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4-Hydroxy TEMPO Attenuates Dichlorvos Induced Microglial Activation and Apoptosis

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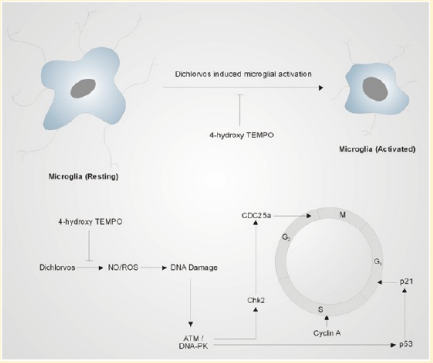
ABSTRACT: Microglial cells have been implicated in various neurodegenerative diseases. Previous studies from our lab have shown that dichlorvos (an organophosphate) could induce Parkinson's like features in rats. Recently, we have shown that dichlorvos can induce microglial activation, and if not checked in time could ultimately induce neuronal apoptosis. However, this activation does not always pose a threat to the neurons. Activated microglia also secrete various neuronal growth factors, suggesting that they have beneficial roles in CNS repair. Therefore, it is essential to control their detrimental functions selectively. Here, we tried to find out how microglial cells behave when exposed to dichlorvos in either the presence or absence of potent nitric oxide scavenger and superoxide dismutase mimetic, 4-hydroxy TEMPO (4-HT). Wistar rat pups (1 day) were used to isolate and culture primary microglial cells. We found 4-HT pretreatment successfully attenuated the dichlorvos mediated microglial activation. Moreover, 4-HT pretreatment decreased the up-regulated levels of p53 and its downstream effector, p21. The expression of various cell cycle regulators such as Chk2, CDC25a, and cyclin A remained close to their basal levels when 4-HT pretreatment was given. DNA fragmentation analysis showed significant reduction in the DNA damage of 4-HT pretreated microglia as compared to dichlorvos treated cells. In addition to this, we found 4-HT pretreatment prevented the microglial cells from undergoing apoptotic cell death even after 48 h of dichlorvos exposure. Taken together, our results showed 4-HT pretreatment could successfully ameliorate the dichlorvos induced microglial cell damage.



KEYWORDS: *Microglia, 4-hydroxy TEMPO, dichlorvos, p53, cell cycle, apoptosis*

Organophosphates (OPs) are one of the most widely used pesticides in agriculture, wartime, and domestic insect control. It is therefore virtually impossible to escape from their exposure. The primary target for OPs is acetylcholine esterase (AChE), a β-esterase whose physiological function is to hydrolyze acetylcholine, a major neurotransmitter in peripheral and central nervous systems. However, a number of studies on the cholinesterase-unrelated effects underlying the neurotoxicity of OPs have pinpointed the combination of antimitotic and pro-apoptotic mechanisms.[1,2] Epidemiological studies indicate that pesticides are one of the leading candidates among environmental toxins that may contribute to the pathogenesis of PD.[3] Reports of parkinsonism following pesticide exposure[4,5] make pesticide-induced parkinsonism biologically plausible. Hertzman et al. have also established a significant association between PD and the occupation of handling pesticides in British Columbia.[6] Although the risk of OP exposure as a result of extensive pesticide use is considerably higher for most people, but the increasing concern is the threat from intentional poisonings by rogue governments and terrorist organizations. Several reports have shown that in the 1980s the Iraqi military attacked Iranian military soldiers and Kurdish civilians with OP-based nerve agents responsible for "tens of thousands" of casualties.[7,8]

A previous study from our lab has shown that chronic low dose dichlorvos exposure can induce oxidative stress and α-synuclein expression which might be responsible for observed dopaminergic neuronal cell death, and hence could induce Parkinson's like symptoms in rats.[9] In addition, our lab has also shown that dichlorvos, another known OP that was used in Gulf War (GW), could induce microglial activation in vivo as well as in vitro conditions.[10,11] The presence of activated microglia close to dopaminergic neurons in post-mortem PD patient brains[12,13] and PD animal models[14] suggests a close relationship between neurodegeneration and neuroinflammation in PD. Numerous investigations have proposed a deleterious role of microglial activation in PD based on the vulnerability of dopaminergic neurons to various microglia-derived pro-inflammatory cytokines.[15,16]

As we have already shown, dichlorvos can induce oxidative as well as nitrosative stress in microglia, which might be responsible for observed DNA damage, mitotic arrest, and ultimately apoptotic cell death in these cells. So, in the present study, we hypothesized that if we restrict the production of oxidative/nitrosative stressors, we could possibly prevent the microglial overactivation, and hence its downstream effects. To achieve this, we employed a potent nitric oxide scavenger and superoxide dismutase mimetic, 4-hydroxy TEMPO (4-HT). It is a commercially available nitroxide, and a unique antioxidant

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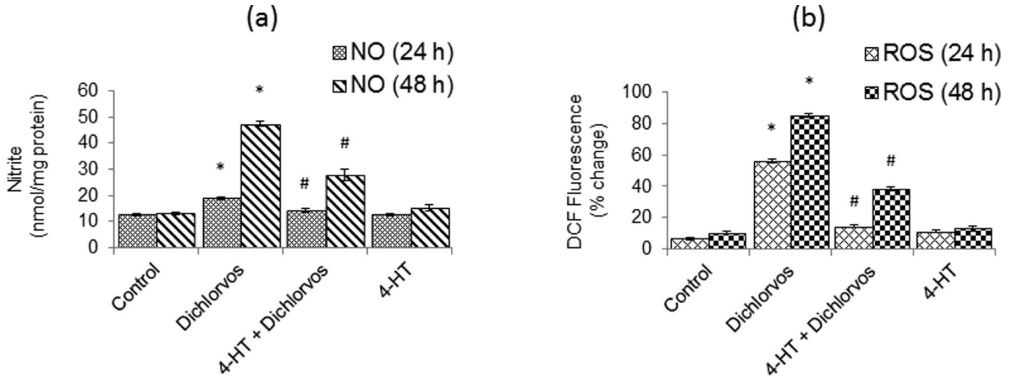

Figure 1. 4-HT attenuates dichlorvos mediated over production of NO and ROS. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV. (a) NO and (b) ROS levels were estimated after 24 and 48 h of dichlorvos treatment. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

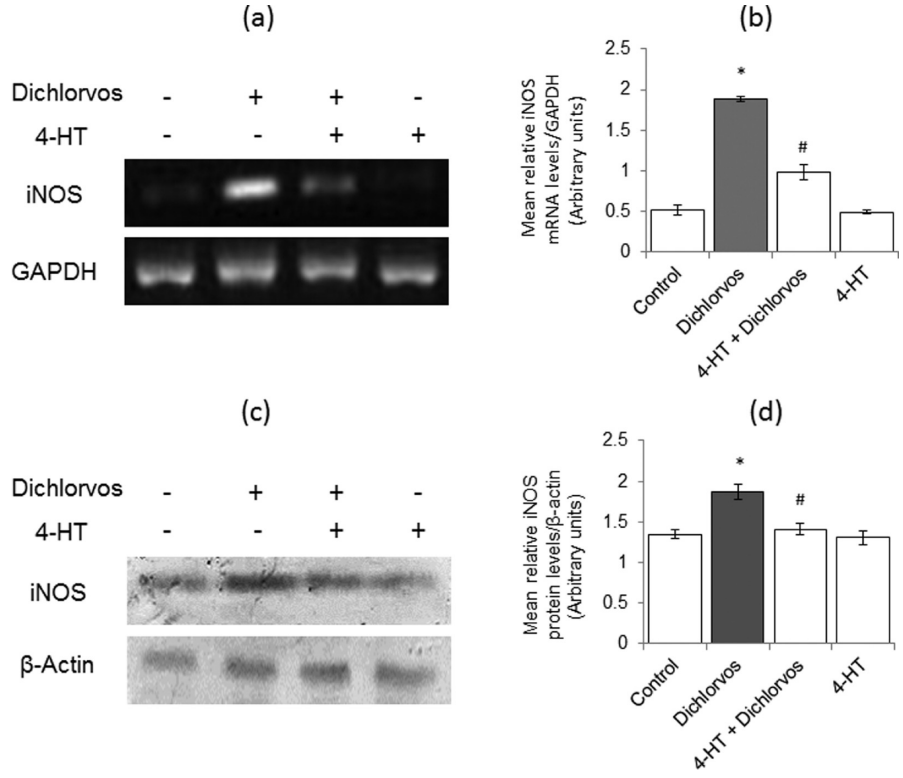

Figure 2. 4-HT attenuates dichlorvos induced expression of iNOS. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV. iNOS mRNA (a and b) and protein (c and d) levels were estimated after 24 h of dichlorvos treatment. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

which possesses several advantages that make it an excellent candidate to provide cellular protection.[17] In contrast to other antioxidants that are generally active against only one species of radicals, 4-HT possesses superoxide dismutase (SOD)-like activity, which dismutates the oxygen radicals and also reduces the NO levels.[18] Importantly, it has a catalytic mode of action and replenishes its antioxidant capacity, in contrast to other antioxidants, which eventually exhaust their antioxidant capacity.[19] All these properties make 4-HT a potential candidate to prevent nitrosative/oxidative stress generated by dichlorvos in microglial cells and therefore might be useful against microglia mediated neuronal damage.

RESULTS AND DISCUSSION

In the present study, we have shown that dichlorvos can induce microglial activation and stimulates the production of ROS, NO and CD11b cell surface expression. Increased expression of these molecules could be responsible for the observed cell cycle arrest in microglia; however, prolonged (48 h) dichlorvos exposure resulted in p53 mediated apoptotic cell death. However, we have shown that by limiting the production of ROS and NO via 4-HT, we could prevent the dichlorvos induced microglial apoptosis.

4-HT Attenuates Dichlorvos Mediated Overproduction of NO and ROS. To begin with, we first estimated the nitric oxide levels using the enzymatic property of nitrate

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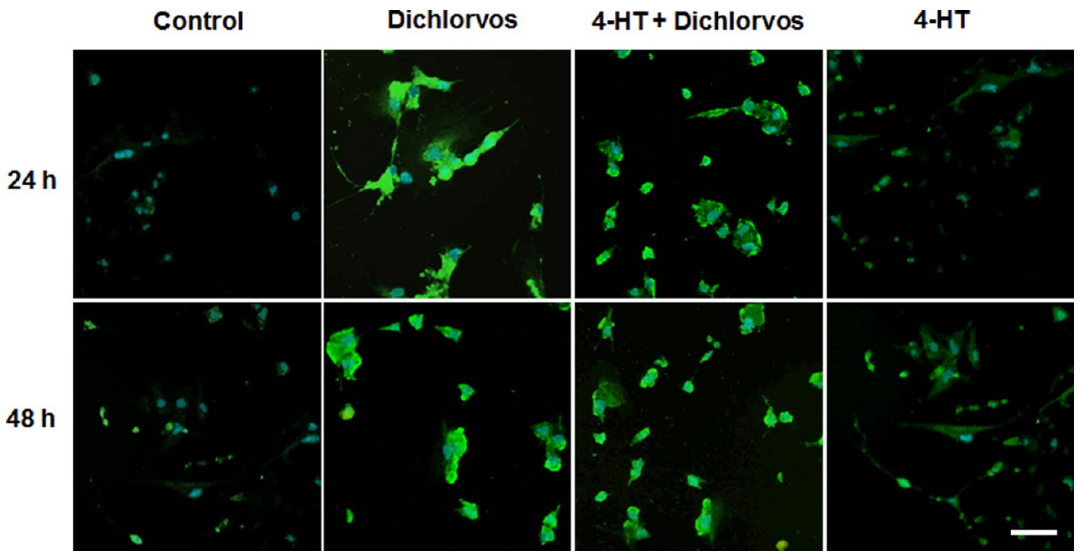

Figure 3. 4-HT attenuates dichlorvos induced expression of CD11b on microglial cells. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV. CD11b levels were analyzed by immunofluorescence detection after 24 and 48 h of dichlorvos treatment. Nuclei were stained with DAPI. Magnification, 100×.

reductase, which converts nitrate to nitrite, and ROS levels were estimated by using H_2DCFDA dye. On administering respective treatments, culture media were collected from each group after 24 h as well as 48 h. When media from 24 h dichlorvos treated cells were analyzed, we found a small, but significant increase in NO and almost 8-fold increase in ROS levels as compared to control cells. However, 48 h of dichlorvos treatment resulted in drastic increase in both NO (∼4-fold) as well as ROS (∼8-fold) levels. On the other hand, 4-HT pretreatment significantly decreased the dichlorvos induced NO production and almost restored the ROS levels in the culture (Figure 1). NO, produced by activation of NOS (nitric oxide synthase), has been proposed as a cytotoxic molecule that is responsible for neuronal cell death.[20] Therefore, next we wanted to check whether dichlorvos could affect the expression of inducible NOS and whether 4-HT could ameliorate this dichlorvos induced expression.

4-HT Pretreatment Prevents the Dichlorvos Induced Expression of iNOS and CD11b. Semiquantitative PCR and Western blot results revealed a significant increase in the expression of iNOS after the dichlorvos exposure both at transcriptional as well as translational levels. However, 4-HT pretreatment successfully attenuated the dichlorvos induced iNOS expression (Figure 2). Our results are in agreement with previous reports which showed mevinphos (OP) induced toxicity via NO, produced by activated iNOS.[21] In addition to this, it has been observed that 4-HT can block the enhanced NMDA-induced neurotoxicity in rat cultured cortical brain cells following induction of inducible NOS, suggesting that $ONOO^-$ was the damaging species.[21,22] Thus, these results suggested that 4-HT has the potential to prevent dichlorvos induced production of NO and ROS in microglial cells.

Our next objective was to check whether 4-HT has the potential to attenuate dichlorvos induced microglial activation. Among different markers, CD11b has immense biological significance.[23,24] It acts as a binding protein for intracellular cell adhesion molecule-1 and complement C3bi. It is reported that, in various neuroinflammatory diseases, increased Cd11b expression corresponds to the severity of microglial activation.[25] In agreement with our previous results, we observed marked attenuation of dichlorvos induced CD11b expression levels when microglia were pretreated with 4-HT (Figure 3). These findings are in line with previous reports, which have shown that microglia in brain regions are susceptible to OPs and rapidly become active on exposure.[26,27] However, our results are in contrast to recent finding by Ojo et al. in which chlorpyrifos (an OP) was unable to induce microglial activation.[28] Moreover, it has also been shown that 4-HT can prevent isoliquiritigenin induced CD11b expression in human myelogenous leukemia cell line HL-60.[29]

Under stress conditions, NO can combine with O_2^- to form peroxynitrite, which can induce DNA damage, protein degradation, and S-nitrosylation of cysteine residues in proteins that leads to cell damage and often death.[30] We have shown that prolonged exposure of dichlorvos can induce DNA damage in microglial cells.[11] Dichlorvos has also been shown to exert cytotoxic effects in rat hepatocytes,[31] as well as in rat tracheal epithelial cell line.[32] DNA strand breakage caused by dichlorvos is correlated with its DNA alkylating property in cultured Chinese hamster cells.[33] Moreover, dichlorvos also found to induce DNA damage in human lymphocytes[34] and micronucleus induction in CHO cells.[35] Therefore, next we wanted to check whether 4-HT pretreatment could prevent the dichlorvos induced DNA damage in microglial cells.

4-HT Pretreatment Attenuates Dichlorvos Induced DNA Fragmentation and Apoptosis in Microglial Cells. To check the extent of DNA damage induced by dichlorvos, we stained the microglial nuclei with DAPI. It is a blue fluorescent nucleic acid stain that preferentially stains double-stranded DNA (dsDNA). DAPI staining revealed a significant increase (>8-fold) in the small nuclear fragment formation in microglial cells after 48 h of dichlorvos treatment when compared to control cells. However, 4-HT pretreatment significantly prevented the dichlorvos induced nuclear fragmentation (Figure 4). In order to confirm our results, next we assessed the magnitude of damage caused by dichlorvos treatment on the genomic assembly. The DNA isolated from the dichlorvos treated microglial cells showed numerous oligonucleosomal fragments/ladders when subjected to agarose gel electrophoresis. As it is evident from Figure 5, 48 h of dichlorvos

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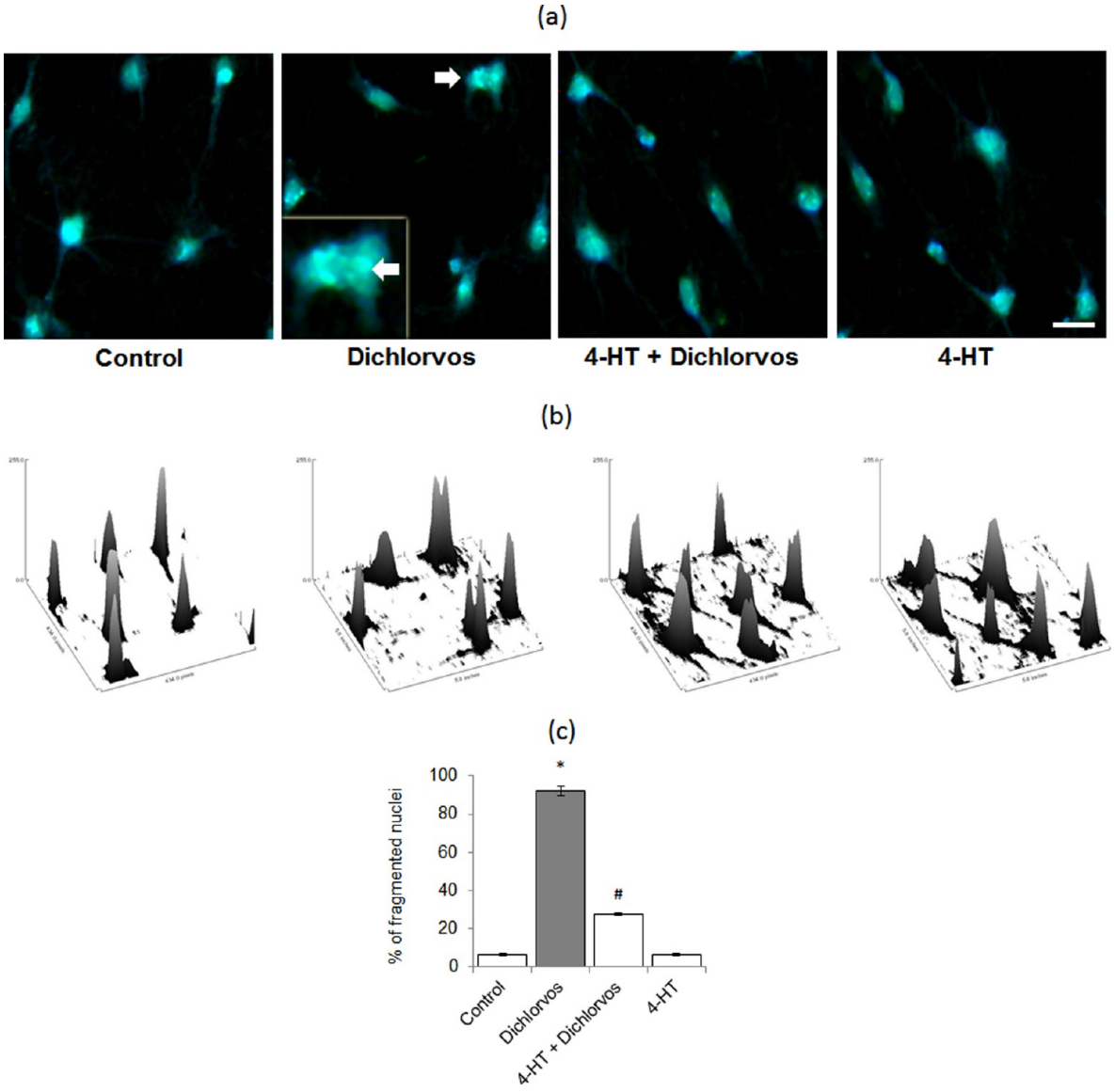

Figure 4. DAPI staining for fragmented nuclei detection in microglial cells. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV. (a) Microglial cells were stained with nucleus specific dye, DAPI. (b) Surface plots of microglial cells, treated with dichlorvos and or with 4-HT, were constructed by using Image-J software. Single peak represents the intact nucleus whereas multifurcated peaks represent fragmented nucleus. (c) Histogram shows the percentage of cells having fragmented nuclei after 48 h of dichlorvos treatment. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group. Magnification, 100×.

exposure (lane 2) resulted in increased DNA fragmentation in microglial cells when compared to control cells (lane 1). However, 4-HT pretreated microglial cells (lane 3) showed clear attenuation of the dichlorvos induced DNA fragmentation. Therefore, both DAPI staining and DNA fragmentation analysis revealed that 4-HT can prevent the dichlorvos induced DNA damage in microglial cells. This could be attributed to its strong antioxidant properties which prevented the formation of NO and ROS in microglial cells. Our results are in agreement with the work of Chan et al., which showed that 4-HT prevented the O_2^- production as well as DNA fragmentation when injected into the rostral ventrolateral medulla of lipopolysaccharide treated rats.[36] Moreover, it has been shown that 4-HT completely prevented the formation of microcystin-LR-induced DNA strand breaks and oxidized purines in HepG2 cells.[37]

The cellular response to DNA damage involves the activation of checkpoint pathways that impose the delay of cell-cycle progression and control DNA repair and replication.[38] Key molecules for the initiation of the damage response are ATM (ataxia telangiectasia mutated) and DNA-PK (DNA protein kinase). Therefore, next we wanted to check whether 4-HT could prevent the dichlorvos induced DNA damage response in microglial cells. However, one of the major constraints that we faced during this study was the limited number of microglial cells to extract the proteins for blotting assays, as microglia constitute only 8−10% of total glial cell population in mammalian brain. The yield after isolation and purification from single culture was very small, so we had to pool cells from at least 4−5 cultures. We have applied many improvements in our primary culture protocol but only managed to get a very limited amount of protein. The situation becomes worse with

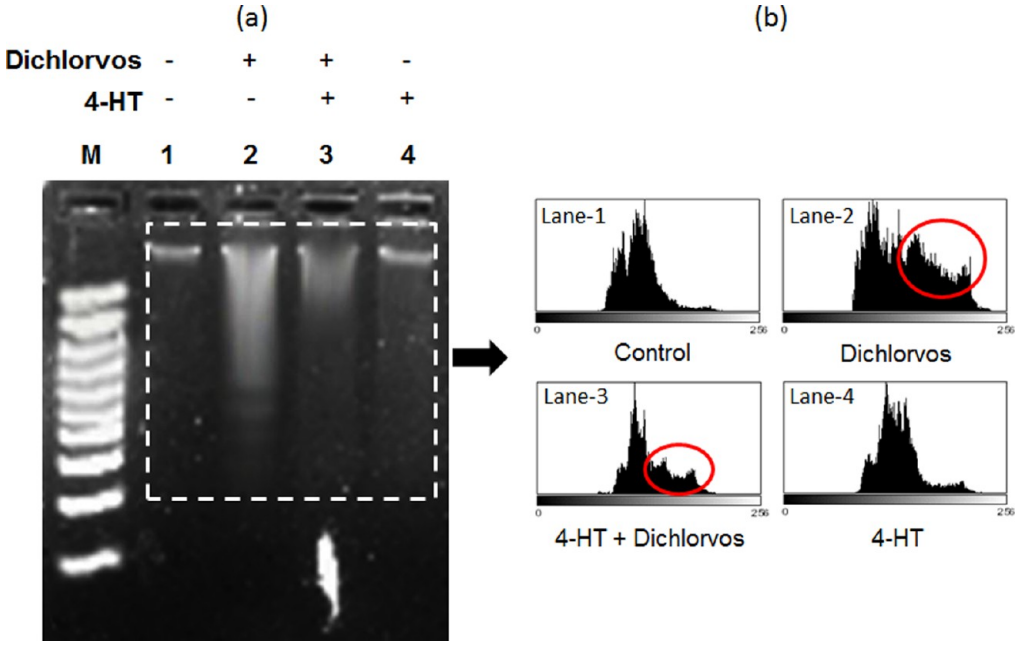

Figure 5. DNA fragmentation analysis. DNA was isolated from microglial cells after 48 h of treatment. The obtained DNA was subjected to agarose (1.8%) gel electrophoresis. (a) Lane 1, 100 bp ladder; lane 2, DNA from control cells; lane 3, DNA from dichlorvos treated cells; lane 4, DNA from dichlorvos and 4-HT pretreated cells; lane 5, DNA from 4-HT treated cells. (b) Surface plots of DNA isolated from microglial cells in each lane were constructed by using Image-J software. Numbers of peaks are proportional to the extent of DNA fragmentation.

the dichlorvos treated cultures as the number of viable cells is reduced. In addition, the abundance of proteins involved in the cell cycle regulation and DNA repair system is much less, which makes the situation even more difficult to obtain the protein samples. Therefore, we have tried to provide the best possible images that we could get under these limitations.

4-HT Pretreatment Prevents the Dichlorvos Induced Expression of DNA Repair System. To assess the effect of dichlorvos treatment on ATM and DNA-PK mRNA expression, semiquantitative PCR analysis was performed. After dichlorvos treatment, a significant increase in the expression of both ATM and DNA-PK mRNA was observed when compared to control. However, 4-HT pretreatment resulted in a significant decrease in the ATM and DNA-PK mRNA expression when compared to dichlorvos treated cells (Figure 6a–c). To assess the change in protein levels of DNA repair enzymes, Western blot analysis was performed. Results showed significant increase in the ATM (~2-fold) and DNA-PK (~2-fold) protein expression after dichlorvos treatment. However, when cells were pretreated with 4-HT, significantly less expression in the ATM and DNA-PK protein levels were observed as compared with dichlorvos treated cells (Figure 6d–f). ATM is a key player in DSBs responses, being activated by these breaks and phosphorylating key downstream proteins, leading to cell cycle checkpoint arrest and/or apoptosis.[39]

It was observed that following DNA damage, ATM undergoes rapid autophosphorylation and dimer dissociation, leading to its activation.[40] Among other targets, activated ATM phosphorylates p53 at serine 15[41] and indirectly, via Chk2, at serine 20.[42] Both events contribute to nuclear accumulation of p53 and transcriptional induction of p21, an inhibitor of the cyclin-dependent kinase 2/cyclin E complex required for cell-cycle progression from G1 to S phase.[43,44] Until now, we have shown that dichlorvos can induce DNA damage and 4-HT pretreatment could successfully attenuate this damage. Next, we wanted to study the effect of dichlorvos and 4-HT on main genes which get overexpressed in response to DNA damage, that is, p53 and p21.

4-HT Pretreatment Prevents the Dichlorvos Induced Expression of p53 and p21. The change in expression of different cell cycle checkpoints after dichlorvos treatment was analyzed by semiquantitative PCR and Western blotting. Semiquantitative PCR result showed significant increase in the expression of p53 (>2-fold) and p21 (>2-fold) mRNA when microglia were treated with dichlorvos. However, in 4-HT pretreated microglia the expression of p53 and p21 mRNA was significantly decreased when compared to dichlorvos treated cells (Figure 7a–c). To assess the change in their protein levels, Western blot analysis was performed. Results showed a significant increase in the expression of p53 (>3-fold) and p21 (~2-fold) after dichlorvos treatment. However, pretreatment with 4-HT showed significant decrease in p53 and p21 protein expression in microglia as compared to dichlorvos treated cells (Figure 7d–f). Our findings are in line with the results of the previous report which showed treatment of TPC-1 cells with rotenone increased the levels of p53 together with its phosphorylation at serine 15 and its nuclear accumulation.[45] A recent study showed paraquat exposure can also lead to NO mediated p53 activation in Siah cells.[46]

On receiving DNA damage, mammalian cells maintain their genomic integrity by activating G1 or G2 cell-cycle checkpoints or by inducing apoptosis.[47] Therefore, next we wanted to check how the 4-HT pretreatment affect the cell cycle distribution in microglia exposed with dichlorvos.

4-HT Pretreatment Prevents the Dichlorvos Induced Cell Cycle Arrest. Flow cytometry results showed a significant increase in the G1 (65.07%) and G2/M (22.54%) cell population whereas significant decrease in S phase (5.54%) population, when microglia were treated with dichlorvos as compared to control G1 (60.74%), S (14.82) and G2/M (9.49%) cell population. However, significant decrease in the microglial cell number was observed in the G1 (61.77%) and

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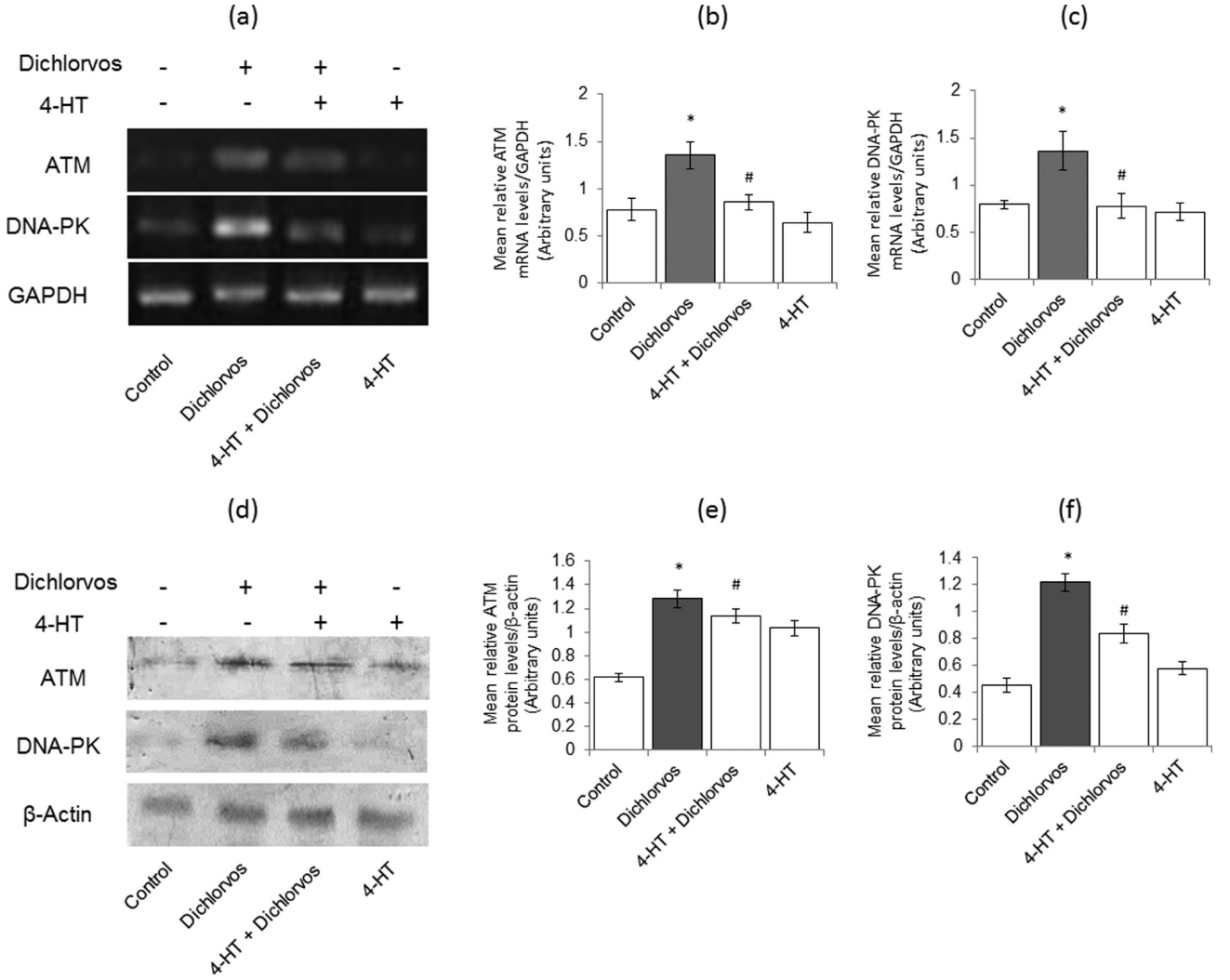

Figure 6. 4-HT pretreatment prevents the dichlorvos induced expression of DNA repair system. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV and subjected to respective treatment for 24 h. (a–c) Semiquantitative PCR results showing ATM and DNA-PK mRNA levels in the presence of dichlorvos and or with 4-HT. (d–f) Western blot analysis showing ATM and DNA-PK protein levels in the presence of dichlorvos and or with 4-HT. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

G2/M (11.64%) phase, whereas no significant increase in S phase (6.92%) population was observed in 4-HT + dichlorvos treated cells when compared with dichlorvos alone treated cells (Figure 8).

Cell cycle arrest is the compensatory mechanism of overcoming the cellular damage induced by a toxicant. Dichlorvos is known to induce DNA damage in various cells, so next we checked how microglia tried to prevent this damage and whether 4-HT pretreatment could attenuate these effects. Phosphorylation of Chk2 at Thr68 by ATM is the first step in its activation.[48,49] It is followed by homodimerization and trans-phosphorylation at Thr383 and Thr387 resulting in full activation.[50] Once activated, Chk2 regulates cell cycle checkpoint arrest through phosphorylation of CDC25a, CDC25b, and CDC25c, thus inhibiting their activity.[51] CDC25a is required for the activation of Cdk1 at the G2/M boundary, resulting in a G2 arrest. Several investigators reported that NO in proliferating cells inhibits cell proliferation by suppressing the cyclin A associated kinase activity through up-regulation of p21 protein.

4-HT Pretreatment Attenuates the Dichlorvos Mediated Change in the Expression of Cell Cycle Mediators. Semiquantitative PCR results showed a significant increase in the expression of Chk 2 (>2-fold) and CDC25a (~2-fold) mRNA whereas significant decrease was observed in Cyclin A (~2-fold) mRNA expression when microglia were treated with Dichlorvos. However, 4-HT pretreated cells showed a significant decrease in the expression of Chk 2 and CDC25a mRNA levels, whereas a small but significant increase in cyclin A mRNA expression was observed when compared with dichlorvos treated cells (Figure 9a–d). To assess the change in protein expression of these cell cycle mediators, Western blot analysis was performed. Results showed a significant increase in the expression of Chk 2 (~2-fold) and CDC25a (<2-fold), whereas a significant decrease in cyclin A protein levels was observed after dichlorvos treatment. However, 4-HT pretreated cells showed significant decrease in Chk 2 and CDC25a protein expression, whereas small but significant increase in Cyclin A protein expression was observed when compared with dichlorvos treated cells (Figure 9e–h). Cyclin A binds to S phase cyclin dependent kinase 2 (Cdk2) and is required for the

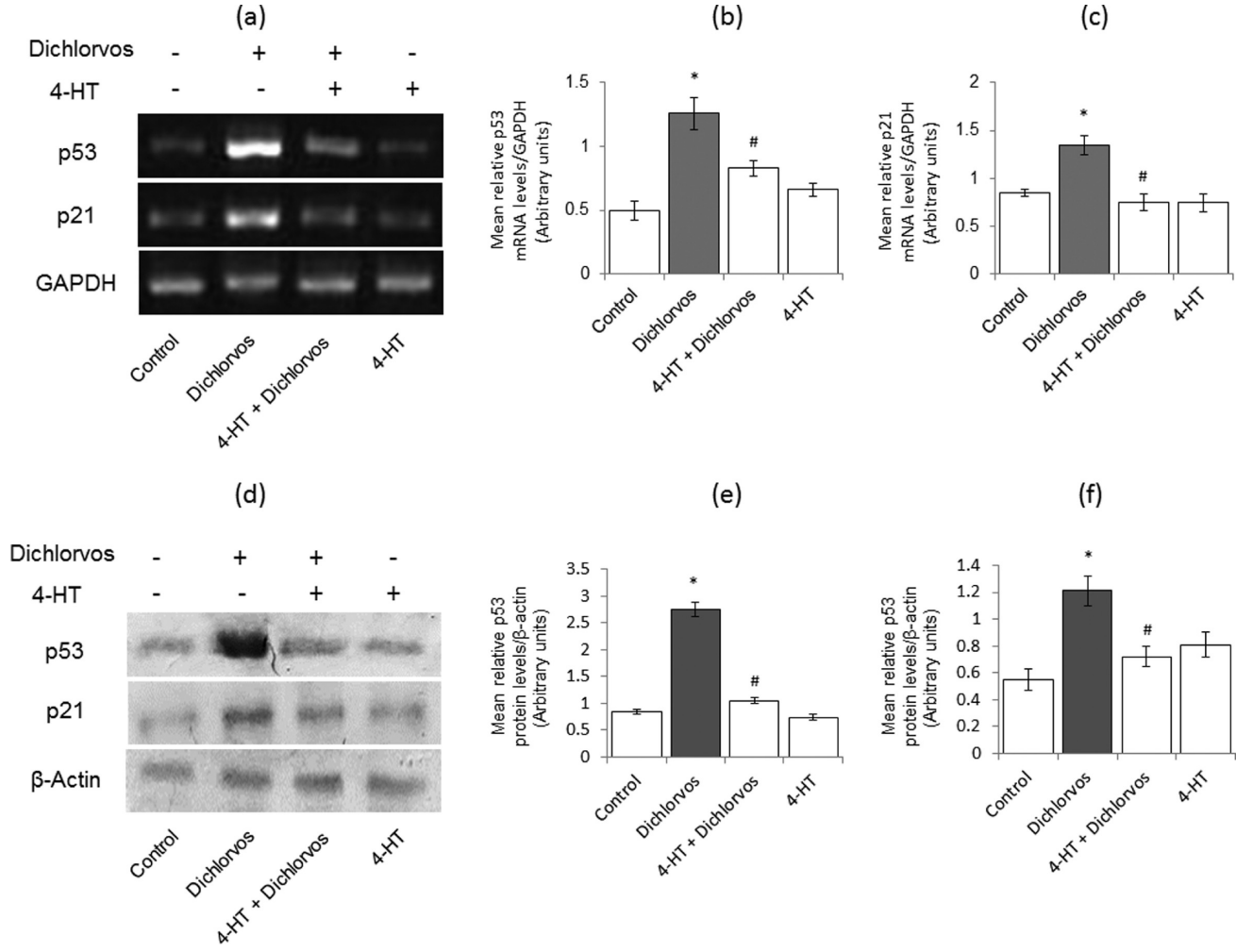

Figure 7. 4-HT pretreatment prevents the dichlorvos induced expression of p53 and p21. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV and subjected to respective treatment for 24 h. (a–c) Semiquantitative PCR results showing p53 and p21 mRNA levels in the presence of dichlorvos and or with 4-HT. (d–f) Western blot analysis showing p53 and p21 protein levels in the presence of dichlorvos and or with 4-HT. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

cell to progress through the S phase.[52] Our results are in agreement with previous reports which showed checkpoint activation by genotoxins like OPs results in either cell cycle arrest and repair of the damage, senescence, or apoptosis.[53,54] In addition, a recent report also showed that 4-HT treatment successfully attenuated the docosahexaenoic acid induced cell cycle arrest and apoptosis in human pulmonary artery smooth muscle cells.[55]

Doherty et al. have demonstrated that the incidence of micronuclei increases after exposure to trichlorfon in human lymphoblastoid cells, which suggests that OPs are potentially genotoxic.[56] Internucleosomal fragmentation of genomic DNA has been the biochemical hallmark of apoptosis for many years.[57] In accordance with these reports, our results strongly suggests that microglia might be undergoing apoptotic cell death after dichlorvos treatment. To confirm this, we checked the expression of key molecules involved in the apoptotic process, such as Bax, cytochrome *c*, and caspase 3.

4-HT Pretreatment Attenuates the Dichlorvos Induced Apoptosis in Microglial Cells. Western blot results showed a significant increase in the Bax expression (~ 2-fold) in mitochondrial fractions, increased cytochrome *c* release (~ 2-fold) from mitochondria into the cytosol and significant increase in the active to pro-caspase 3 ratio after 48 h of dichlorvos treatment when compared to control cells. However, significantly less increase in the expression of Bax and cytochrome *c* along with decreased active to pro-caspase 3 ratio was observed in 4-HT pretreated cells as compared to dichlorvos treated cells (Figure 10). Investigation by Kashyap et al. showed monocortophos (OP) exposure altered the expressions of caspase 3 and caspase 9, genes involved in apoptosis signaling cascade, in PC12 cells.[58] Moreover, paraquat and rotenone induce cytochrome *c* release[59,60] and caspase-9 activation, which are preceded by the induction/activation of proapoptotic Bax and Bak.[61] In addition, it has been observed that 4-HT pretreatment successfully inhibited the plumbagin and juglone induced accumulation of Bax in the mitochondrial membrane, cytochrome *c* release, as well as caspase-3 activation in human peripheral blood lymphocytes.[63]

To our knowledge this is the first report about the 4-HT mediated attenuation of various molecules involved in the cell cycle arrest and apoptotic microglial cell death induced by dichlorvos. In addition, the data presented here provide a useful in vitro model to study the implication of associative processes

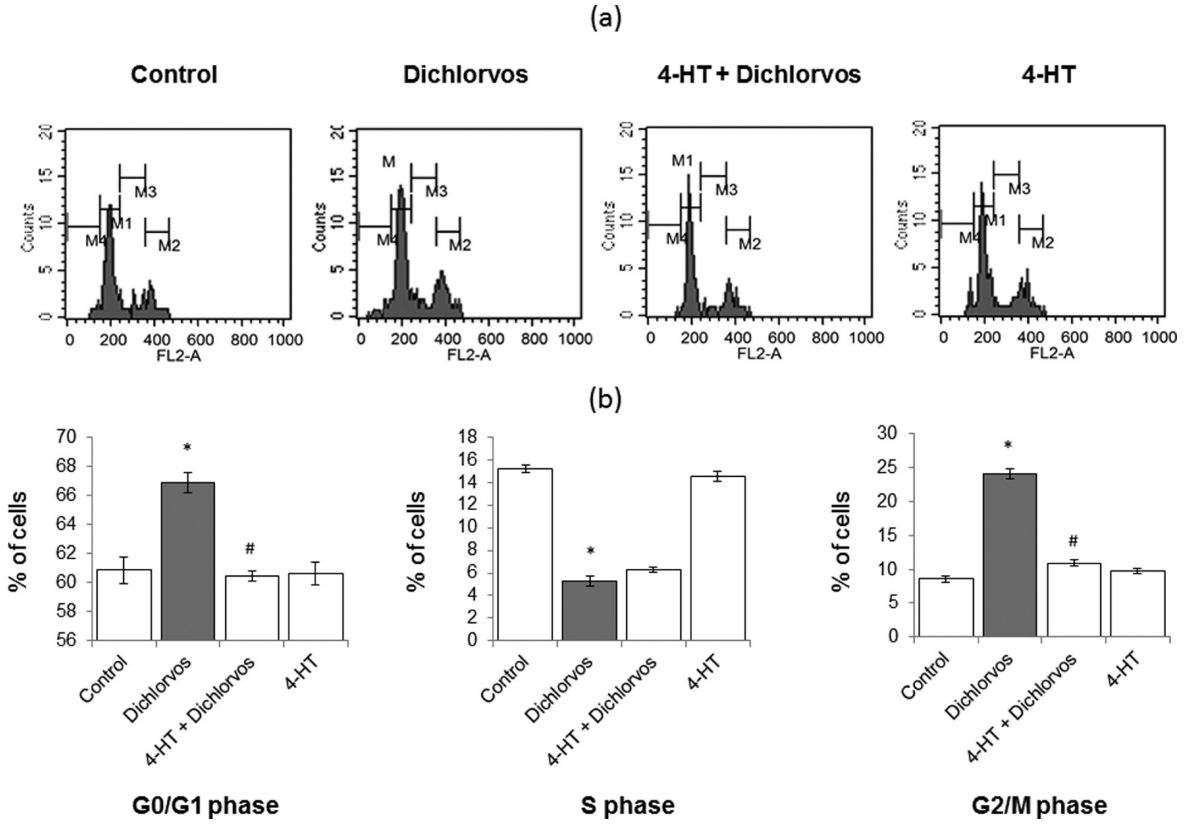

Figure 8. 4-HT pretreatment prevents the dichlorvos induced cell cycle arrest. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV. (a) Flow cytometry results show the distribution of microglial cell in different phases of cell cycle when treated with dichlorvos and or with 4-HT for 24 h. (b) Bar graphs show percentage of cells in each phase of cell cycle after dichlorvos exposure. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

in the development of OP-related sickness syndromes. The present study showed that microglial cells increased the expression of various cell cycle checkpoints and halted cell cycle progression either at G1, S, or G2/M phase to buy some time to repair dichlorvos induced DNA damage, whereas 4-HT pretreatment prevented the damaging effects of dichlorvos induced NO and ROS on DNA and other cellular components. Therefore, in the absence of significant DNA damage, DNA damage repair system was not activated, and cell cycle progression was almost comparable to control. Moreover, 4-HT pretreatment also prevented the dichlorvos induced apoptosis in microglial cells, and therefore could be used as potential therapeutic agent in microglia induced neurodegenerative disorders.

METHODS

Reagents and Antibodies. Minimum essential medium (MEM) was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS) and 0.25% trypsin was purchased from Invitrogen (Carlsbad, CA), while 96- and 6-well tissue culture plates, 25 cm^2 T-flasks, and cell scrapers were purchased from Greiner bio-one (St. Gallen, Switzerland). Syringe filters (0.2 μL) were purchased from Millipore (Billerica, MA). Griess reagent, H_2DCFDA and Dichlorvos were procured from Sigma-Aldrich (St. Louis, MO). Primary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), 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 of one day were sacrificed according to the guidelines of the 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.[11] Briefly, cerebral tissue was isolated aseptically and the meninges were removed. Each dissected brain was cut into small fragments and triturated with a pipet, followed by 5−10 passes through a 20 gauge needle. The dissociated cells were seeded in 25 cm^2 plastic flasks at the density of 2×10^6 cells in MEM, supplemented with 60 U/mL of penicillin, 50 mg/mL of streptomycin, and maintained at 37 °C in an atmosphere of 95% air and 5% CO_2 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.

Selection of Dose. *Dichlorvos.* Based on pharmacokinetic models and biomonitoring data, Buratti et al. have proposed that OP concentrations around 10 μM (in vitro; corresponding to 2.6, 2.5, and 1.6 μg/mL of methyl Parathion, methyl Paraoxon. and Dimefox, respectively) reflect comparable conditions as observed in general humans OP exposure cases.[64] Moreover, the concentrations higher than 100 μM reflect acute accidental intoxication. Therefore, to mimic the in vivo conditions, one third of the IC_{50}, that is, 10 μM (2.21 μg/mL), of dichlorvos was used to treat microglia in this study.

4-Hydroxy TEMPO. As evident from MTT assay results, 4-HT pretreatment provided maximum protection (>90% viability) at 50 μM concentration; and when this concentration was employed for inhibiting NO, a significant decline in microglial NO levels was

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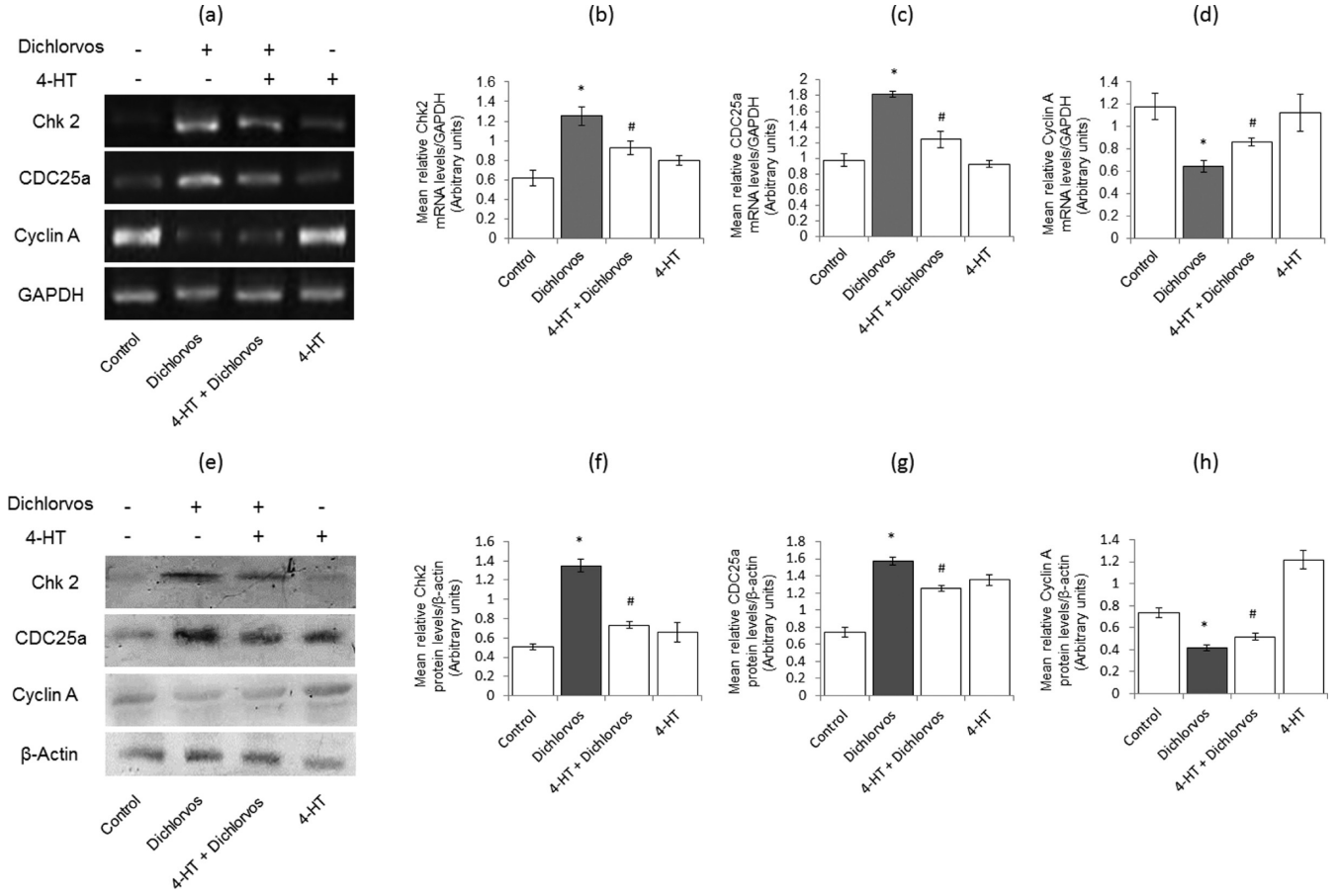

Figure 9. 4-HT pretreatment attenuates the dichlorvos mediated change in the expression of cell cycle mediators. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV and subjected to respective treatment for 24 h. (a) Semiquantitative PCR results showing (b) Chk2, (c) CDC25a, and (d) cyclin A mRNA levels in the presence of dichlorvos and or with 4-HT. (e) Western blot analysis showing (f) Chk2, (g) CDC25a, and (h) cyclin A protein levels in the presence of dichlorvos and or with 4-HT. Data were expressed as mean ± SEM of three independent experiments. *$p \leq 0.05$, significantly different from the control group. #$p \leq 0.05$, significantly different from dichlorvos group.

observed when compared to dichlorvos treated cells (data not shown). Therefore, we used 50 μM concentration of 4-HT against 10 μM dichlorvos in subsequent experiments.

Experimental Design. The whole study was divided into the following parts:

Control Group. Primary microglial cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV.

Dichlorvos Group (24 h). Primary microglial cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV and then treated with 10 μM of dichlorvos for 24 h.

Dichlorvos Group (48 h). Primary microglial cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV and then treated with 10 μM of dichlorvos for 48 h.

4-HT + Dichlorvos Group. Primary microglial cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV and subjected to 4-HT treatment (50 μM) prior 30 min of dichlorvos (10 μM) exposure (24 or 48 h).

4-HT Group. Primary microglial cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV and pretreated with 50 μM of 4-HT.

Measurement of Nitrite Accumulation. The concentration of nitrite, a stable oxidation product of NO, was estimated by Griess assay. Microglial cells (2×10^4 cells/well) were grown in 96-well tissue culture plates in the presence or absence of 10 μM dichlorvos. Using a micropipet, 50 μL of culture supernatant was transferred to 96-well microtiter plates, and mixed with 50 μL of the Griess reagent (0.1% *N*-[1-naphthyl]ethylenediamine dihydrochloride and 1% sulfanilamide in 2% phosphate solution). After incubation for 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 as described previously.[65] 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 diacetae (H_2DCFDA). After 20 min incubation in the 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.

Immunofluorescence. Microglia were cultured in 6 well tissue culture plates (Nalge Nunc International, Naperville, IL). After 7 DIV, cells were fixed with acetone/methanol (1:1) at −20 °C for 30 min. Cells were carefully washed three times with phosphate buffered saline (PBS) and blocked with blocking buffer (PBS with 1% BSA) at room temperature for 1 h followed by incubation with mouse monoclonal CD11b IgG2a primary antibody (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA) at 4 °C overnight. Cells were again subjected to three PBS washes and then incubated for 1 h at room temperature with FITC labeled rabbit anti-mouse secondary antibodies (1:1000,

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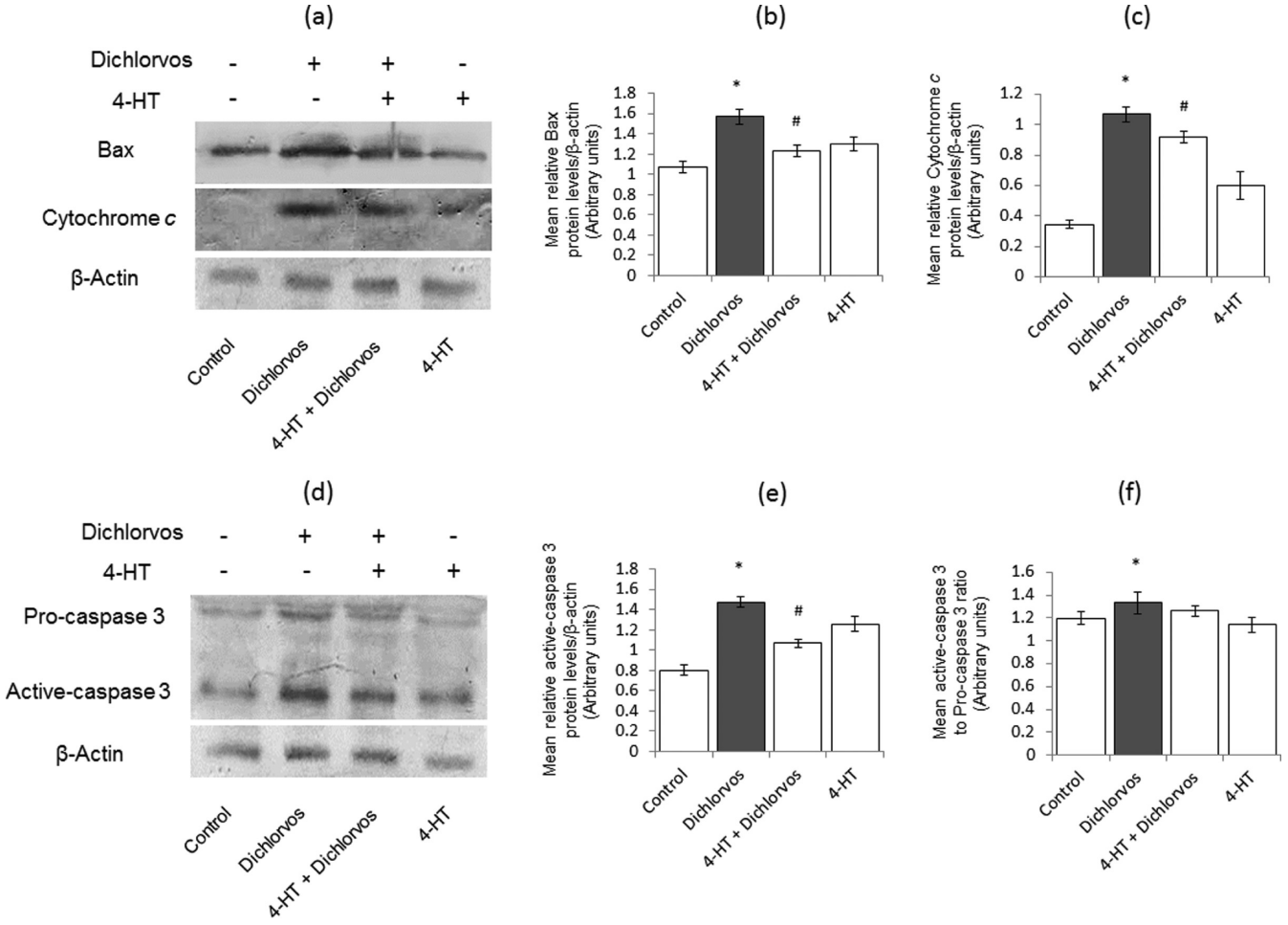

Figure 10. Western blot analysis of Bax, cytochrome *c*, and caspase-3. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 3 DIV and subjected to respective treatment for 48 h. (a) Western blot analysis showing levels of (b) Bax in the mitochondrial fraction, (c) cytochrome *c* release in cytosolic fraction, and (d–f) levels of active caspase-3 and ratio of active to pro-caspase-3 in cytosolic fractions of microglial cells. Data were expressed as mean ± SEM of three independent experiments. $^{*}p \leq 0.05$, significantly different from the control group. $^{\#}p \leq 0.05$, significantly different from dichlorvos group.

Table 1. Primer Sequences, PCR Conditions and Sizes of PCR Products

gene	forward sequence	reverse sequence	T_m (°C)	product size (bp)
p53	GCTTCGAGATGTTCCGAGAG	AGGATGCAGAGGCTGTCAGT	57.8	380
p21	TCAGTGGACCAGAAGGGAAC	GGTCCCCATCCCAGATAAGT	57.1	197
Chk2	TGGCAAGGAATGGATAGGAG	GTGAGAGGTCGTGCAACTGA	57.8	197
Cdc25A	TGTACGGGAACGAGATAGGC	TCTTGGTGCGGAACTTCTTT	57.4	182
cyclin A	TTTGGGCTTTCCCTCTTTCT	ACATCCGCACCGTTAACTTC	57.4	194
ATM	GGCTACCAAGACCGGACATA	TCCAAATGTCATGGCTTTCA	58.2	194
DNA-PK	TGAAGTGCTCTGGGTCTGTG	TTCAGGTGCATCAAGTCGAG	57.3	186
GAPDH	CACTGTGCCCATCTATGAGGG	TCCACATCTGCTGGAAGGTGG	61.8	496

Banglore Genei, India). Finally, cells were analyzed by fluorescent microscopy (Olympus, Lake Success, NY).

Semiquantitative PCR. Cells (1×10^6 cells/well) were cultured in 6-well tissue culture plates. Total RNA was extracted from cells of different groups using a Total RNA extraction kit (Taurus Scientific, India). Extracts were assayed to determine the quality and concentration of the RNA using a spectrophotometer. Extracts were stored at −20 °C. Isolated RNA was then digested with DNase (Promega, Germany) to destroy contaminating DNA, and cDNA was synthesized with RevertAid H Minus M-muLV Reverse Transcriptase (Fermentas, Germany). A total of 10 ng of cDNA was subjected to reverse transcriptase PCR amplification. For primer sequences, PCR conditions, and sizes of PCR products, please refer to Table 1.

DAPI Staining. DAPI, a DNA-binding fluorescent dye, was used to detect any abnormality in the architecture of chromatin. After treatment with dichlorvos (10 μM) and 4-HT (50 μM) for 48 h, the cells were washed three times with PBS, fixed in 1:1 acetone/methanol solution for 10 min at −20 °C, and then stained with 500 ng/mL DAPI for 10 min. Cells were observed under a fluorescent microscope, and images were analyzed via ImageJ Software (version 1.32, NIH, Bethesda, MD).

DNA Fragmentation. DNA was isolated from microglial cells grown in 6-well tissue culture plates (2×10^6 cells/well) using Apoptotic-Ladder Kit (G-Biosciences, Maryland Heights, MO). In brief, the cells were harvested and washed with PBS followed by centrifugation to get a pellet. The pellet was resuspended in PBS and

dispensed into a microfuge tube. Cells in suspension were again centrifuged to get pellet and added 150 μL of nucleosomal buffer to the pellet. Cells were suspended by pipetting up and down for a few times, again centrifuged at 15 000*g* for 5 min in the cold, and transferred the supernatant into a clean tube, followed by re-extraction of the nuclear pellet by repeating the previous steps. Then, we pooled the collected supernatant into a single tube and added 30 μL of the DNA stripping solution. We mixed the provided proteinase K solution by tapping the tubes and added 10 μL to the sample solution. The tubes were inverted a few times to mix the solution. The tubes were incubated at 55 °C for 90 min and allowed to cool at room temperature. We then added 150 μL of precipitation buffer and gently tapped the tube to mix properly. We centrifuged the tubes at 15 000*g* for 10 min and transferred the supernatant to a clean tube, and then 500 μL of ethanol was added and mixed gently. After incubation at −20 °C for 1 h, the sample was centrifuged at 15 000*g* for 10 min, and then the nucleosomal DNA precipitate was recovered. Supernatant was discarded and centrifuged again for 10 s. The pellet was allowed to evaporate and dry by a brief incubation at 35−40 °C. Finally, the pellet was resuspended in 25−30 μL of TE buffer and incubated at 35 °C for 10−15 min or until the DNA pellet was fully hydrated and subjected to 1.8% agarose gel electrophoresis.

Preparation of Mitochondrial and Cytosolic Fractions for Western Blotting. The cytosolic and mitochondrial fractions were prepared by the method of Tang et al.[65] At the time of fractionation the integrity of mitochondria was checked by assessing the respiratory control ratio and marker enzymes (data not shown). The microglia were homogenized (20 strokes) in 500 μL of buffer A (20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 220 mM mannitol, 68 mM sucrose, 1 mM leupeptin, 5 μg/mL pepstatin A, 5 μg/mL aprotinin, 0.5 mM PMSF). The homogenate was then centrifuged at 1000*g* for 10 min at 4 °C. The resulting supernatant contained the cytosolic fractions and the pellets contained enriched mitochondrial fractions. The cytosolic fractions were stored at −80 °C until further analysis. Pellets containing mitochondria were treated with the lysis buffer (1× 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 20 min. The lysate was centrifuged at 10 000*g* at 4 °C for 30 min. The resulting supernatant was kept as solubilized mitochondrial enriched fraction and stored at −80 °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 μg of protein were boiled in laemmli buffer for 5 min and subjected to electrophoresis (12% SDS-PAGE) followed by transferring to nitrocellulose membrane. The blots were blocked with 5% nonfat dry milk for 5 h, the membranes were then incubated with primary iNOS (1:200)/p53 (1:500)/p21 (1:500)/Chk2 (1:500)/CDC25a (1:500)/cyclin A (1:1000)/ATM (1:500)/DNA-PK (1:500)/Bax (1:100)/cytochrome *c* (1:100)/caspase-3 (1:100)/β-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. The membrane was again incubated for 1 h at 37 °C with horseradish peroxidase (HRP) conjugated secondary antibody. After 1 h of incubation, the blots were again washed with PBS plus 0.1% Tween-20 for 30 at 5 min interval of time. Immunoreactive proteins were visualized by DAB (diaminobenzidine) system. The densitometry analysis of the protein bands was carried out using AlphaEase FC 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.[66]

Statistical Analysis. For all experiments, data were analyzed from at least three independent experiments, each with at least duplicate determinations. A nonparametric method, Mann−Whitney U-Test, was applied to compare the statistical difference between the control and dichlorvos group as well as dichlorvos and 4-HT + dichlorvos group. Statistically significant values represent a p level of 0.05 or below. Error bars represent standard error of means (SEM).

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Author Contributions

A.S. and K.D.G. planned the experiments. D.R.S. and W.Y.W. helped A.S. in executing the experiments. A.S. and K.D.G. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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