

Original Article



Protective Effects of N-Acetylcysteine on Dipentyl Phthalate Induced Cognitive Dysfunction and Brain Oxidative Stress in Mice

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ABSTRACT

Background: Dipentyl phthalate (DPeP) is a plasticizer compound commonly used in polyvinylchloride plastic to enhance softness and flexibility. They are not bound covalently to plastic polymers; therefore, they can dissolve into the environment and adversely affect the health of humans and animals.

Objectives: The aim of this study was to investigate the effect of DPeP on cognition and protective effects of N-acetylcysteine (NAC) on DPeP induced alteration in cognitive behaviour and oxidative stress markers in mice.

Methods: Mice were orally treated with 2 doses (33 mg/kg and 100 mg/kg) of DPeP for 28 days. Cognitive functions were assessed using spatial navigation tasks on the Morris water maze and the step-down latency in the passive avoidance apparatus. Oxidative stress was assessed by examining the levels of malondialdehyde, glutathione, ferric reducing antioxidant power, and 8-hydroxy-deoxyguanosine levels in the whole brain of mice.

Results: There was a significant increase in latency in spatial navigation tasks and a significant decline in the step-down latency in passive avoidance apparatus in the DPeP-treated group compared to the control groups. There was also a significant increase in the levels of oxidative stress following DPeP administration as seen with the rise in the levels of malondialdehyde, 8-hydroxy-deoxyguanosine, and a fall in glutathione and ferric reducing antioxidant power levels.

Conclusion: The present study demonstrated that DPeP adversely affects learning and memory functions in mice by oxidative stress-mediated neuronal damage. These effects were attenuated by pretreatment with N-acetylcysteine.

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Introduction

Phthalates are pervasive environmental contaminants that constitute a large class of plasticizers that are combined with polyvinyl chloride products to enhance their softness and flexibility. These are diesters of 1,2-benzenedicarboxylic acid (phthalic acid), used by the chemical industry in a wide range of consumer products, such as building materials, food packaging, medical applications, and cosmetics [1, 2]. Phthalate esters are not covalently bound to these consumer products and may percolate in the surrounding environment [3]. Humans may get exposed to such phthalate esters either directly via oral, inhalational, and dermal routes, or indirectly from the contaminated environment [4]. Several lines of evidence suggest that the metabolites of these plasticizers have been widely detected in human urine across the world, indicating its widespread exposure to humans [5-7]. Children, in particular, are at higher risk of exposure since phthalates are common constituents of many items, such as feeding bottles and mouth toys [8]. Phthalate exposure is a concern for human health as they are known to have deleterious effects on various organ systems, including the endocrine, reproductive, and central nervous system [9-12].

The central nervous system, in particular, is more susceptible to harm from phthalate exposure as mature neurons cannot proliferate for self-repair. There is growing epidemiological evidence that demonstrates the link between phthalate exposure with attention deficit hyperactivity syndrome in childhood and cognitive dysfunction among adults [10, 13, 14]. Furthermore, studies in animals manifested that exposure to diethylhexylphthalate during the gestational period in rats is associated with a decline in the levels of free cholesterol, sphingomyelin, and docosahexaenoic acid that are essential components for the development of the nervous system [15]. Similarly, a study demonstrated that another phthalate i.e., DPeP, is also associated with reproductive and developmental toxicity; however, the data regarding its neurodevelopmental toxicity is limited [16].

The deleterious effects of phthalates may occur probably because of the generation of reactive oxygen species (ROS) which are formed during its metabolism and may lead to altered cell signaling, mitochondrial dysfunction, and carcinogenesis [9, 17]. A recent study demonstrated that phthalate esters could induce oxidative stress by activating peroxisomal proliferation and increasing the production of ROS [18]. Similarly, another study stated that phthalates trigger Deoxyribonucleic Acid (DNA)

damage in the sperm, testes, and human embryonic kidney cell culture line [19, 20]. In addition, phthalates can activate peroxisome proliferator-activated receptors, increase fatty acid oxidation, and the occurrence of malformations [9]. Additionally, both in vivo and in vitro studies have indicated a positive correlation between phthalate exposure and oxidative stress [21, 22].

There is evidence suggesting that antioxidants, such as vitamin E, could have the potential to ameliorate phthalate-induced memory deficit by limiting the decline in reduced glutathione (GSH) and the induction of malondialdehyde (MDH) levels in the experimental model [22]. Similarly, N-acetylcysteine (NAC) is another antioxidant that supplies the cysteine required for the synthesis of GSH. Previous studies have shown that oral administration of NAC protects cognitive deficits, GSH reduction, and type-2 ryanodine receptor decrease induced by amyloid beta oligomers, which may be the causative factors for Alzheimer disease in the rat model [23]. Thus, oxidative stress could be regarded as one of the key upstream events in phthalate toxicity; however, there is limited understanding of the cognitive impairment caused by phthalate exposure [24, 25]. In view of such evidence, the present study aims to evaluate the effects of DPeP exposure on the cognitive profile and oxidative stress markers in mice. Additionally, the effects of the co-administration of NAC with DPeP are assessed on these parameters.

Material and Methods

Study animals

Male Swiss albino mice, weighing from 20 to 25 g, were acquired from the central animal house of the University College of Medical Sciences and GTB Hospital, Delhi. The mice were kept under controlled environmental surroundings (22°C±1°C of temperature with 50%±2% of relative humidity, maintained with a natural light/day cycle). These mice were kept under hygienic conditions in polypropylene cages with soft bedding with free access to pellet diet and water for the whole duration of the experiment. Before the experimentation, the animals were acclimatized to the laboratory conditions. Animal procurement was done after taking permission from the Institutional Animal Ethics Committee, UCMS vide letter no. IAEC/2011/75 and CPCSEA (Committee for the Purpose of Control and supervision of Experiments on Animals). Meanwhile, the guidelines were followed for the care of animals.

Drugs treatment schedule

DPeP (Sigma St. Louis, MO, USA; purity > 97.0%) was dissolved in corn oil (vehicle for DPeP) and orally administered at a dose of 33 mg/kg and 100 mg/kg body weight for 28 days. NAC (SRL, Mumbai, purity=99.0%) was given orally at a dose of 100 mg/kg after dissolving in distilled water 1 h before DPeP, daily for 28 days. The animals were divided into two sets of 7 groups (I to VII) with 6 mice, each mouse for each experimental test of cognition. Groups I and II were treated orally with distilled water and corn oil, respectively. Groups III and IV were treated orally with DPeP at a dose of 33 mg/kg/day and 100 mg/kg/day, respectively. Group V received NAC at a dose of 100mg/kg/day. Groups VI and VII received DPeP at a dose of 33 mg/kg/day and 100 mg/kg/day along with 100 mg/kg/day of NAC, respectively [26, 27].

Tests to assess cognition

Step-down latency in passive avoidance apparatus

The passive avoidance apparatus consisted of a wooden block placed at the center of a grid floor to be served as a shock-free zone. On the 20th day of the experiment, the mice were placed in the shock-free zone and an electric shock (20 V) was given through the grid floor on stepping down from the shock-free zone. The mice were given 3 sets of such training at 1 h intervals. The acquisition of step-down latency (SDL) was recorded after 1 h of the third training session without giving a shock. On the 21st and 28th days of the experiment, the procedure was repeated without giving a shock. The time taken for the animals to step down was recorded as the first and the second retention latency. For the animals that did not step down during the cut-off time of 180 s, the SDL was considered as 180 s [28].

Spatial navigation task

The morris water maze (MWM) was used to evaluate the acquisition and retention of the spatial navigation task. The animals were given training sessions to swim toward a visible platform in a circular pool with a diameter of 150 cm. Water (28°C±2°C) was filled up to a height of 30 cm. The water was made opaque by a non-toxic dye. The pool had 4 quadrants (N, S, E, and W) and 4 locations around its edges that were equally spaced, and used for testing as starting points. A circular platform of 9 cm diameter was placed at the center of one of the quadrants about 2 cm above the water level for testing the acquisition latency and then immersed 2 cm below the water level to test the retention latency. On the 20th day of the experiment, the mice were trained by

giving 4 trials with different starting points. The mice were released for the trials while facing toward the wall of the pool and the latency to find the escape platform was recorded to a maximum of 180 s. The mice that could not locate the platform within the given time were given guidance to reach the platform and were kept there for at least 30 s. The time taken by the animals to reach the platform was taken as the initial acquisition latency (IAL). The animals were returned to their home cages after the trials, and a 5 min gap was given between the subsequent trials. On the 21st day, the animals were released randomly at one of the edges of the pool, and the time taken to find the platform was recorded as first retention latency (1st RL). Similarly, the time taken to find the hidden platform on the 28th day was recorded and labeled as the second retention latency (2nd RL) [29, 30].

Locomotor activity

Both the horizontal and the vertical locomotor activity was measured by an activity cage (UGO Basile). The activity was recorded for 5 min on the 20th, 21st, and 28th days. As the animal moved about a clear acrylic cage, it interrupted one or more infrared beams (IR). The electronic unit of the activity cage recorded the beam interruptions and they were used to analyze and assess the activity of animals as described by Bareggi et al. (2003) [31].

Biochemical tests

On the 29th day, following assessment of behavioral parameters, animals were euthanized by giving deep halothane anesthesia [32]. Their whole brain was dissected out, washed using ice-cold sodium phosphate buffer, weighed, and finally stored over ice. The sodium phosphate buffer (KH₂PO₄ and Na₂HPO₄) was used to homogenize the brain tissue. Then, centrifugation was done at 3000 rpm for 15 min, and the following oxidative stress markers were estimated with the supernatant within the next 12 h of dissection.

Measurement of malondialdehyde

Malondialdehyde (MDA) was estimated as described by Okhawa et al. (1979) [33]. The processed tissue sample (0.5 mL) were added to acetic acid 1.5 mL (20%) pH 3.5, 1.5 mL thiobarbituric acid (0.8%), and 0.2 mL sodium lauryl sulfate (8.1%). The sample was then boiled for 60 min at 100°C, cooled, and then 5mL of n-Butanol. pyridine (15:1 v/v) and 1 mL of distilled water were added. The mixture was then vortexed. Centrifugation of the mixture was done at 4000 rpm for 10 min. The organic layer was withdrawn and using a spectrophotometer its absorbance was measured at 532 nm.

Measurement of reduced glutathione

GSH was estimated according to the method described by Ellman et al. (1959) [34]. Centrifugation of the prepared homogenate was done with 5% trichloroacetic acid to remove the proteins. A volume of 2 mL of phosphate buffer (pH=8.4), 0.5 mL of 5,5'-dithiobis (2-nitrobenzoic acid), along with 0.4 mL of double-distilled water were then added to 0.1 mL of this homogenate. The mixture was vortexed and then its absorbance was measured at 412 nm within 15 min.

Measurement of ferric reducing antioxidant power level

The FRAP assay was done according to the method described by Benzie et al. (1996) [35]. This method involves the reduction of the complex of Fe³⁺-TPTZ (2, 4, 6-tri-(2-pyridyl)-s-triazin) to the ferrous form at low pH by low molecular weight antioxidants. A total of 100 µL of supernatant or plasma, 3 mL of the FRAP reagent (25 mL 0.3 M sodium acetate buffer, pH 3.6; 2.5 mL 0.01 M TPTZ in 0.04 M HCl; 2.5 mL 0.02 M FeCl₃.6H₂O; preheated to 37°C) was added and incubated for 5 min at 37°C and then its absorbance was recorded at 593 nm using a spectrophotometer. The value of the FRAP was expressed as µmol/mL of homogenate.

Measurement of 8-hydroxy-deoxyguanosine level

The 8-OH-dG levels in the mice brain were estimated using a commercially available murine 8-OH-dG EIA kit from Cayman chemical company, USA, according to the instructions given by the manufacturer.

Statistical analysis

The data were recorded as Mean±Standard Error of the Mean (SEM) and analyzed using a 1-way analysis of variance followed by the post hoc Tukey test. The P value of less than 0.05 was considered significant for all the experiments.

Results

Effects of dipentyl phthalate and dipentyl phthalate plus n-acetylcysteine treatment on memory task using passive avoidance test

The treatment of the mice with 33 mg/kg and 100 mg/kg doses of DPeP led to a significant decrease in the mean acquisition of SDL in passive avoidance tasks compared to the control and vehicle-treated mice (P<0.05).

There was a significant reduction in the retention latencies in both DPeP-treated groups on day 21 (P<0.01 vs control and P<0.05 vs vehicle) and day 28 (P<0.001) compared to the control and vehicle groups. Pretreatment of NAC (100 mg/kg) with DPeP (33 mg/kg) treated mice significantly prolonged the retention latencies on day 21 and day 28 compared to the group that was treated only with DPeP (33 mg/kg) (P<0.05) (Figure 1).

Effects of dipentyl phthalate and dipentyl phthalate plus n-acetylcysteine treatment on memory task using spatial navigation test

The treatment of the mice with 33 mg/kg and 100 mg/kg doses of DPeP caused a significant increase in the mean acquisition of latency in spatial navigation tasks compared to the control (P<0.05) and vehicle (P<0.01) groups on the 20th day, suggesting the DPeP-induced impairment in the acquisition of spatial navigation task. On the 20th day, the pretreatment of NAC (100 mg/kg) with the DPeP (33 mg/kg) group significantly reduced the mean acquisition latency to reach the platform compared to the group treated only with DPeP (33mg/kg) (P<0.01). A significant increase in retention latencies (1st RL and 2nd RL) on day 21 and day 28 were noted for DPeP (33 mg/kg) and DPeP (100 mg/kg) treated groups compared to retention latencies on days 21 and 28 of the control (P<0.01) and vehicle (P<0.05) groups. Administration of NAC (100 mg/kg) in DPeP (33 mg/kg) treated mice showed a significant decline in the 1st RL and the 2nd RL compared to the DPeP (33 mg/kg) group (P<0.05) which suggests an overall improvement in the performance for the spatial navigation task (Figure 2).

Locomotor activity

None of the studied groups showed any significant difference in either horizontal or vertical locomotor activity when compared to the control and vehicle groups on the 20th, 21st, and 28th days.

Effects of dipentyl phthalate and dipentyl phthalate plus n-acetylcysteine treatment on malondialdehyde and glutathione levels

Administration of DPeP (33 mg/kg and 100 mg/kg) resulted in a marked rise in the MDA levels and a decline in the GSH levels compared to the control and vehicle-treated groups. NAC pretreatment with DPeP (33mg/kg) and DPeP (100mg/kg) led to a significant decrease (P<0.001) in MDA levels compared to the groups treated only with DPeP (33mg/kg) and DPeP (100mg/kg). Co-administration of NAC with DPeP (33mg/kg) in mice

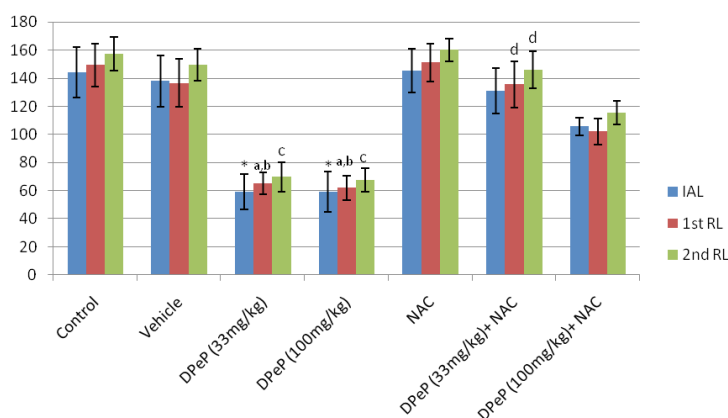


Figure 1. Effects of DPeP and DPeP+NAC treatment on step down latency in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; IAL, initial acquisition latency; RL, retention latency.

Notes: Values are presented as Mean±Standard Mean Error; (n = 6); IAL: $F_{6,35} = 6.64$; 1st RL: $F_{6,35} = 8.61$; 2nd RL: $F_{6,35} = 14.99$; * $P < 0.05$ as compared to the control/vehicle group; ^a $P < 0.01$ as compared to the control group; ^b $P < 0.05$ as compared to the vehicle group; ^c $P < 0.001$ as compared to the control/vehicle group; ^d $P < 0.05$ as compared to the DPeP (33 mg/kg) group.

showed a significant increase in the GSH levels compared to the group only treated with DPeP (33mg/kg) ($P < 0.05$) (Figure 3 and 4).

the groups treated only with DPeP 33mg/kg and DPeP 100mg/kg (Figure 5).

Effects of dipentyl phthalate and dipentyl phthalate plus n-acetylcysteine treatment on ferric reducing antioxidant power levels

Effects of dipentyl phthalate and dipentyl phthalate plus n-acetylcysteine treatment on 8-hydroxydeoxyguanosine levels

DPeP 33 mg/kg ($P < 0.05$) and DPeP 100 mg/kg ($P < 0.001$) resulted in a marked decline in the brain FRAP levels compared to the control and vehicle groups. The administration of NAC with DPeP 33 mg/kg ($P < 0.05$) and DPeP 100mg/kg ($P < 0.01$) in the mice showed a significant increase in the brain FRAP levels compared to

The DPeP-treated mice (33 mg/kg and 100 mg/kg) showed a significant increase in the brain 8-OH-dG levels compared to the control and vehicle groups. Co-administration of NAC with DPeP (33 mg/kg and 100 mg/kg) in mice showed a significant decline ($P < 0.05$) in the brain 8-OH-dG levels compared to the groups treated only with DPeP (33 mg/kg and 100 mg/kg) (Figure 6).

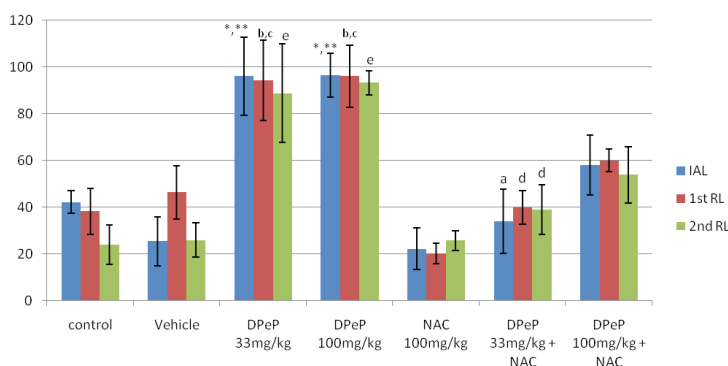


Figure 2. Effects of DPeP and DPeP + NAC treatment on the morris water maze in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; IAL, initial acquisition latency; RL, retention latency.

Notes: Values are represented as Mean±Standard Mean Error; (n=6); IAL: $F_{6,35} = 7.44$; 1st RL: $F_{6,35} = 7.41$; 2nd RL: $F_{6,35} = 7.17$; * $P < 0.05$ as compared to the control group; ** $P < 0.01$ as compared to the vehicle group; ^a $P < 0.01$ as compared to the DPeP (33 mg/kg) treated group; ^b $P < 0.05$ as compared to the vehicle group; ^c $P < 0.01$ as compared to the control group; ^d $P < 0.05$ as compared to the DPeP (33 mg/kg) treated group; ^e $P < 0.001$ as compared to the control/vehicle group.

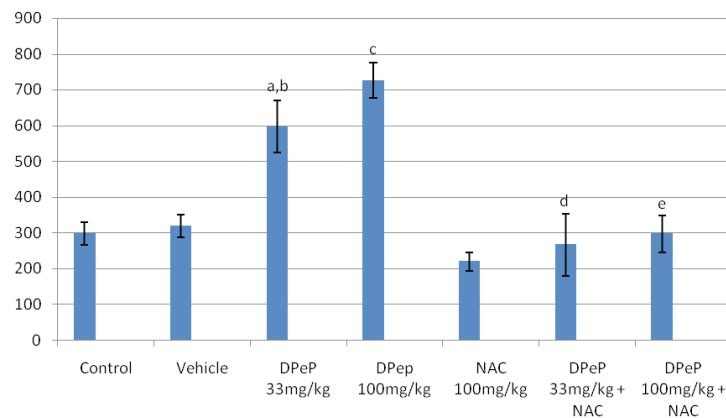


Figure 3. Effects of DPeP and DPeP + NAC treatment on MDA levels in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; MDA, malondialdehyde.

Notes: Values are presented as Mean±Standard Mean Error; (n=6); $F_{6,35} = 12.59$; ^aP<0.001 as compared to the control group; ^bP<0.01 as compared to the vehicle group; ^cP<0.001 as compared to the control/vehicle group; ^dP<0.001 as compared to the DPeP (33 mg/kg) treated group; ^eP<0.001 as compared to the DPeP (100 mg/kg) group.

Discussion

The present study aimed to evaluate the effects of DPeP, a phthalate ester, on cognition in mice. The cognitive functions in the mice were assessed by studying the step-down latency in the passive avoidance apparatus and measuring the latency to reach the platform in the MWM. The role of oxidative stress in the development of phthalate-induced change in cognitive functions was also measured by estimating the levels of MDA, GSH, FRAP, and 8-OH-dG in the whole brain tissue of the mice.

A significant decline in the acquisition and retention of SDL in the passive avoidance test was observed in

the DPeP-exposed mice compared to the control group. There was also a significant improvement in the time taken to finish the spatial navigation task. This deficit in learning and memory following the DPeP treatment suggests the deleterious effects of DPeP on the brain of the mice. Similar findings were also observed by Ma P et al. in 2015 who studied the effects of Diisononyl Phthalate (DINP) on cognition in mice and reported that DINP impaired cognitive abilities and resulted in oxidative stress-mediated brain injury as well [25]. Another study by Yan B et al. (2019) also reported DEHP-induced memory deficit in the mice along with an escalation in the oxidative stress and apoptosis of hippocampal neurons in the central nervous system [36].

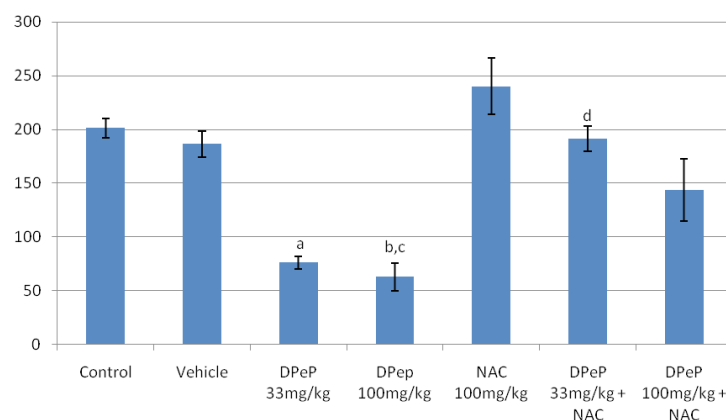


Figure 4. Effects of DPeP and DPeP+NAC treatment on GSH levels in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; GSH, glutathione.

Notes: Values are presented as mean±standard mean error; (n=6); $F_{6,35} = 14.76$; ^aP<0.05 as compared to the control/vehicle group; ^bP<0.01 as compared to the control group; ^cP<0.05 as compared to the vehicle group; ^dP<0.05 as compared to the DPeP (33 mg/kg) treated group.

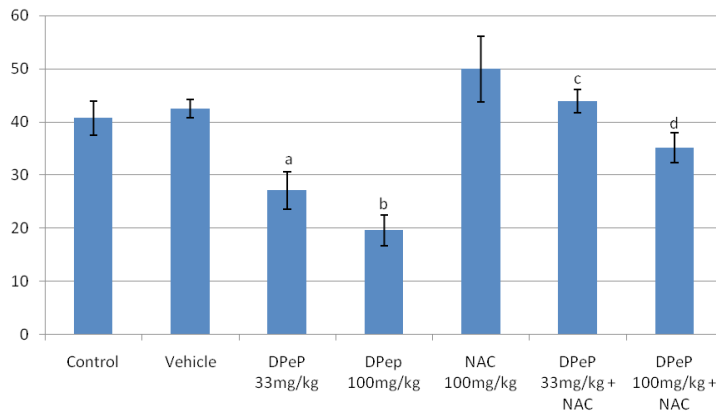


Figure 5. Effects of DPeP and DPeP + NAC Treatment on FRAP levels in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; FRAP, ferric reducing oxidizing power.

Notes: Values are presented as Mean±Standard Mean Error; (n = 6); $F_{6,35} = 9.07$; ^a P<0.05 as compared to the vehicle group; ^b P<0.001 as compared to the control/vehicle group; ^c P<0.05 as compared to the DPeP (33 mg/kg) treated group; ^d P<0.01 as compared to the DPeP (100 mg/kg) group.

In the present study, the DPeP treatment also resulted in a significant rise in the brain MDA and 8-OH-dG levels along with a decrease in the GSH and FRAP levels when compared to the control groups. This finding suggests an increase in the brain oxidative stress in mice and possible neuronal damage as indicated by the rise in the 8-OH-dG levels. The study by Ma et al. in 2015 on the mice model also reported an increase in the MDA levels and 8-OH-dG levels following exposure to DINP and its metabolites [25, 37].

NAC, an N-acetyl derivative of L-cystine, is a potent antioxidant. It is a thiol compound that interacts directly with ROS and protects the cells against oxidative damage [38]. Pretreatment with NAC in DPeP (33 mg/kg)

treated mice significantly reversed the cognitive impairment when compared to the group treated only with DPeP (33 mg/kg). We observed a significant increase in the SDL in the passive avoidance test and a significant decrease in the time to reach the platform in the spatial navigation task when compared to the group treated only with DPeP (33mg/kg). These findings suggest that NAC protected DPeP-induced cognitive impairment. A previous study by Huang Q et al. (2018) also suggests that NAC has the potential to be used as a therapeutic option to ameliorate mild cognitive impairment in the mice model [39].

In this study, pretreatment with NAC in the mice administered with a high dosage of DPeP (100mg/kg) showed

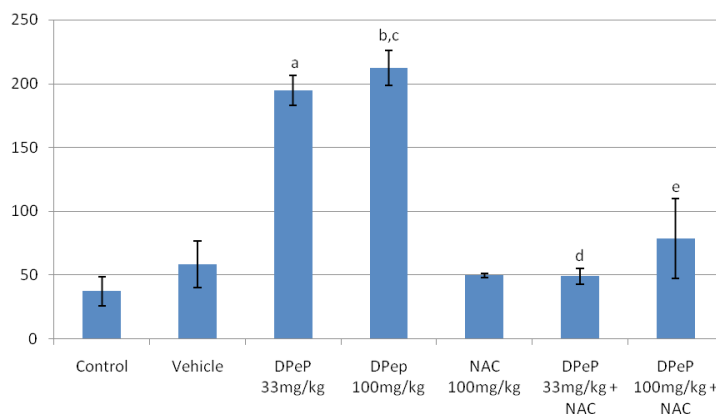


Figure 6. Effects of DPeP and DPeP + NAC treatment on 8-OH-dG levels in mice

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Abbreviations: DPeP, dipentyl phthalate; NAC, N-acetylcysteine; 8-OH-dG, 8-hydroxy-deoxyguanosine.

Notes: Values are presented as mean±standard mean error; (n=6); $F_{6,35} = 21.09$; ^a P<0.05 as compared to the control/vehicle group; ^b P<0.05 as compared to the vehicle group; ^c P<0.01 as compared to the control group; ^d P <0.05 as compared to the DPeP (33 mg/kg) treated group; ^e P<0.05 as compared to the DPeP (100 mg/kg) treated group.

improvement in the SDL or the spatial navigation task when compared to the DPeP (100mg/kg) groups; however, this was not statistically significant. This may be because a higher dose of NAC may be needed to protect against a high dosage of DPeP. Furthermore, the pretreatment with NAC resulted in a significant reduction in the brain MDA and 8-OH-dG levels along with an increase in the GSH and FRAP levels when compared to the DPeP groups. These findings suggest that DPeP-induced toxicity is because of the increase in oxidative stress in the brain and these effects may be mitigated to an extent by pretreatment with NAC. A study by Gu J et al. 2020 showed that NAC can attenuate the Bisphenol AF-induced cardiotoxicity in the Zebrafish model by reducing the MDA levels and inhibiting the ROS by increasing the activity of superoxide dismutase and catalase enzymes [40]. Another study by Lie et al. 2019 found that ROS generation was significantly inhibited by NAC and prevented the Bisphenol AF-induced DNA destruction in MCF-7 human breast cancer cells [41]. Similarly, Garg G et al. 2018 demonstrated that NAC can attenuate neurodegeneration and oxidative stress in the rat brain by increasing the FRAP levels and reducing the MDA levels [42].

Previous studies in the literature have focused primarily on a few traditional phthalates, such as DEHP, dibutylphthalate, diethylphthalate, and benzylbutylphthalate; many of these phthalates are now well-recognized as health hazards and lately, countries across the world have come up with regulations to ban or restrict their use in various consumer products [11]. However, many other phthalates like DPeP are still commercially used as plasticizers and limited data are available on their health effects. There is an urgent need to understand what kind of health risk is posed by DPeP and other phthalates which are still used in many consumer products and whether we need to replace them with safer alternatives.

Conclusion

The administration of DPeP-induced learning and memory deficits in the mice, probably because of oxidative stress-mediated neuronal damage, and pretreatment with NAC was associated with amelioration of the DPeP-induced effects.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by Institutional Animal Ethics Committee (IAEC), University College of Medical Sci-

ences (Code: IAEC/2011/75) and CPCSEA guidelines were followed for the care of animals.

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Authors' contributions

Animal experimentation, data collection, and manuscript writing: Sandhya Rani Gautam; Conceptualization of the study, critical review, data analysis: Seema Jain; Conceptualization of the study, methodology, supervision: Pramod Kumari; Biochemical testing, materials, supervision: Basudev Banerjee; Manuscript writing, critical review, data collection: Proteesh Rana.

Conflict of interest

The authors declared no conflict of interest.

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