

# Attenuation of Dichlorvos-Induced Microglial Activation and Neuronal Apoptosis by 4-Hydroxy TEMPO

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**Abstract** The neurotoxic consequences of acute high-level as well as chronic low-level organophosphates exposure are associated with a range of abnormalities in nerve functions. Previously, we have shown that after 24 h of dichlorvos exposure, microglia become activated and secrete pro-inflammatory molecules like nitric oxide, tumour necrosis factor- $\alpha$  and interleukin-1 $\beta$ . Here, we extended our findings and focused on the neuronal damage caused by dichlorvos via microglial activation. For this, neurons and microglia were isolated separately from 1-day-old Wistar rat pups. Microglia were treated with dichlorvos for 24 h and supernatant was collected (dichlorvos-induced conditioned medium, DCM). However, when 4-hydroxy TEMPO (4-HT) pretreatment was given, we observed significant attenuation of dichlorvos-induced microglial activation; we also collected the supernatant of this culture (4-HT + DCM, TDCM). Next, we checked the effects of DCM on neurons and found heavy loss in viability as evident from NF-H immunostaining and MTT results, whereas dichlorvos alone-treated neurons showed comparatively less damage. However, we observed significant increase in neuronal viability when cells were treated with TDCM. Semi-quantitative PCR and western blot results revealed significant increase in p53, Bax and cytochrome *c* levels along with caspase 3 activation after 24 h of DCM treatment. However, TDCM-treated neurons showed significant decrease in the expression of these pro-apoptotic molecules. Taken together, these findings suggest that 4-HT can significantly attenuate dichlorvos-induced microglial activation and prevent apoptotic neuronal cell death.

**Keywords** 4-Hydroxy TEMPO · Dichlorvos · Microglia · Pro-inflammatory cytokines

## Introduction

Epidemiological studies have repeatedly indicated that pesticide exposure is a significant risk factor for various neurodegenerative diseases in which microglial cells become activated and played an important role in the disease pathology [1]. Until now, organophosphates (OPs)-mediated toxicity is primarily linked with neurons, but it is time to make a shift in our current approach and other cells of CNS should also be examined. Recent report from our lab has shown that dichlorvos (an OP) exposure can lead to activation-induced cell death in microglia preceded by increased production of various pro-inflammatory molecules like nitric oxide (NO), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) [2]. In addition to this, several studies based on neuron – microglia interrelationships have preferentially enlightened the side of neuronal damage exacerbation from activated microglia [3].

One of the outcomes of microglia activation is the production of NO, a well-known pathophysiological agent, associated with hypertension, diabetes and heart failure [4]. NO can also react with superoxide anions to form peroxynitrite, which has strong oxidant properties and can damage cellular components when protein nitration takes place [5]. Munch et al. tried to determine the effects of secreted factors from activated murine N-11 microglia on viability and morphology of neurons using the differentiated neuroblastoma cell line Neuro2a [6]. The results suggest that even in the absence of significant cell death, inflammatory processes, which are partly transmitted via NO metabolites, may affect intrinsic functions of neurons such as neurite extension that are essential components of neuronal morphology and

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thus may contribute to degenerative changes in Alzheimer's disease.

Kim et al. has shown the importance of NO in diisopropyl-fluorophosphate (OP)-mediated induction of seizures in rats [7]. Although the concentration and source of NO can determine the response of a cell, however, pro-apoptotic and anti-apoptotic response appears to be specific for different cell types. 4-Hydroxy TEMPO (4-HT) is a commercially available potent NO scavenger and superoxide dismutase mimetic and possesses several advantages that makes it an excellent candidate to provide cellular protection [8]. Recent study has shown that the administration of 4-HT before disease onset to females of an ALS rat model moderately extended the survival of the animals while protecting their cellular and molecular structures against damage [9]. Moreover, in a spinal cord injury model, 4-HT pretreatment has shown to inhibit the expression of COX-2 and iNOS, and decreases NO production with reduction of injured area [10].

Based on these reports, strategy of employing 4-HT to protect dichlorvos-induced nitrosative/oxidative stress via microglial activation appears to be logical. Moreover, literature is almost silent about the role of microglial activation in neurodegeneration induced by OPs. Therefore, our aim was to check the protective efficacy of 4-HT against dichlorvos-induced microglial activation and neuronal cell death.

## Materials and Methods

### Reagents and Antibodies

Minimum essential medium (MEM) was purchased from Hyclone (Utah, USA). Fetal bovine serum (FBS) and 0.25 % trypsin were purchased from Invitrogen (California, USA), while 96- and 6-well tissue culture plates, 25 cm<sup>2</sup> T-flasks and cell scrapers were purchased from Greiner Bio-One (St. Gallen, Switzerland). Syringe filters (0.2 µL) were purchased from Millipore (Massachusetts, USA). Griess reagent, dichlorvos, fluorocitrate and minocycline were procured from Sigma-Aldrich (Missouri, USA). Neurofilament heavy chain protein (NFH), p53, Bax, cytochrome *c* and caspase 3 primary antibodies were purchased from Santa Cruz Biotechnologies (California, USA), whereas secondary antibodies were purchased from Bangalore Genei (Bangalore, India). All other chemicals used in this study were of tissue culture grade.

### Microglial Cell Culture

For all experiments, Wistar rats were used, which were bred and kept under constant conditions (12 h light/12 h dark cycle)

in the central animal house of the Institute. Neonatal pups (1 day old) were sacrificed as per the guide lines of Institutional Animal Ethical Committee. The "Principles of laboratory animal care" (NIH publication no. 85–23, revised 1996) guidelines were strictly followed for all experiments. All efforts were made to minimize the number of animals used and their suffering. Microglial cells were obtained from the mixed brain cell culture of newborn pups as previously described [2]. Briefly, cerebral tissue was isolated aseptically and the meninges were removed. Each dissected brain was cut into small fragments and triturated with a pipette, followed by 5–10 passes through a 20 gauge needle. The dissociated cells were seeded in 25 cm<sup>2</sup> plastic flasks at the density of  $2 \times 10^6$  cells in MEM, supplemented with 60 U/mL of penicillin and 50 mg/mL of streptomycin and maintained at 37 °C in an atmosphere of 95 % air and 5 % CO<sub>2</sub> for 7 days in vitro (DIV). After 7 DIV, flasks were shaken carefully and the cells in suspension were collected by centrifugation. The cells were transferred to the tissue culture plates and incubated for 24 h. Microglia were found to be the predominant cellular population as analyzed by flow cytometry using antibodies against CD11b marker.

### Neuronal Cell Culture

One-day-old pups of male Wistar rats were sacrificed, and brains were rapidly extracted on ice. The cortices were dissected out off meninges, and the cortical fragments then incubated with 0.25 % trypsin and 20 µg/mL Dnase I in phosphate buffer saline at 37 °C for 15 min. The cortical fragments were dissociated to single cells by continuous pipetting, and the cells obtained were suspended in culture medium and plated onto collagen I-coated plates. The cells were cultured and maintained at 37 °C in an atmosphere of 95 % air and 5 % CO<sub>2</sub> for 7 DIV. To eliminate chances of glial contamination, fluorocitrate (10 fmol) and minocycline (1 pmol) were added into the culture medium. After 7 DIV, cells were immunostained with NFH antibody to characterize the neuronal population in the culture.

### Determination of Cell Viability

Cell viability was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, after 24 h of dichlorvos treatment (0 to 50 µM), the cells ( $\sim 2 \times 10^4$  cells/mL) were incubated with 0.5 mg/mL MTT for 1 h at 37 °C and then solubilized by adding a solution containing 50 % dimethylformamide and 20 % sodium dodecyl sulphate (pH 4.7). The amount of MTT formazan produced was determined by measuring its

absorbance at a test wavelength of 570 nm and a reference wavelength of 655 nm.

#### Selection of Dose

*Dichlorvos*: based on pharmacokinetic models and biomonitoring data, Buratti et al. (2007) have proposed that OP concentrations around 10  $\mu\text{M}$  (in vitro; corresponding to 2.6, 2.5 and 1.6  $\mu\text{g}/\text{mL}$  of methyl parathion, methyl paraoxon and dimefox, respectively) reflect comparable conditions as observed in general humans OP exposure cases. Moreover, the concentrations higher than 100  $\mu\text{M}$  reflect acute accidental intoxication [11]. Therefore, in order to mimic the in vivo conditions, one third of I.C.<sub>50</sub>, i.e. 10  $\mu\text{M}$  (2.21  $\mu\text{g}/\text{mL}$ ), of Dichlorvos was used to treat microglia in this study.

*4-Hydroxy TEMPO*: As evident from MTT assay results, 4-HT pretreatment provide maximum protection (>90 % viability) at 50  $\mu\text{M}$  concentration and when this concentration was employed for inhibiting NO, significant decline in microglial NO levels was observed when compared to dichlorvos-treated cells. Therefore, we used 50  $\mu\text{M}$  concentration of 4-HT against 10  $\mu\text{M}$  dichlorvos in subsequent experiments.

#### Experimental Design

Whole study was divided into following parts:

1. Microglial cell culture
2. Neuronal cell culture

##### 1. Microglial Culture

- Control group: Primary microglial cells were cultured and maintained in MEM supplemented with 10 % FBS for 3 DIV.
- Dichlorvos group: Primary microglial cells were cultured and maintained in MEM supplemented with 10 % FBS for 3 DIV and then treated with 10  $\mu\text{M}$  of dichlorvos for 24 h.
- 4-HT + dichlorvos group: Primary microglial cells were cultured and maintained in MEM supplemented with 10 % FBS for 3 DIV and pretreated with 50  $\mu\text{M}$  of 4-HT followed by 10  $\mu\text{M}$  of dichlorvos for 24 h.
- 4-HT group: Primary microglial cells were cultured and maintained in MEM supplemented with 10 % FBS for 3 DIV and pretreated with 50  $\mu\text{M}$  of 4-HT.

##### 2. Neuronal Culture

- Control group: Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 %  $\text{CO}_2$  at 37 °C for 7 DIV
- Dichlorvos-induced culture medium group (DCM): Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 %  $\text{CO}_2$  at 37 °C for 7 DIV and then treated with conditioned medium (DCM) recovered from 24 h dichlorvos (10  $\mu\text{M}$ )-treated microglial culture.
- 4-HT + DCM group (TDCM): Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 %  $\text{CO}_2$  at 37 °C for 7 DIV and then treated with conditioned medium (TDCM) recovered from 24 h 4-HT (50  $\mu\text{M}$ ) + dichlorvos (10  $\mu\text{M}$ )-treated microglial culture.
- 4-HT group: Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 %  $\text{CO}_2$  at 37 °C for 7 DIV and then treated with 50  $\mu\text{M}$  4-HT.

#### Measurement of Nitrite Accumulation

The concentration of nitrite, a stable oxidation product of NO, was estimated by Griess assay. Microglial cells ( $2 \times 10^4$  cells/well) were grown in 96-well tissue culture plates in the presence or absence of 10  $\mu\text{M}$  dichlorvos. Using micropipette, 50  $\mu\text{L}$  of culture supernatant was transferred to 96-well microtiter plates and mixed with 50  $\mu\text{L}$  of the Griess reagent (0.1 % *N*-[1-naphthyl]ethylenediamine dihydrochloride and 1 % sulfanilamide in 2 % phosphate solution). After the incubation of 10 min at room temperature, the absorbance was measured with a microplate reader at a test wavelength of 540 nm and a reference wavelength of 655 nm. The standard curve was constructed with the culture medium containing known concentrations of sodium nitrite and employed to calculate the concentration of nitrite in samples.

#### Measurement of ROS Levels

ROS levels were measured by the method of Socci et al. [12]. Briefly, mitochondria were isolated from microglial cells and added to respiration buffer containing 5 mM pyruvate, 2.5 mM malate and 10 mM of dichlorodihydrofluorescein diacetate. After 20 min incubation in dark, mitochondria were pelleted by centrifugation and extra mitochondrial dye was

washed off. Fluorescence was quantified using a Cary Eclipse fluorimeter (Varian, Palo Alto, USA) (excitation 488 nm, emission 525 nm) and related to total protein content.

#### Semi-quantitative PCR

Cells ( $1 \times 10^6$  cells/well) were cultured in six-well tissue culture plates. Total RNA was extracted from cells of different groups using Total RNA extraction kit (Taurus Scientific, India). Extracts were assayed to determine the quality and concentration of the RNA using spectrophotometer. Extracts were stored at  $-20^\circ\text{C}$ . Isolated RNA was then digested by DNase (Promega, Germany) to destroy contaminating DNA, and cDNA was synthesized with RevertAid™ H Minus M-muLV Reverse Transcriptase (Fermentas, Germany). Ten nanograms of cDNA was subjected to Reverse Transcriptase PCR amplification. For primer sequences, PCR conditions and sizes of PCR products, please refer to Table 1.

#### Cytokine ELISA Immunoassays

TNF- $\alpha$  and IL-1 $\beta$  gene expressions, estimated from semi-quantitative PCR, were evaluated for protein expression using ELISA. For ELISA, microglial cells ( $2 \times 10^4$  cells/well) were cultured in 96-well plates. The conditioned medium was collected from microglial cells treated with dichlorvos for different time intervals. The levels of TNF- $\alpha$  and IL-1 $\beta$  were measured using conventional sandwich ELISA kits from R&D Systems (Minnesota, USA). Assays were performed according to the manufacturer's instructions.

#### DAPI Staining

DAPI, a DNA-binding fluorescent dye, was used to detect the nucleus in neuronal cells. After treatment with either DCM or TDCM for 24 h, the cells were washed three times with phosphate-buffered saline (PBS), fixed in 1:1 acetone/methanol solution for 10 min at  $-20^\circ\text{C}$ , then stained

with 500 ng/mL DAPI for 10 min. Cells were observed under a fluorescent microscope.

#### NFH Immunostaining

Neuronal cells ( $5 \times 10^4$  cells/well) were cultured in six-well tissue culture plates. After 3 DIV, media were replaced and cells were treated with dichlorvos (10  $\mu\text{M}$ ) and/or 4-HT (50  $\mu\text{M}$ ) and fixed with acetone/methanol (1:1) at  $-20^\circ\text{C}$  for 30 min. Cells were carefully washed three times with PBS and blocked with blocking buffer (PBS with 1 % BSA) at room temperature for 1 h followed by incubation with the mouse monoclonal anti-NFH primary antibody (1:500) at  $4^\circ\text{C}$  overnight. Cells were again subjected to three PBS washes and then incubated for 1 h at room temperature with FITC-labelled rabbit anti-mouse secondary antibodies (1:10,000). Finally, cells were analyzed by fluorescence microscopy.

#### Preparation of Mitochondrial and Cytosolic Fractions for Immunoblotting

The cytosolic and mitochondrial fractions were prepared by the method of Tang et al. [13]. At the time of fractionation, the integrity of mitochondria was checked by assessing respiratory control ratio and marker enzymes (data not shown). The microglia were homogenized (20 strokes) in 500  $\mu\text{L}$  of buffer A (20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM  $\text{MgCl}_2$ , 220 mM mannitol, 68 mM sucrose, 1 mM leupeptin, 5  $\mu\text{g}/\text{mL}$  pepstatin A, 5  $\mu\text{g}/\text{mL}$  aprotinin, 0.5 mM PMSF). The homogenate was then centrifuged at  $1,000 \times g$  for 10 min at  $4^\circ\text{C}$ . The resulting supernatant contained the cytosolic fractions and the pellets contained enriched mitochondrial fractions. The cytosolic fractions were stored at  $-80^\circ\text{C}$  until further analysis. Pellets containing mitochondria were treated with the lysis buffer (1 $\times$  PBS, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 250 mM sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM DTT and protease inhibitor) and were incubated on ice for

**Table 1** Primer sequences, PCR conditions and sizes of PCR products

Gene	Forward primer	Reverse primer	Tm ( $^\circ\text{C}$ )	Product size (bp)
TNF- $\alpha$	GGGCTCAGAATTTCCAACAA	GAGACAGCCTGATCCACTCC	55.0	190
IL-1 $\beta$	GCAATGGTCGGGACATAGTT	GAATGTGCCACGGTTTTTCIT	57.0	189
P53	GCTTCGAGATGTTCCGAGAG	AGGATGCAGAGGCTGTCACT	58.2	194
Bax	ATGGAGCTGCAGAGGATGAT	GATCAGCTCGGGCACTTTAG	57.3	186
Cytochrome <i>c</i>	TCAATGATGCTGCCTTTCAC	TTTCCGAATGGGCATTTAAG	57.1	197
Caspase 3	GCTACGATCCACCAGCATTT	ATGCCACTCTCCTTTCCTT	57.4	194
GAPDH	CACTGTGCCATCTATGAGGG	TCCACATCTGCTGGAAGGTGG	61.8	400

20 min. The lysate was centrifuged at  $10,000\times g$  at  $4\text{ }^{\circ}\text{C}$  for 30 min. The resulting supernatant was kept as solubilized mitochondrial-enriched fraction and stored at  $-80\text{ }^{\circ}\text{C}$  until further use.

### Immunoblot Analysis

Cells were homogenized, and protein was extracted. The protein was isolated from mitochondrial and cytoplasmic fractions of each group. Samples containing  $75\text{ }\mu\text{g}$  of protein were boiled in Laemmli buffer for 5 min and subjected to electrophoresis (12 % SDS-PAGE) followed by transfer to nitrocellulose membrane. The blots were blocked with 5 % non-fat dry milk for 5 h; the membranes were then incubated with primary NFH/p53/Bax/cytochrome *c*/caspase 3/ $\beta$ -actin (1:100) at room temperature for 3 h. After incubation, the nitrocellulose membrane was washed with PBS plus 0.1 % Tween-20 for 30 min at 5-min interval of time. Membrane was again incubated for 1 h at  $37\text{ }^{\circ}\text{C}$  with horseradish peroxidase-conjugated secondary antibody. After 1 h of incubation, the blots were again washed with PBS plus 0.1 % Tween-20 for

30 min at 5-min interval of time. Immunoreactive proteins were visualized by diaminobenzidine system. The densitometry analysis of the protein bands was carried out using AlphaEaseFC software to compare the relative expression of proteins.

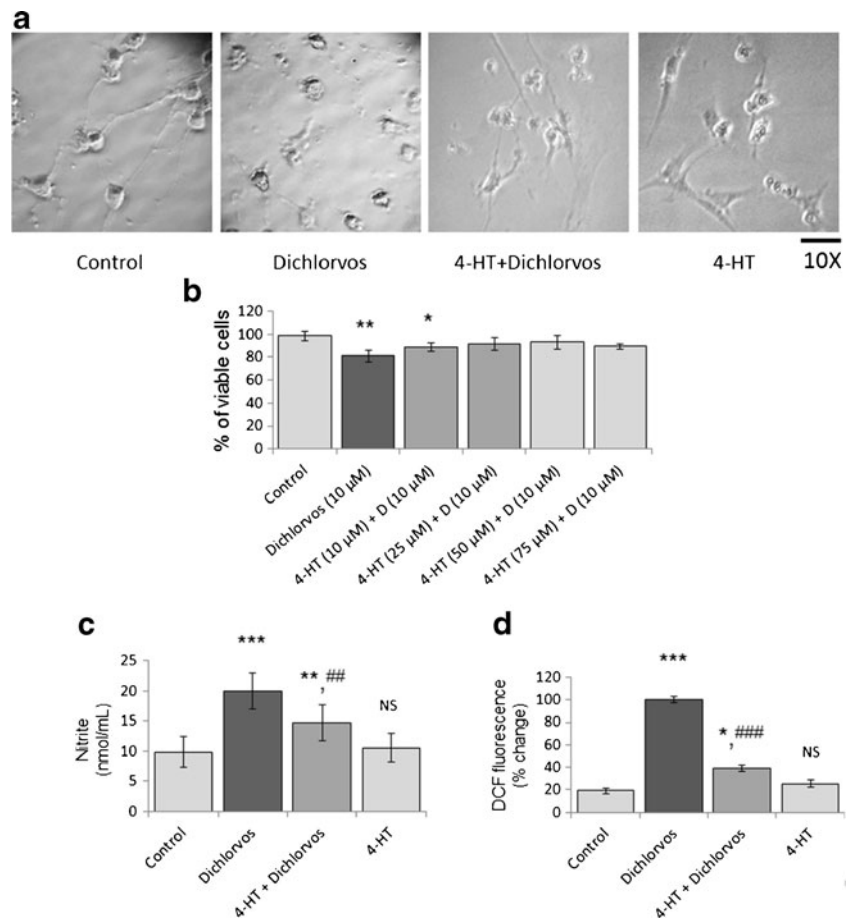
### Protein Estimation

Protein was determined by the method of Lowry et al. using bovine serum albumin as standard [14].

### Statistical Analysis

For all experiments, data were analyzed from at least three independent experiments, each with at least duplicate determinations. Student's *t* test was applied to compare the statistical difference between control and dichlorvos-treated cells. Statistical analysis of the data was performed using Sigma Stat 3.5 software with statistically significant values representing a *p* level of 0.05 or below. Error bars represent standard error of means (SEM).

**Fig. 1** Effect of 4-HT pretreatment on dichlorvos-induced viability loss, NO and ROS production. Primary microglial cells were cultured in minimum essential medium supplemented with 10 % fetal bovine serum and incubated in 5 %  $\text{CO}_2$  at  $37\text{ }^{\circ}\text{C}$  for 3 DIV. **a** Light micrographs showing morphological changes in microglial cells under different treatment conditions. **b** Cell viability was measured by MTT assay after 24 h of treatment as described under “Materials and Methods”. **c** NO levels were measured by Griess assay and **d** ROS levels were measured by DCF-DA dye, after 24 h of dichlorvos ( $10\text{ }\mu\text{M}$ ) and 4-HT ( $50\text{ }\mu\text{M}$ ) exposure. Data were expressed as mean  $\pm$  SEM of three independent experiments. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  significantly different from the control group; ### $p < 0.001$ , ## $p < 0.01$ , significantly different from the dichlorvos group; NS, non-significant





## Results

### Effect of 4-HT Pretreatment on Dichlorvos-Induced Microglial Viability Loss

Light microscopic examination revealed marked change in the microglial morphology, i.e. from resting to activate state, when exposed with dichlorvos. However, 4-HT pretreatment resulted in very less or no change in the morphology of microglial cells (Fig. 1a). Results of MTT assay showed that cell viability was significantly decreased when microglia were exposed to 10  $\mu$ M dichlorvos for 24 h. However, when cells were pretreated with 4-HT (50  $\mu$ M), significant increase (>90 %) in their viability was observed (Fig. 1b).

### Effect of 4-HT Pretreatment on Dichlorvos-Induced NO and ROS Production in Microglial Cells

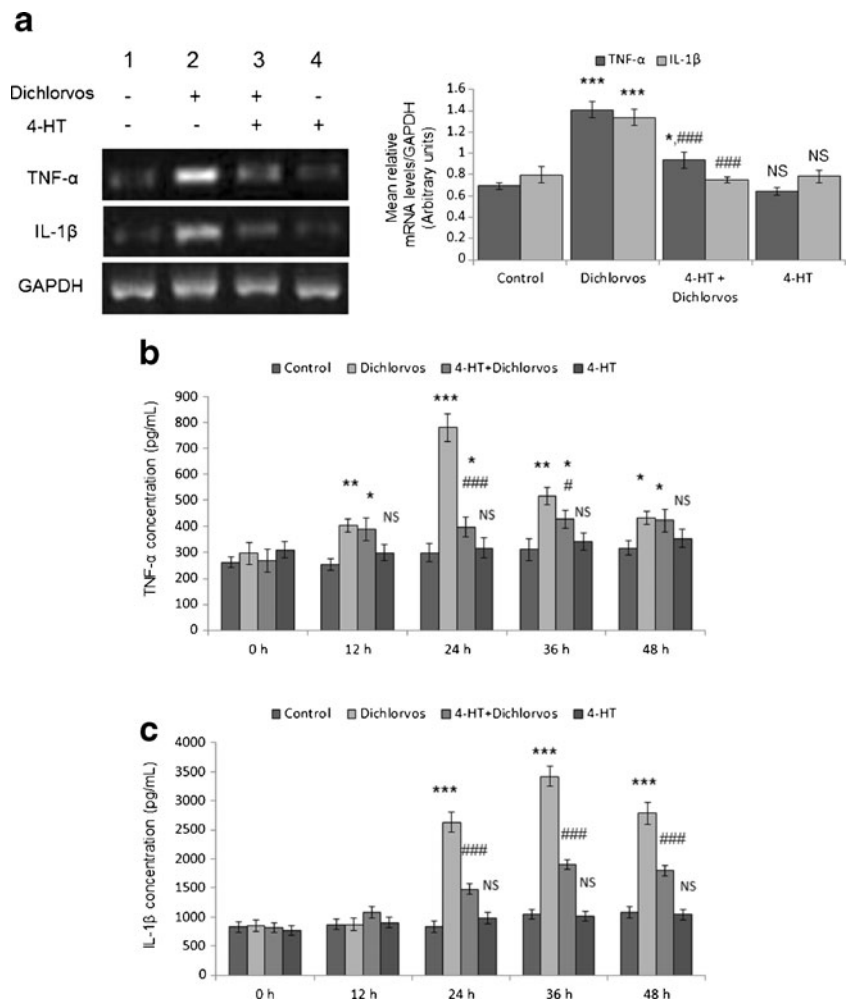
Next, we estimated the NO and ROS levels in microglia after the dichlorvos exposure in presence and absence of 50  $\mu$ M 4-

HT. We observed significant increase in the production of both NO and ROS after 24 h of dichlorvos treatment. However, significant reduction in NO as well as ROS production was observed when 4-HT pretreatment was given to the microglial cells (Fig. 1c, d).

### Effect of 4-HT Pretreatment on Dichlorvos-Induced TNF- $\alpha$ and IL-1 $\beta$ Levels in Microglial Cells

To find out whether 4-HT pretreatment attenuates dichlorvos-mediated changes in pro-inflammatory cytokine levels of microglial cells, for this, expression of TNF- $\alpha$  and IL-1 $\beta$  mRNAs were estimated. Semi-quantitative PCR results showed significant ( $p < 0.001$ ) upregulation of TNF- $\alpha$  (~2-fold) and IL-1 $\beta$  (~1.5-fold) mRNA levels in dichlorvos-treated microglial cells when compared to control. However, 4-HT-pretreated microglia showed significant decrease in the mRNA expression of both TNF- $\alpha$  and IL-1 $\beta$  (Fig. 2a). In addition, sandwich ELISA results showed significant increase in secreted TNF- $\alpha$  (~3-fold) and IL-1 $\beta$  (~2-fold) levels after

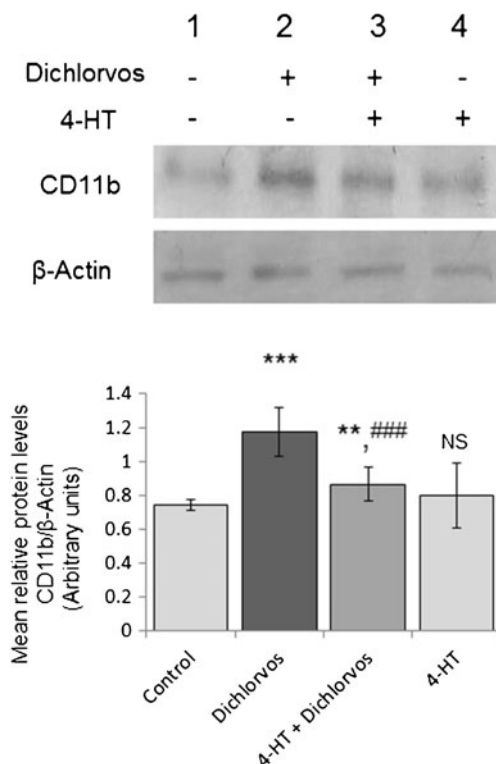
**Fig. 2** Effect of 4-HT pretreatment on dichlorvos-induced TNF- $\alpha$  and IL-1 $\beta$  levels in microglial cells. Primary microglial cells were cultured in minimum essential medium supplemented with 10 % fetal bovine serum and incubated in 5 % CO<sub>2</sub> at 37 °C for 3 DIV. **a** Semi-quantitative PCR analysis was performed to measure mRNA levels of TNF- $\alpha$  and IL-1 $\beta$  after 24 h of dichlorvos (10  $\mu$ M) treatment either in presence or absence of 4-HT (50  $\mu$ M). **b, c** Secreted TNF- $\alpha$  and IL-1 $\beta$  levels were checked at 12 h intervals using sandwich ELISA. Data were expressed as mean  $\pm$  SEM of three independent experiments. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  significantly different from the control group; ### $p < 0.001$ , # $p < 0.05$  significantly different from the dichlorvos group; NS, non-significant



24 h of dichlorvos treatment. However, 4-HT pretreatment resulted in significant reduction in TNF- $\alpha$  and IL-1 $\beta$  secreted levels when compared to their levels in dichlorvos-treated cells (Fig. 2b, c).

#### Effect of 4-HT Pretreatment on Dichlorvos-Induced CD11b Expression in Microglial Cells

To understand whether dichlorvos exposure results in microglial activation, microglial activation marker, i.e. CD11b, expression was assessed. We also examined the effect of 4-HT on dichlorvos-mediated expression of CD11b in rat primary microglia. Dichlorvos exposure increased the expression of CD11b as revealed by western blot analysis in primary rat microglia. However, 4-HT markedly suppressed the dichlorvos-mediated stimulation of CD11b expression in primary microglial cells (Fig. 3).



**Fig. 3** Effect of 4-HT pretreatment on dichlorvos-induced CD11b expression in microglial cells. Primary microglial cells were cultured in minimum essential medium supplemented with 10 % fetal bovine serum and incubated in 5 % CO<sub>2</sub> at 37 °C for 3 DIV. Cells were treated with dichlorvos (10  $\mu$ M) either in presence or absence of 4-HT (50  $\mu$ M). Western blot analysis was performed to measure CD11b protein levels after 24 h of treatment. Data were expressed as mean  $\pm$  SEM of three independent experiments. \*\*\* $p$ <0.001, \*\* $p$ <0.01 significantly different from the control group; ### $p$ <0.001 significantly different from the dichlorvos group; NS, non-significant

#### Effect of DCM and TDCM Treatment on Neuronal Viability

Primary neuronal cells were cultured in the minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C. Neurons were characterized by staining with neuron-specific NFH antibody. Viability of primary neuronal cells was checked after DCM or dichlorvos treatment at every 12 h interval. Results of MTT assay revealed time-dependent decrease in the viability of neurons when exposed to DCM. However, significant less reduction in cellular viability was observed when neurons were treated with dichlorvos alone as compared to DCM treated neurons (Fig. 4a). Light microscopy and NFH immunostaining also showed significant reduction in the neuronal cell number after DCM treatment. However, TDCM-treated neurons showed significant increase in the viability as well as restoration of normal morphology (Figs. 4b and 5).

#### Effect of DCM and TDCM Treatment on p53 Expression in Neurons

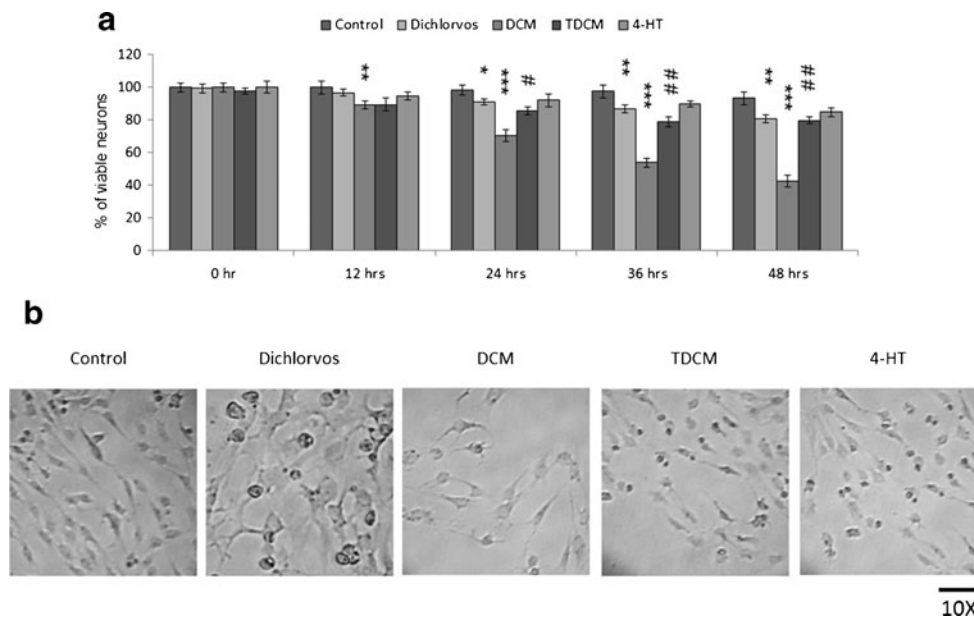
Semi-quantitative PCR results showed significant increase (~2.5-fold increase) in the expression of p53 mRNA when neurons were treated with DCM. Further, to assess the change in protein levels of p53, western blot analysis was performed. Results showed a significant increase (~2-fold increase) in the expression of p53 protein when neurons were treated with DCM as compared to control cells. However, TDCM-treated neurons showed significant decrease in p53 mRNA as well as protein expression when compared to DCM-treated cells (Fig. 6).

#### Effect of DCM and TDCM Treatment on Bax Expression in Neurons

Semi-quantitative PCR results showed significant increase (~2-fold increase) in the expression of Bax mRNA when neurons were treated with DCM. To assess the change in protein levels of Bax in mitochondrial fraction, western blot analysis was performed. Results showed a significant increase (~0.64-fold increase) in the expression of Bax protein when neurons were treated with DCM as compared to control cells. However, TDCM-treated neurons showed significant decrease in Bax mRNA as well as protein expression when compared to DCM-treated cells (Fig. 7).

#### Effect of DCM and TDCM Treatment on Cytochrome *c* Expression in Neurons

Semi-quantitative PCR results showed significant increase (~3-fold increase) in the expression of cytochrome *c* mRNA when neurons were treated with DCM. To assess the change in protein levels of cytochrome *c* in cytosolic fraction,



**Fig. 4** Effect of DCM and TDCM treatment on neuronal viability. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. **a** MTT analysis showing percentage of viable neurons after DCM/TDCM treatment. *DCM* = dichlorvos-treated microglial culture medium, *TDCM* = 4-HT + DCM. **b** Panels

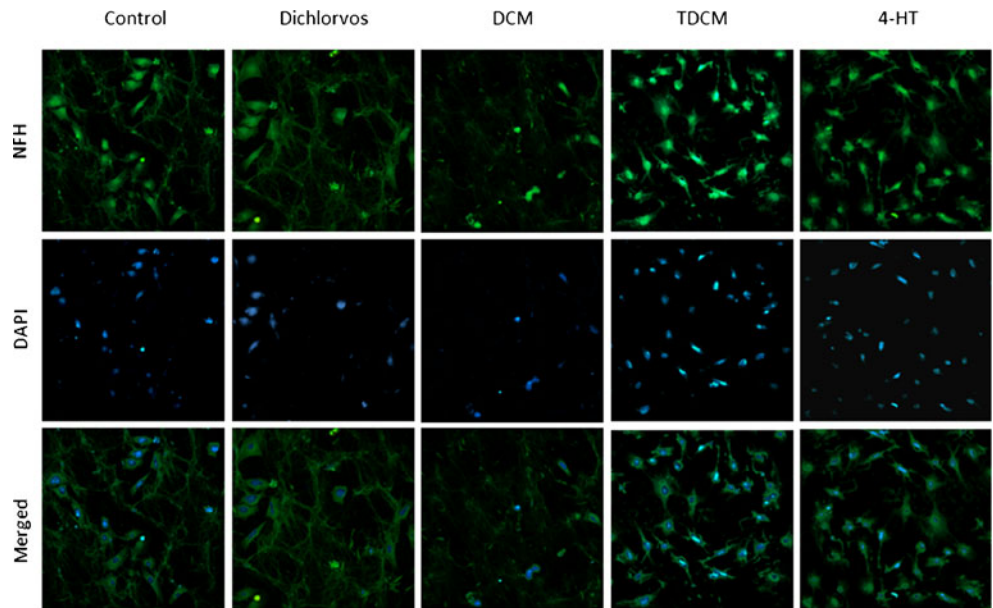
show light micrographs of primary neuronal cells. Magnification = ×10. The values are mean ± SEM of three independent experiments. \*\*\**p*<0.001, \*\**p*<0.01, \**p*<0.05, significantly different from control. ##*p*<0.01, #*p*<0.05, significantly different from dichlorvos. *NS*, non-significant when compared to control

western blot analysis was performed. Results showed a significant increase (~0.65-fold increase) in the expression of cytochrome *c* protein when neurons were treated with DCM as compared to control cells. However, significant reduction in the release of cytochrome *c* from mitochondria to cytosol was observed when neurons were treated with TDCM as compared to DCM-treated cells (Fig. 8).

Effect of DCM and TDCM Treatment on Caspase 3 Expression in Neurons

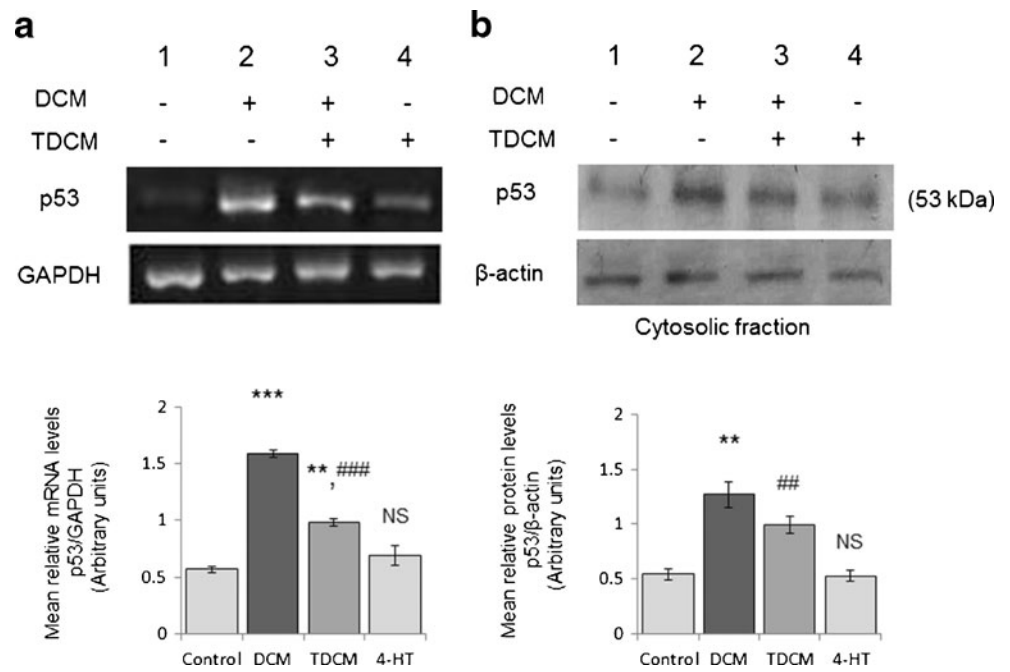
Semi-quantitative PCR results showed significant increase (~3.5-fold increase) in the expression of caspase 3 mRNA when neurons were treated with DCM. To assess the change in protein levels of caspase 3 in cytosolic fraction, western

**Fig. 5** Effect of DCM and TDCM treatment on NFH staining. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. Panels show NFH and DAPI staining of primary neuronal cells. Magnification = ×10





**Fig. 6** Effect of DCM and TDCM treatment on p53 expression in neurons. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. The cells were pretreated with TDCM. **a** Semi-quantitative PCR analysis of p53 mRNA. **b** Western blot analysis of p53 protein. The values are mean ± SEM of three independent experiments. \*\*\**p*<0.001, \*\**p*<0.01, significantly different from control. ###*p*<0.001, ##*p*<0.01, significantly different from dichlorvos. NS, non-significant when compared to control

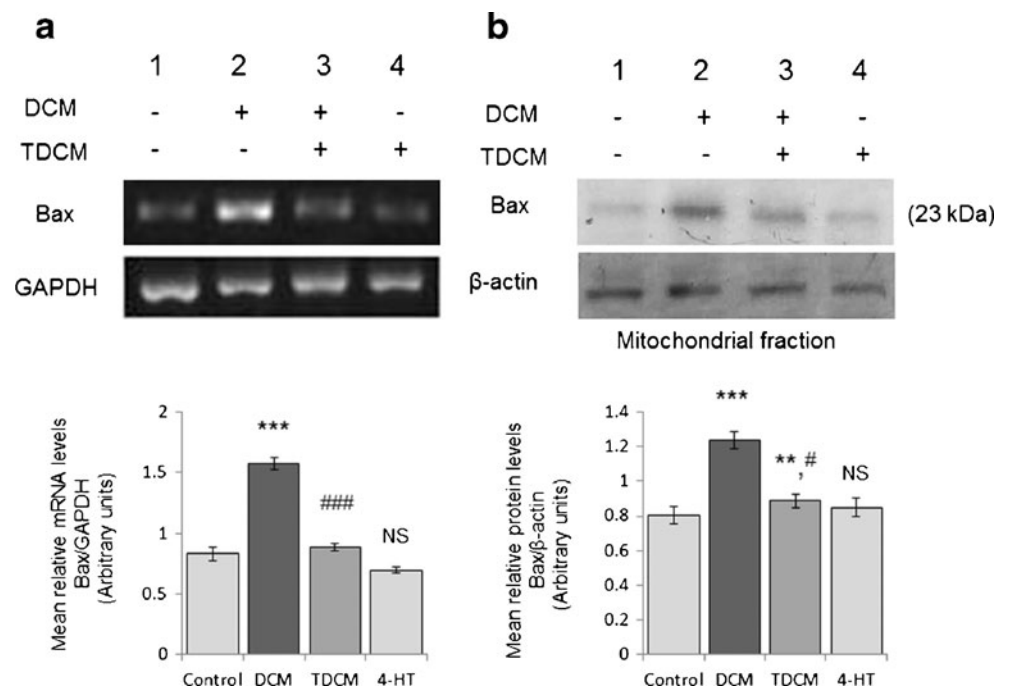


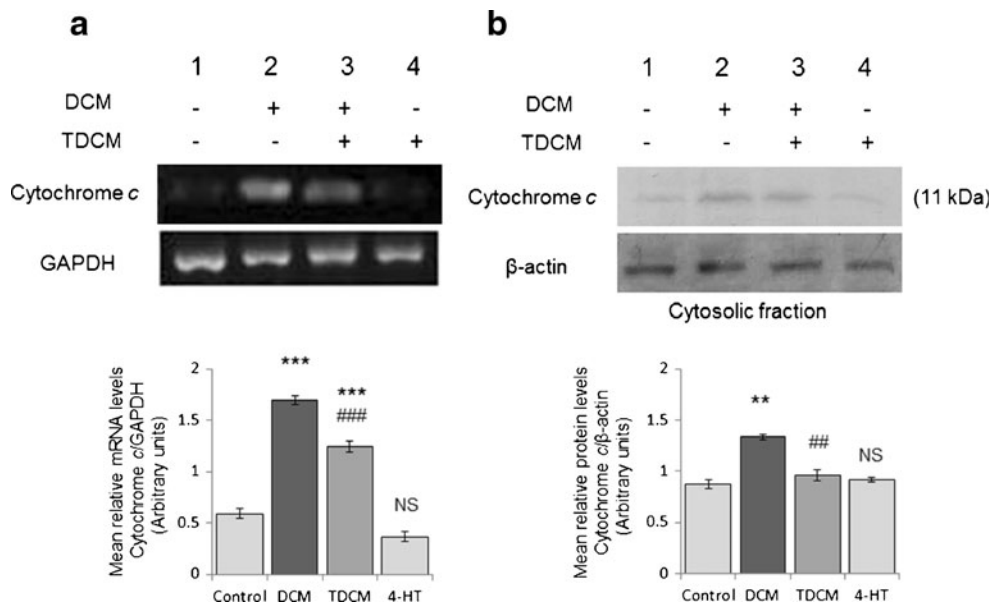
blot analysis was performed. Results showed a significant increase (~0.72-fold increase) in the expression of active caspase 3 protein when neurons were treated with DCM as compared to control cells. However, TDCM-treated neurons showed significant decrease in caspase 3 mRNA as well as protein expression when compared to DCM-treated cells (Fig. 9).

**Discussion**

Until now, literature is almost silent about the role of OPs in microglia-induced neuronal damage. Therefore, we wanted to study the deleterious effects of dichlorvos-induced microglial activation on neuronal cells. For this, we first treated the microglial cells with dichlorvos and collected the microglial

**Fig. 7** Effect of DCM and TDCM treatment on Bax expression in neurons. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. The cells were pretreated with TDCM. **a** Semi-quantitative PCR analysis of Bax mRNA. **b** Western blot analysis of Bax protein. The values are mean ± SEM of three independent experiments. \*\*\**p*<0.001, \*\**p*<0.01, significantly different from control. ###*p*<0.001, #*p*<0.05, significantly different from dichlorvos. NS, non-significant when compared to control



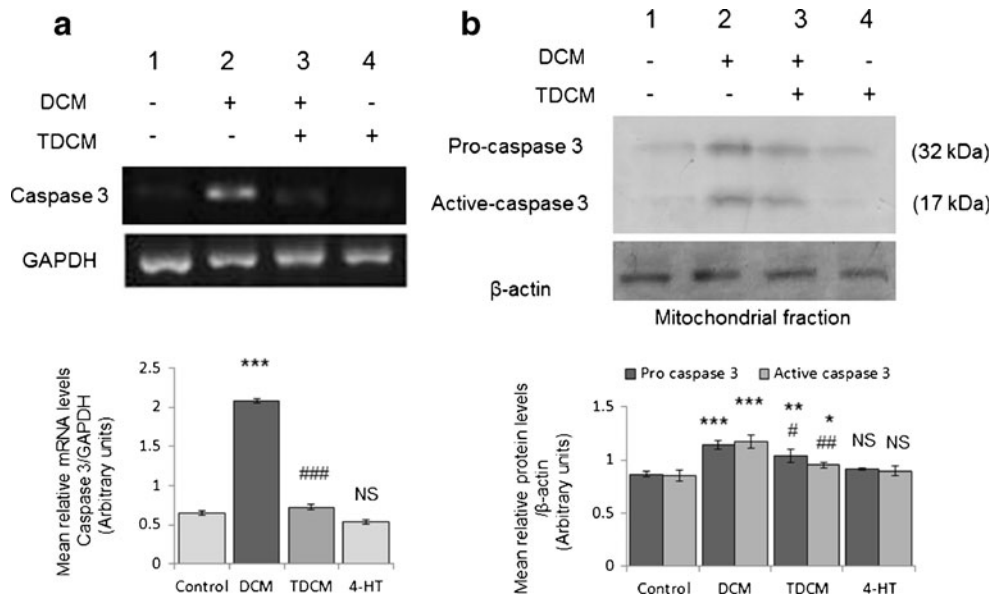


**Fig. 8** Effect of DCM and TDCM treatment on cytochrome *c* expression in neurons. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. The cells were pretreated with TDCM. **a** Semi-quantitative PCR analysis of

cytochrome *c* mRNA. **b** Western blot analysis of cytochrome *c* protein. The values are mean  $\pm$  SEM of three independent experiments. \*\*\* $p$ <0.001, \*\* $p$ <0.01, significantly different from control. ### $p$ <0.001, ## $p$ <0.01, significantly different from dichlorvos. NS, non-significant when compared to control

supernatant (DCM) which contains various pro-inflammatory molecules. Then, we treated the primary neuronal cells either with DCM or with dichlorvos alone. We found significant decrease in the viability of neurons after 24 h of DCM exposure when compared with control or with dichlorvos alone. In order

to find out the mode of neuronal cell death, expression levels of various pro-apoptotic molecules were checked. We observed significant increase in p53, Bax, cytochrome *c* and caspase 3 levels after DCM exposure as compared to control. However, neuronal cells treated with TDCM showed significant decrease



**Fig. 9** Effect of DCM and TDCM treatment on caspase 3 expression in neurons. Primary neuronal cells were cultured in minimum essential medium supplemented with Neuro basal medium and B27 supplement and incubated in 5 % CO<sub>2</sub> at 37 °C for 7 DIV. The cells were pretreated with TDCM. **a** Semi-quantitative PCR analysis of caspase 3 mRNA. **b**

Western blot analysis of caspase 3 protein. The values are mean  $\pm$  SEM of three independent experiments. \*\*\* $p$ <0.001, \*\* $p$ <0.01, \* $p$ <0.05, significantly different from control. ### $p$ <0.001, ## $p$ <0.01, # $p$ <0.05, significantly different from dichlorvos. NS, non-significant when compared to control

in the levels of p53, Bax, cytochrome *c* and caspase 3. These findings suggest that oxidative/nitrosative stress along with pro-inflammatory molecules released by microglial cells in response to dichlorvos treatment can induce apoptotic cell death in neurons and 4-HT pretreatment can effectively attenuate this neuronal damage by preventing the microglial activation.

In the mature brain and under physiological conditions, resting microglia acquire the characteristic ramified morphological appearance and serve the role of immune surveillance and host defense. Microglia, however, are particularly sensitive to changes in their microenvironment and readily become activated in response to infection or injury. These cells appear to be more responsive than neurons or astrocytes to the presence of toxicants and the resultant microglial activation often results in increased neurotoxicity [15]. Our light microscopic results were also in line with the previous reports and showed marked change from ramified (resting state) to amoeboid (active state) morphology of microglia when treated with dichlorvos. However, 4-HT pretreated cells showed very less or no change in the microglial morphology. Moreover, activated microglia upregulate a variety of surface receptors, including the major histocompatibility complex and complement receptors. Since decades, CD11b integrin has been used as a well-established marker of microglial activation [16]. So, we also checked the expression of microglial activation marker, CD11b, and found significant increase in its expression after 24 h of dichlorvos treatment. However, 4-HT pretreatment effectively prevented the upregulation of microglial CD11b expression levels when compared with dichlorvos-treated cells. These results are in agreement with previous reports which showed that in various neuroinflammatory diseases, increased Cd11b expression corresponds to the severity of microglial activation [17, 18].

Besides morphological changes and surface molecule upregulation, activated microglia secrete a host of soluble factors. The majority of factors produced by activated microglia, however, are pro-inflammatory and neurotoxic. These include the cytokines TNF- $\alpha$  and IL-1 $\beta$  and free radicals such as NO and ROS. Studies using cell culture and animal models have demonstrated that excessive quantities of these factors produced by activated microglia can be deleterious to neurons [19, 20]. Recently, we have shown that dichlorvos exposure can induce the production of above-mentioned pro-inflammatory molecules in microglial cells. Therefore, our next objective was to check whether 4-HT pretreatment could attenuate the production of these molecules in microglial cells after the dichlorvos treatment. We found significant decrease in the dichlorvos-induced ROS production when microglia were pretreated with 4-HT, suggesting that it would be effective against downstream targets of ROS which are responsible for neuronal injury. Next, Griess assay was performed to measure the NO levels; we found time-dependent increase in NO levels when cells were treated with

dichlorvos. However, 4-HT pretreated cells showed significant reduction in the NO levels when compared with dichlorvos-treated cells. These results suggest that 4-HT, which is a potent NO scavenger and SOD mimetic, can effectively downregulate the dichlorvos-induced NO and ROS production in microglial cells.

Next, we assessed the levels of pro-inflammatory cytokines; semi-quantitative PCR analysis and ELISA results showed that 4-HT pretreatment can significantly attenuated the production of the TNF- $\alpha$  and IL-1 $\beta$  when compared with dichlorvos-treated cells. However, the mechanism how 4-HT controls the expression of these cytokines is not very clear, but this could be attributed to reduced oxidative and nitrosative stress which in turn responsible for decreased microglial activation. Similar findings, although in different tissue, have shown that pretreatment with 4-HT resulted in significant attenuation of LPS-induced lung injury as well as inhibition of LPS-mediated increase in TNF- $\alpha$ , IL-1 $\beta$  and NO levels in lung tissue [21]. Furthermore, 4-HT can successfully mitigate oxidative stress, improve mitochondrial structural integrity and restore intra-cellular and intra-organelle redox status, in cardiac tissue [22].

Our next objective was to check how the primary neuronal cells behave when grown in activated microglial environment. As we have already shown that microglial cells secrete various pro-inflammatory molecules in culture medium when treated with dichlorvos. So, we collected the conditioned medium obtained from the primary microglial cells treated with dichlorvos (DCM). Then, we checked the viability of neurons when exposed either with DCM or with dichlorvos. Results of light microscopy, immunofluorescence and MTT analysis showed significant decrease in the neuronal cell viability after DCM treatment when compared to controls. Although dichlorvos alone-treated neurons also showed decrease in their viability, but the extent was significantly less than that of DCM-treated neurons, suggesting clear involvement of molecules present in DCM. However, when neurons were exposed with TDCM, significant attenuation of loss in neuronal viability was observed. These results are in line with previous findings of Monnet-Tschudi et al. which showed, toxic exposure to isolated neurons caused death of only ~30 % of the neurons, whereas all neurons died in co-cultures of neurons with microglial cells [23].

Previous studies from our lab have shown that low level chronic exposure to dichlorvos can induce apoptotic neuronal cell death in a rodent model [1, 24]. In addition, many in vitro and in vivo findings suggest that OPs can target glial cells as well [25, 26]. Using serum-free aggregating brain cell cultures, glial reactivity was studied in response to various neurotoxicants ranging from OP pesticides (parathion and chlorpyrifos) to heavy metals (mercury) [23]. The exacerbated neurotoxicity was accompanied by the increased release of TNF- $\alpha$ , indicating increased microglial activation

[27]. Several studies have shown that the pro-inflammatory molecules secreted by microglia can induce neuronal apoptosis in vitro as well as in vivo [28].

Based on this background, we intended to check whether DCM exposure could also induce apoptosis in primary neuronal cells. We found significant increase in p53 and Bax mRNA as well as protein expression when neurons were exposed to DCM as compared to control. These results are in line with previous findings of Yang and Tiffany-Castiglioni which showed that paraquat-induces cell death in dopaminergic neurons through a mechanism in which p53 and mitochondrial apoptotic pathway are linked [29]. Increased cytochrome *c* release from mitochondria and increased active caspase 3 expression in the cytosolic fraction were observed when neurons were treated with DCM as compared to control. However, TDCM-treated neurons showed significant decrease in the p53, Bax, cytochrome *c* and caspase 3 mRNA as well as protein levels when compared with DCM-treated neurons. These findings added new dimension to the molecular mechanism of dichlorvos-induced neurotoxicity and also supplemented our in vivo work which showed similar results [1]. These results suggested that soluble factors like NO, ROS, TNF- $\alpha$  and IL-1 $\beta$  secreted by dichlorvos-induced microglial cells might play an important role along with other pro-inflammatory molecules, in promoting neuronal cell death. Taken together, all these findings suggest that 4-HT could be a potential therapeutic agent to prevent the dichlorvos (OPs)-induced microglial activation and neuronal damage.

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**Conflict of Interest** None

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