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Research Article

ATTENUATION OF OXIDATIVE STRESS AND NEUROTOXICITY BY MK-801 (DIZOCILPINE) ON DIPENTYLPHTHALATE-INDUCED COGNITIVE DYSFUNCTION IN MICE

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ABSTRACT

Objectives: The aim of our research is to study the effect of dipentylphthalate (DPeP), a plasticizer on cognition and various oxidative stress markers in mice, and to explore the modulatory effects of MK-801.

Methods: In the present study, experimental mice were orally treated with two doses (33 and 100 mg/kg) of DPeP for 28 days. Cognitive functions were assessed using spatial navigation task on Morris water maze (MWM) and step-down latency (SDL) in passive avoidance apparatus. Oxidative stress was assessed by examining the levels of malondialdehyde (MDA), glutathione (GSH), ferric-reducing antioxidant power (FRAP), and 8-hydroxy-deoxyguanosine (8-OH-dG) levels in whole brain of mice.

Results: DPeP exposure led to a statistically significant increase of latency in spatial navigation task and significant decline in the SDL in passive avoidance apparatus when compared to the control groups. Oxidative stress markers showed a significant increase following DPeP administration as seen with rise in levels of MDA, 8-OH-dG, and a fall in GSH and FRAP levels.

Conclusion: The present data suggest that DPeP could adversely affect learning and memory functions in mice by an oxidative stress-mediated neuronal damage and pre-administration of MK-801 has the potential to attenuate these effects.

Keywords: Phthalate, Oxidative stress, MK-801, Reactive oxygen species.

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INTRODUCTION

The plastic industry has been expanding exponentially over decades. Along with it, the uses of many compounds which are added to these plastic products to render them soft and more resilient are also on an increasing trend. One such diverse group of additives includes plasticizers. Dipentylphthalate (DPeP) is one of the phthalate plasticizers used in a variety of goods such as medical devices, catheters, blood bags, toys, and cosmetics [1,2]. There is an emergence of public concern due to the detrimental effects with the widespread use of these additives on human health. This is particularly noticed as they are not covalently bound to these polymers, thus can migrate and ooze over time into the environment and contaminate soil, water, dust, and food [3-5]. Various epidemiological studies displayed deleterious health hazards on neurological development, reproduction, endocrine system, and a line of allergies on exposure to these diverse groups of phthalates [6,7]. In a line of the previous studies, exposure to phthalates and their metabolites in human assessed from serum, urine, breast milk, adipose tissue, cord blood, and placenta appeared high compared to the safe limit indicating its extensive exposure to mankind [8-10].

Worldwide, regulatory agencies came into action to limit the use and application of these harmful additives to various plastic products. Phthalate plasticizers, in particular, are regulated with the Consumer Product Safety Act in Canada and the Consumer Product Safety Improvement Act (CPSIA) in the U.S [11,12]. Still, a congruous international approach for regulating these chemicals is missing, especially with regard to children's toys and cosmetics [13]. Furthermore, a comprehensive risk assessment is required to measure the hazards and adverse health outcomes accurately in humans.

N-methyl-D-aspartate (NMDA), an ionotropic glutamate receptor, is considered an important molecule to regulate crucial roles in pathophysiology of numerous disease conditions such as Parkinsonism, Alzheimer's, and schizophrenia [14]. It also plays a pivotal role in various functions of brain namely memory formation, motor control, anxiety, and depression [15]. However, NMDA receptor activation due to overstimulation by glutamate can cause neuronal damage due to excitotoxicity and can lead to cognitive impairment in various neurodegenerative disorders such as Alzheimer's disease and schizophrenia [16-18].

MK-801 (dizocilpine) is a non-competitive inhibitor of NMDA receptor. The previous literature states that selective antagonism of NMDA receptors by MK-801 has been shown to attenuate the cognitive dysfunction induced by exposure to 1-bromopropane in Wistar rats [19]. These findings are further strengthened by other experimental studies demonstrating the potential of MK-801 in providing protection against methamphetamine-induced neurotoxicity by blocking microglial activation in cultured mouse microglial cells [20]. Another set of observations have shown promising role of another NMDA antagonist, memantine in improving cognition in patients of Alzheimer's disease [21,22]. These findings give us insight into the fact that NMDA antagonist could have the ability to improve cognition in neurodegenerative disorders. In view of these animal and clinical observations, much importance is being made to define the cellular and molecular mechanism underlying the neuroprotective role of NMDA antagonists, MK-801.

Piracetam, a well-established nootropic agent, is a derivative of the neurotransmitter Y-aminobutyric acid. It is a prototype drug of this class which improves cognitive functions such as learning and memory [23].

With this background, we investigated the prospective of MK-801, a non-competitive NMDA antagonist on dipentylphthalate-induced cognitive impairment in mice. A line of behavioral tests such as

step-down latency (SDL) in passive avoidance apparatus and Morris water maze were used to study the neuroprotective role of MK-801 in mice. Brain oxidative stress parameters were also evaluated by measuring malondialdehyde (MDA), non-protein thiol (GSH), ferric reducing antioxidant power (FRAP), and 8-hydroxy-deoxyguanosine (8-OH-dG) levels in mice brain.

METHODS

Animals

Male Swiss albino mice (weight 20–25 g) were obtained from the Central Animal House of the University College of Medical Sciences (UCMS) and GTB Hospital, Delhi. This study was performed in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and with prior approval from the Institutional Animal Ethics Committee (IAEC), UCMS. All the animals were kept in polypropylene cages with soft bedding and acclimatized in before the laboratory conditions with 22±1°C of temperature with 50±2% of relative humidity with natural light/day cycle.

Drugs treatment schedule

Corn oil (vehicle for DPeP) was used as dissolving agent for DPeP (Sigma St. Louis, MO, USA; purity >97.0%) and administered at a dose of 33 mg/kg and 100 mg/kg body weight orally for 28 days. MK-801 (Dizocilpine; Sigma-Aldrich, USA) was dissolved in distilled water and given at a dose of 0.5 mg/kg/day i.p. 30 min prior DPeP, daily for 28 days. Control animals received corn oil only. Piracetam (UCB India Pvt. Limited) was used as positive control and given at a dose of 200 mg/kg/day orally after dissolving in distilled water 60 min before DPeP.

Animals were divided into two set of seven groups (I–VII) with six mice each for each experimental test of cognition. Groups I and II were treated orally with corn oil and piracetam, respectively. Groups III and IV were treated orally with DPeP at the dose of 33 mg/kg/day and 100 mg/kg/day, respectively [24]. Group V received MK-801 in the dose of 0.5 mg/kg/day. Groups VI and VII received DPeP at the dose of 33 and 100 mg/kg/day along with MK-801, respectively.

Test for the assessment of cognition

Step-down latency (SDL) in passive avoidance apparatus

Passive avoidance apparatus comprised a wooden block that is placed at the center of a grid floor and served as a shock free zone (SFZ). On the 20th day of experiment, mice were placed on SFZ, and on stepping down from the SFZ, an electric shock (20 V) was given through the grid floor. Mice were given three such trainings at an interval of 1 h. Acquisition step-down latency (SDL) was recorded after 1 h of third training session without giving shock. On the 21st and 28th day of experiment, the procedure was repeated without giving shock. The time taken for the animals to step down was recorded as the 1st and 2nd retention latency, respectively. A cutoff time of 180 s was taken as the SDL for those animals which did not step down during this cutoff time [25].

Morris water maze (MWM)

The acquisition and retention of a spatial navigation task were evaluated using MWM. Training sessions were given to swim to a visible platform in a circular pool of diameter 150 cm. It was filled with water (28±2°C) up to a height of 30 cm and the water in the pool was make opaque by adding a non-toxic dye. The pool was divided into four equally spaced quadrants (N, S, E, and W) around the edge of the pool that was used as starting points for the test. A circular platform of 9 cm diameter was placed at the center of one of the quadrants. For testing of acquisition latency, it was placed about 2 cm above the water level and then immersed 2 cm below the water level to test for retention latency. On the 20th day of experiment, four training sessions were given to the animals with different starting points each time. Animals were returned to their home cages after the trials, and a 5 min gap was given between the subsequent trials. 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. Those mice which could not able to escape onto the platform within this time frame were guided to the platform and allowed to remain there for 30 s. The time taken by animal to reach the platform was taken as the initial acquisition latency (IAL). On the 21st and 28th day of experiment, the animals were released randomly at one of the edges of the pool and time taken to find the platform was recorded as first retention latency (1st RL) and second retention latency (2nd RL), respectively [26,27].

Locomotor activity

With the help of an activity cage (UGO Basile), both horizontal and vertical locomotor activities were recorded for a period of 5 min on the 20th, 21st, and 28th days. As the animal moved in a clear acrylic cage, they interrupted one or more infrared (IR) beams. These beam interruptions were counted and recorded by the electronic unit and were used to assess and analyze the animal activity as described by Bareggi *et al*. [28].

Biochemical tests

On day 29, animals were euthanized by giving halothane anesthesia following behavioral assessment [29]. Their whole brain was dissected out and washed using ice-cold sodium phosphate buffer, weighed, and stored over ice.

Assessment of oxidative stress markers

Procedures for the estimation of brain oxidative stress markers were carried out within next 12 h of dissection. Homogenization of the brain tissue was done using sodium phosphate buffer (KH2PO4 and Na2HPO4). Then, it was centrifuged at 3000 rpm for 15 min and the supernatant was used for the estimation of MDA, GSH, FRAP, and 8-OH-dG levels. MDA was estimated following a method described by Okhawa *et al.* (1979) [30]. GSH was determined according to the method described by Ellman *et al.* [31]. FRAP assay was done according to the method described by Benzie *et al.* [32]. 8-OH-dG levels in mice brain were estimated using a commercially available murine 8-OH-dG EIA kit from Cayman Chemical Company, USA, as per the manufacturer's instructions.

Statistical analysis

Data were expressed as mean±standard error of mean (SEM) and analyzed using one way ANOVA followed by *post hoc* Tukey's test. $p<0.05$ was taken as statistically significant in all the experiments.

RESULTS

Effects of DPeP and MK-801 pre-treatment on memory performance using passive avoidance apparatus

Treatment with 33 mg/kg and 100 mg/kg doses of DPeP resulted in significant decrease in the mean acquisition SDL in passive avoidance task in mice when compared to the control ($p<0.05$) and standard treatment experimental group ($p<0.001$).

There was a significant decline in retention latencies in both the DPeP (33 mg/kg and 100 mg/kg) treated groups on day 21 and day 28 as compared to the control ($p<0.05$) and standard group ($p<0.001$). Furthermore, pre-treatment of 33 mg/kg DpeP treated mice with MK-801 significantly prolonged the retention latency on day 21 ($p<0.05$) and day 28 ($p<0.001$) when compared to DPeP 33 mg/kg alone treated group (Fig. 1).

Effects of DPeP and MK-801 pre-treatment on memory performance using MWM

Treatment of mice with DPeP (33 mg/kg and 100 mg/kg) caused a significant increase in mean acquisition latency in spatial navigation task in MWM when compared to the control and standard drug-treated groups ($p<0.01$) on the 20th day of experiment.

There was a significant increase in retention latencies on day 21 (1st RL) and day 28 (2nd RL) with the DPeP (33 mg/kg) and DPeP (100 mg/kg) treated groups as compared to retention latencies on day 21 and day 28 of the control and standard ($p<0.05$) groups. Pre-administration

with MK-801 in DPeP (33 mg/kg) treated mice displayed a significant decrease in the 1st RL and 2nd RL when compared to DPeP (33 mg/kg) alone treated mice ($p<0.05$). These data suggest that pre-treatment with MK-801 has the potential to reduce the DPeP-induced cognitive impairment in mice. No statistically significant difference was found when MK-801 alone treated mice were compared with the control groups (Fig. 2).

Effects of DPeP and MK-801 pre-treatment on oxidative stress markers

MDA and GSH levels

There was a significant increase in MDA levels ($p<0.001$) and decrease in GSH levels ($p<0.05$) in the brain of mice administered with DPeP (33 and 100 mg/kg) when compared to the control and standard groups. MK-801 pre-administration with DPeP induced a significant decrease in MDA levels when compared to the DPeP 33 mg/kg and DPeP 100 mg/kg alone treated groups ($p<0.001$). In addition, a significant rise in GSH levels in mice treated with MK-801 along with DPeP 33 mg/kg was found when compared to the DPeP 33 mg/kg alone treated group ($p<0.01$) (Figs. 3 and 4).

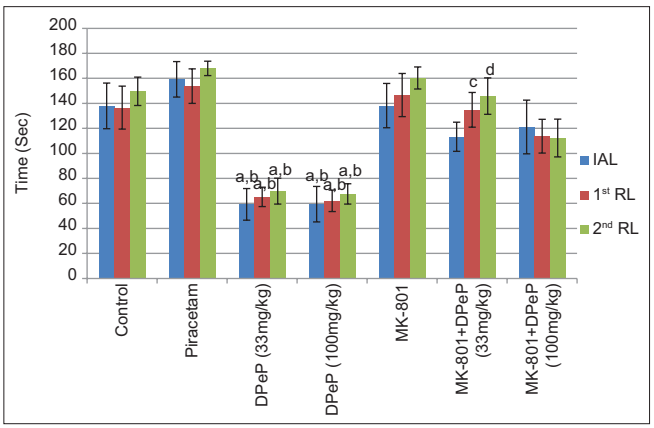

Fig. 1: Effect of DPeP and MK-801 pre-treatment on step down latency in mice. DPeP: Dipentylphthalate; IAL: Initial acquisition latency; RL: Retention latency. Values are mean±standard error of mean; (n=6) [a]p<0.05 as compared to the control group; [b]p<0.001 as compared to the standard group; [c]p<0.05 as compared to the DPeP (33 mg/kg) treated group; and [d]p<0.001 as compared to the DPeP (33 mg/kg) treated group

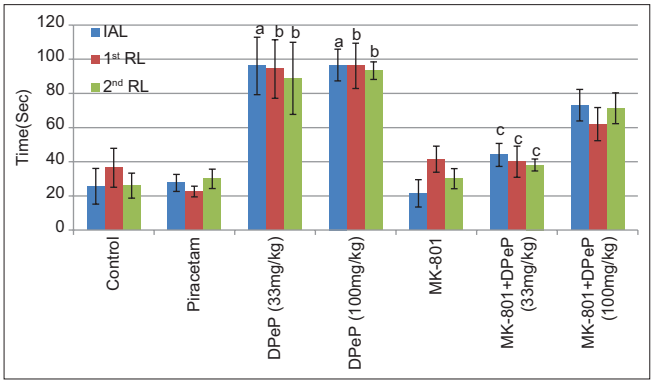

Fig. 2: Effect of DPeP and MK-801 pre-treatment on spatial navigation task in mice. DPeP: Dipentylphthalate; IAL: Initial acquisition latency; RL: Retention latency. Values are mean±standard error of mean; (n=6) [a]p<0.01 as compared to the control and standard group; [b]p<0.05 as compared to the control and standard group; and [c]p<0.05 as compared to the DPeP (33 mg/kg) treated group

FRAP and 8-OH-dG levels

FRAP levels were significantly declined in DPeP 33 mg/kg ($p<0.05$) and DPeP 100 mg/kg ($p<0.001$) treated mice as compared to the control and standard groups. MK-801 administration along with DPeP 33 mg/kg resulted in a significant increase in brain FRAP levels as compared to the DPeP 33 mg/kg alone treated group ($p<0.05$) (Fig. 5).

Mice treated with both the doses of DPeP (33 and 100 mg/kg, respectively) showed a significant increase in brain 8-HG-OG levels as compared to the control and standard groups ($p<0.05$). Coadministration of MK-801 with DPeP (33 and 100 mg/kg) in mice caused a decline in the brain 8-OH-dG levels, although not reaching statistically significant difference versus control group (Fig. 6).

Locomotor activity

There was no significant difference in either horizontal or vertical locomotor activity when compared to the control group on day 20, day 21, and day 28.

DISCUSSION

DPeP, a phthalate plasticizer, is extensively used in chemical manufacturing industry as solvents in dyes, air fresheners, cosmetics, and medical equipment [1]. The previous study by Silva *et al.* have reported that mono-4-hydroxypentyl phthalate ester, a major urinary biomarker of DPeP, is detected in about 29% of human urine samples indicating its significant exposure to human [33]. The purpose of the present study was to explore the potential of MK-801 (dizocilpine), a non-competitive NMDA receptor blocker on DPeP-induced cognitive behavioral changes in mice. SDL in passive avoidance apparatus and

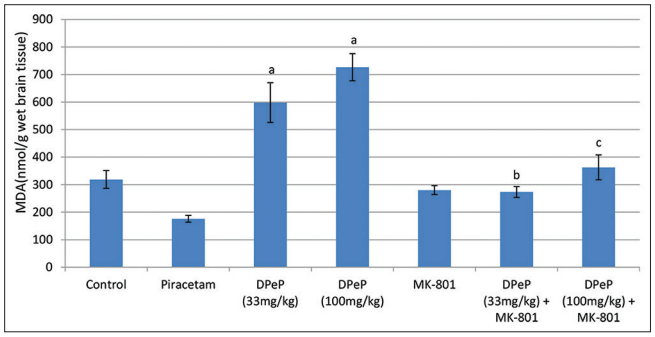

Fig. 3: Effects of DPeP and MK-801 pre-treatment on MDA levels in mice. DPeP: Dipentylphthalate; MDA: Malondialdehyde. Values are mean±standard error of mean; (n=6) [a]p<0.001 as compared to the control and standard group; [b]p<0.001 as compared to the DPeP (33 mg/kg) treated group; and [c]p<0.001 as compared to the DPeP (100 mg/kg) treated group

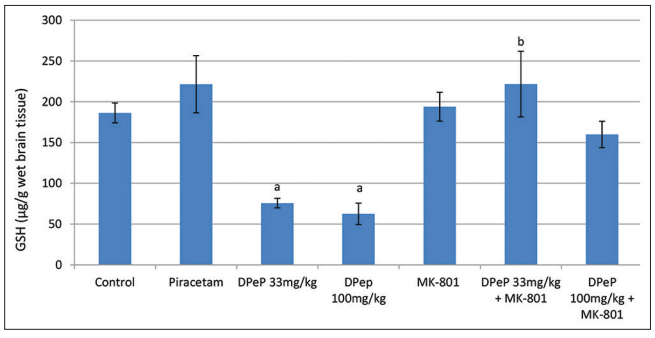

Fig. 4: Effect of DPeP and DPeP + MK-801 treatment on GSH levels in mice. DPeP: Dipentylphthalate; GSH: Glutathione. Values are mean±standard error of mean; (n=6) [a]p<0.05 as compared to the control and standard group and [b]p<0.01 as compared to the DPeP (33 mg/kg) treated group

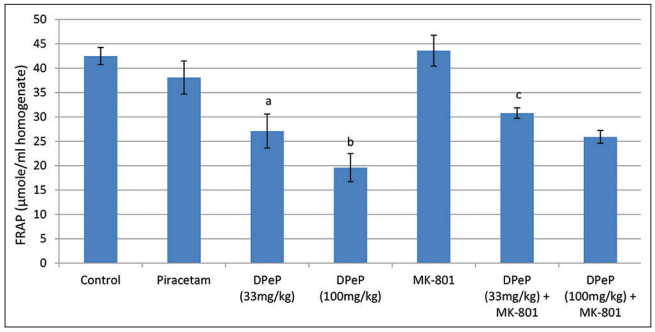

Fig. 5: Effect of DPeP and MK-801 pre-treatment on FRAP levels in mice. DPeP: Dipentylphthalate; FRAP: Ferric reducing antioxidant power. Values are mean±standard error of mean; (n=6) [a]p<0.05 as compared to the control and standard group; [b]p<0.001 as compared to the control and standard group; and [c]p<0.05 as compared to the DPeP (33 mg/kg) treated group

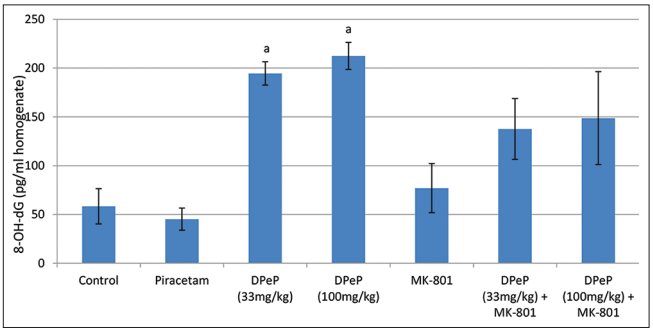

Fig. 6: Effects of DPeP and MK-801 pre-treatment on 8-OH-dG levels in mice. DPeP: Dipentylphthalate; 8-OH-dG: 8-hydroxy-deoxyguanosine. Values are mean±standard error of mean; (n=6) [a]p<0.05 as compared to the control and standard group

spatial navigation task in Morris water maze was used to assess the cognitive behavioral changes in mice. In addition, role of oxidative stress as an underlying mechanism for the development of behavioral changes induced by DPeP was assessed by measuring MDA, GSH, FRAP, and 8-OH-dG levels in the whole brain tissue of mice.

Learning and memory are distinctive ability of cognition that is vital for complex human behavior. In our present study, DPeP administration in mice resulted in a significant decline in the acquisition and retention step-down latency in passive avoidance apparatus. Similarly, a significant increase in escape latency to reach the platform in spatial navigation task was also found in both the groups treated with DPeP. These findings are corroborative with the results of Basha *et al.*, wherein similar increase in the escape latency to reach the platform in spatial navigation task was found on exposure to dibutyl phthalate in rats [34,35]. Similar line of findings was also reported by Yan *et al.* in 2021 who demonstrated learning and memory impairment in mice on exposure to dibutyl phthalate along with a rising trend in oxidative stress markers [36].

Administrations of DPeP in mice also resulted in a significant elevation in the brain MDA and 8-OH-dG levels and a decrease in the GSH and FRAP levels when compared to the control and standard groups. Similar in line, Ma *et al.* in 2015 also showed that phthalate exposure in mice adversely affected the cognitive behavior. In addition, Ma *et al.* also demonstrated an increase in the MDA and 8-OH-dG levels and a decrease in the GSH levels on exposure to diisononyl phthalate (DINP) and its metabolites in mice [37].

To further strengthen the clinical evidence, a line of studies on human population also associated phthalate exposure to increase in oxidative stress markers [38,39]. A majority of phthalate metabolites such as mono-n-butyl phthalate (MBP), mono-iso-butyl phthalate (MiBP), and DEHP were associated with an increase in urinary 8-OH-dG levels, an important biomarker of oxidative stress in a cohort of pregnant women in Puerto Rico [38]. These findings throw light on the possible mechanisms for phthalate-induced behavioral alterations in human that may be relevant to a number of adverse health effects.

NMDA receptor antagonist, MK-801, has a wide range of pharmacological activities such as anticonvulsant, antianxiety, and neuroprotection. Pre-administration with MK-801 in mice treated with DPeP (33 mg/kg) resulted in a significant decline in the time to reach the escape platform in spatial navigation task and an increase in SDL when compared to the DPeP (33 mg/kg) alone treated group, indicating the potential of MK-801 in improving learning and memory of DPeP treated animals. These findings are in corroboration with the results of a study done by Xu *et al.* in 2019 who demonstrated that MK-801 provides protection against 1-BP-induced cognitive dysfunction in Wistar rats by significantly reducing the time to reach the escape platform [19]. However, animals pre-treated with MK-801 in high dose of DPeP (100 mg/kg) demonstrated an increase in SDL and decrease in time to reach the escape platform as compared to the DPeP (100 mg/kg) alone treated group, although not reaching statistically significant difference.

In our study, we also found a significant reduction in brain MDA levels and an increase in GSH and FRAP levels in animals pre-treated with MK-801 as compared to the DPeP (33 mg/kg) alone treated animals. Similar in line, Xu *et al.* demonstrated that MK-801 can attenuate methylmercury-induced neurotoxicity by significantly reducing MDA levels, increasing GSH levels and a reduction in 8-OH-dG immunopositive cells in the cerebral cortex of Wistar rats. These findings further support the neuroprotective effects of MK-801 by alleviating oxidative damage [40]. Another study by Cunha *et al.* also showed that MK-801 can attenuate oxidative damage in Wistar rats by increasing catalase, GSH levels, and superoxide dismutase activity [41]. The present study expands our understanding on the neurobehavioral outcomes on exposure to DPeP. Further, oxidative damage could be one plausible mechanism for phthalate-induced cognitive impairment. It is of utmost importance to understand the mechanistic action of these individual phthalates to facilitate future risk assessment in a more accurate way. Existing regulations over the globe prioritize mainly a small set of phthalates that have already well-established reproductive, neurodevelopment, and endocrine disruptor toxicities [4]. However, long lists of newer entities like DPeP which are of the same class eluded from these regulatory laws and are widely used as an undesirable substitute in the industry offering no benefit to the humankind. New biomonitoring studies should incorporate these newer phthalates over the older ones not only as a human surveillance tool but also for risk assessment and to prioritize our actions for policymaking.

CONCLUSION

In the present study, we found that exposure to DPeP can cause neurobehavioral impairment in Swiss albino mice and resulted in oxidative stress that caused damage to mouse brain. MK-801 has the potential to protect against DPeP-induced cognitive dysfunction.

AUTHORS' CONTRIBUTIONS

All the authors have contributed critically toward the preparation and editing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

CONFLICTS OF INTEREST

All the authors declare that they have no conflicts of interest.

AUTHORS' FUNDING

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