degeneration of dopaminergic neuron (SH-SY5Y) via both caspase dependent and independent pathways. This neuronal cell death can be associated with a number of endosulfan induced neurodegenerative diseases like Alzheimer, Huntington, Parkinson disease etc (Portera-Cailliau et al., 1995; Smale et al., 1995). Similar to API5, HTT is also anti-apoptotic gene which protects CNS (central nervous system) cells from variety of apoptotic stimuli (Rigamonti et al., 2000). HTT is significantly involved in neuronal cell survival and its down-regulation leads to progressive neuro-degeneration (Dragatsis et al., 2000) and apoptotic cell death (Leavitt et al., 2001). Down-regulation of HTT in our study demonstrated the neurodegenerative properties of endosulfan via induction of cell death cascades in neuronal cells.

Endosulfan exposure up-regulates the expression of FOS gene in PC12 neuronal cells (Seth et al., 2002) and up-regulation FOS alters the proliferation and differentiation of epidermal

cells when exposed to dioxin (Tang et al., 2008). Being an organochlorine compound, endosulfan can also exhibit similar effects as dioxin. Similarly, activation of Fas receptor by Fas ligand recruits the FADD to the death domain in the cytoplasmic tail of the receptor, which leads to caspase activation and cell death (Choi et al., 2007). In the present study, both FOS and FADD genes were found to be up-regulated indicating the possibility of altered proliferation and induction of death cascade in neuronal cells.

EGR1 gene is associated with cell proliferation, differentiation and apoptosis. In the nervous system, transcription regulatory factors of EGR family play a pivotal function in coordinating the genes underlying synaptic strength, neuronal plasticity, and learning activities. Exposure to some environmental signals, including ionizing radiation and UV rays has been shown to activate the EGR1 gene which further affects cell growth and apoptosis (Buttiglione et al., 2007). Up-regulation of EGR1 in our study indicated the ability of endosulfan to deregulate neuronal plasticity and synaptic strength of neuronal cells. XRCC5 gene is responsible for DNA double strand break repair and its down-regulation is linked with neuro-degenerative disorders along with the elevated risk of astrocytoma (brain cancer) (Jiang et al., 2006). Epidemiological studies have revealed down regulation of XRCC5 gene in primary brain tumor (Jun et al., 2009). Our results demonstrated down regulation of XRCC5 gene signifying possible mechanism of endosulfan induced neuro-degenerative disorders.

2DE and MALDI-TOF-MS analysis (Fig. 4 and Table 2) reveals differential expression of key proteins; Sidekick-1 (SDK 1) and Sidekick-2 (SDK 2) are involved in regulating the synaptic plasticity and axon guidance of neuronal cells (Kaprielian et al., 2000). Protein

Sidekick-1 expression was down regulated in present work indicates that endosulfan may hamper the process that guide axonal terminals to specific synapses in developing neurons.

G-CSF and its receptor G-CSFR are major growth factors expressed in adult neural stem cells which induce *in vitro* neuronal differentiation (Schneider et al., 2005). G-CSF has also been documented to alter immune function by suppressing the expression of inflammatory cytokines (Maria et al., 2000). Recent studies postulated that G-CSF has neuro-protective function by altering the neuronal zinc-metalloprotease neprilysin (NEP) activity that causes degeneration of fibrillar amyloid β (Takeuchi et al., 2014). The down regulation of G-CSF in present study suggested the induction of neurodegenerative network in neuronal cells via accumulation of fibrillar amyloid β . In contrast to G-CSF, the expression of G protein-regulated inducer of neurite outgrowth 1 (GRIN1) was found to be up regulated in our study. The expression of GRIN1 in Neuro2a and PC12 cells is reported to cause extension of neuritis through G α o Signaling (Nakata and Kozasa, 2004) and responsible for cell differentiation respectively (Suzukawa et al. 2000),

The integrative genomics and proteomic analysis reveals down regulation of API5 gene and ZNF244 protein respectively. Both are associated with apoptotic pathway. API5 is a critical regulator of apoptosis while ZNF244 functions to suppress apoptosis by inhibiting the transport of NF- κ B protein to nucleus. Endosulfan has neurotoxic potential but the molecular basis of its toxicity is still not well explored. The present integrated approach highlighted the possible molecular crosstalk involved in endosulfan toxicity in neuronal cells.

5. Conclusion

Endosulfan is a persistent organic pollutant which is banned for use as a pesticide in many countries due to its persistent nature and extensive use in the past, it can pose serious threats to human and environment even after its discontinuation. To the best of our knowledge, this study presents the first global cellular analysis of acute endosulfan exposure in human neuronal cells. Present study demonstrated integrated genomic and proteomic approach to highlight new genes and proteins, linked for the first time to the molecular mechanism of endosulfan induced neuronal alterations. The identified gene and protein could be useful to understand the molecular mechanism of endosulfan induced neurotoxicity and development of specific biomarkers.

Conflict of interest

The authors declare no conflict of interest.

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Legends

Fig. 1 MTT assay showing cytotoxic effect of endosulfan on SH-SY5Y cell viability. Dose dependent decrease in cell viability compared to control. Results are expressed as mean $\pm$ standard error (n=3) and * indicates statistical significance ($p < 0.05$).

Fig. 2 Gene Ontology (GO) functional annotation cluster analysis was performed by utilizing the DAVID database. Significant biological processes associated with (A) upregulated genes and (B) downregulated genes.

Fig. 3 Validation of microarray data. Bar graph represents the correlation of microarray data with qPCR-based transcript levels for an arbitrarily selected panel of genes. A normalized ratio (Y-axis) of more than 1 indicates up-regulation, whereas a ratio of less than 1 indicates down-regulation. Error bars indicates means $\pm$ SE (n= 6) and * indicates statistical significance ($p < 0.05$).

Fig. 4 Effect of endosulfan on protein expression in SH-SY5Y cells. (A) 2D gel image of the cellular protein profile of the control cells. (B) Enlarged view of equivalent regions from 2DE gel profiles of endosulfan control and exposed cells, showing differentially expressed proteins which have been numbered 1 through 16. The identified protein spots are shown in Table 2.

Fig. 1

Fig. 2 (A)

Fig. 2 (B)

Fig. 3

Fig. 4

Table 1.

List of qPCR primers

Gene ^a	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
XRCC5	TGTACAGCGACAGGTGTTTG	TCCAGGAAGTCAGCCTGTTG	58	204
API5	GGAATACATGCCCCTCCCTC	TGCAAAGTACTGCAGCCTGAT	57	199
HTT	AGGCTCTATTCCCTGCTGGA	GATTCACCAGCTCTGCACCT	57	214
AIFM2	ATGGTTCGGCTGACCAAGAG	GCCACCACATCATTGGCATC	57	202
EGR1	CAGCACCTTCAACCCTCAG	CACAAGGTGTTGCCACTGTT	57	201
FADD	GGCTCGTCAGCTCAAAGTCT	CCTCTTGTACCAGGTCAGCC	57	194
FOS	CCGAGCTGGTGCATTACAGA	ACACACTCCATGCGTTTTGC	57	182
IC (RP II)	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA	54-59	267

^aXRCC5 = X-ray repair complementing defective repair in Chinese hamster cells 5; API5 = apoptosis inhibitor 5; HTT = huntingtin; AIFM2 = apoptosis-inducing factor, mitochondrion-associated 2; EGR1 = early growth response 1; FADD = Fas (TNFRSF6)-associated via death domain; FOS = FBJ murine osteosarcoma viral oncogene homolog; IC = internal control; RPII = RNA polymerase II

Table 2.

Identification of differentially expressed proteins by MALDI-TOF-MS.

Spot ID	Protein name	Accession No.	Fold Change	Biological Function
1	Zinc Finger Protein 224	ZN224_HUMAN	2.11 ↓	Regulation of Transcription
2	MIPOL1	gi 23271558	2.20 ↓	Nasopharyngeal Carcinoma Development, Tumor Suppressor
3	Uncharacterized Protein C22orf34	CV034_HUMAN	1.73 ↓	Unknown
4	Platelet Glycoprotein IX	GPIX_HUMAN	1.68 ↓	Blood coagulation, Cell Adhesion
5	Zinc Finger Protein 451	ZN451_HUMAN	2.32 ↓	Regulation of Transcription
6	Mesogenin-1	MSGN1_HUMAN	2.01 ↓	Segment Specification, Cell Differentiation, Somatogenesis
7	Protein Sidekick-2	SDK2_HUMAN	1.59 ↓	Cell Adhesion , Neuron Development
8	Immunoglobulin Heavy Chain Variable Region	gi 247424500	1.53 ↑	Immune Response
9	Probable Tubulin Polyglutamylase TTLL1	TTLL1_HUMAN	1.83 ↑	Protein Polyglutamylation, Axoneme Assembly
10	Polycystic Kidney Disease Protein 1-Like 1	PK1L1_HUMAN	1.95 ↑	Renal Tubular Development, Calcium Homeostasis, Neuropeptide Signaling Pathway
11	G protein Regulated Inducer of Neurite out Growth 1	gi 31418165	1.76 ↑	Neuron Projection Development

12	Granulocyte Colony-Stimulating Factor	gi 297660573	2.94 ↓	Negative Regulation of Neuron Death, Immune Response, Cytokine-Mediated Signaling Pathway, Granulocyte Differentiation
13	Alpha-Tubulin	gi 340021	1.54 ↑	Microtubule-Based Process, Mitotic Cell Cycle
14	Coronin-1A	gi 5902134	2.00 ↓	Actin Cytoskeleton Organization, Innate Immune Response
15	Propionyl CoA Carboxylase Alpha Subunit	gi 265482	1.52 ↓	Vitamin Metabolic Process, Cellular Lipid Metabolic Process
16	Aspecific BCL-2 ARE Binding Protein 3	gi 27948572	1.70 ↑	Unknown

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Table 3.

Validation of selected genes from microarray data by qPCR.

Gene ID ^a	Biological fuction	Fold change in qPCR	Fold change in microarray	Regulation
XRCC5	Innate immune response, positive regulation of neurogenesis	4.90	3.70	Down
API5	Negative regulation of apoptotic process	2.50	1.70	Down
HTT	Neuron apoptotic process	1.40	1.50	Down
AIFM2	p53-dependent apoptosis response	1.50	1.50	Up
EGR1	Early response transcription factor (Transcriptional regulator of several hundred genes)	4.49	3.30	Up
FADD	Apoptotic signaling pathway	1.60	1.59	Up
FOS	Inflammatory response, apoptosis, cell proliferation and differentiation	8.10	6.00	Up

^aGene names are given in Table 1.