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# Cerium oxide nanoparticles alleviate pesticide-induced cholinergic dysfunction in the rat hippocampus

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**Abstract**
**Objective:** This research explored the neuroprotective potential of cerium oxide nanoparticles ($CeO_2$ NPs) against chlorpyrifos (CPF)-induced cholinergic dysfunction in the rat hippocampus, assessing acetylcholinesterase (AChE) and muscarinic M1 receptor (CHRM1) gene expression, plus oxidative stress markers.
**Methods:** Forty adult male Wistar rats were randomly assigned to four groups (n=10): control (vehicle), CPF-only (5 mg/kg/day oral gavage), $CeO_2$ NPs-only (0.5 mg/kg/day intraperitoneal), and CPF + $CeO_2$ NPs (co-treatment for 28 days). Hippocampal AChE and CHRM1 expression were quantified by qRT-PCR. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured via commercial kits.
**Results:** CPF reduced AChE and CHRM1 mRNA levels by 42% and 31% vs. controls ($p < 0.001$), with elevated lipid peroxidation (MDA: 356 ± 42 vs. 98 ± 11 nmol/mg protein; $p < 0.001$) and reduced antioxidant enzymes (SOD: −43%; CAT: −43%; GPx: −45%). $CeO_2$ NPs co-treatment significantly attenuated these effects, restoring AChE and CHRM1 expression to 76% and 71% of control ($p < 0.01$ vs. CPF-only). MDA decreased by 48% ($p < 0.001$), and antioxidant enzyme activities recovered by 74–87% ($p < 0.01$). $CeO_2$ NPs alone caused no adverse effects.
**Conclusions:** $CeO_2$ nanoparticles effectively attenuate CPF-induced hippocampal cholinergic dysfunction by preserving cholinergic gene expression and redox homeostasis. These findings support $CeO_2$ NPs as a promising approach for treating organophosphate neurotoxicity.

**Keywords:** Cerium oxide nanoparticles; Chlorpyrifos; Acetylcholinesterase; Oxidative stress; Neuroprotection

## Plain English Summary

Pesticides such as Chlorpyrifos may harm the brain, causing harmful oxidative stress and interfering with chemical communication between nerve cells. This research looked at whether cerium oxide nanoparticles could prevent the brain from this damage in rats. The results showed that these nanoparticles performed as expected and reduced brain cell damage and maintained the function of important chemical messengers in the brain, as well as restoring the brain's natural antioxidant defences. This suggests that cerium oxide nanoparticles could potentially be developed as a treatment for people who are exposed to harmful pesticides.

## Introduction

Organophosphate (OP) pesticides are widely used to support agriculture throughout the world, even though the neurotoxic effects are well established. These compounds exert their major toxic effect through prolonged inactivation of acetylcholinesterase (AChE), the enzyme that is responsible for the hydrolysis of acetylcholine at the synaptic junctions. This inactivation elevates acetylcholine levels and leads to an acute

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cholinergic crisis characterised by overactivation of muscarinic and nicotinic receptors (1). However, apart from this acute mechanism, there is growing evidence that chronic subthreshold exposure to organophosphates leads to enduring neurological changes via oxidative stress, mitochondrial dysfunction, and long-term changes in neuronal connectivity, which has been termed Organophosphate-Induced Delayed Neurotoxicity (OPIDN) (2).

Chlorpyrifos (CPF; O, O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) constitutes one of the most widely employed organophosphate insecticides worldwide and is widely used as a model compound to study pesticide-triggered brain toxicity. Such epidemiological research has attributed chronic CPF exposure as part of ambient air pollution to neurodevelopmental disorders, cognitive issues, and high neurodegeneration rates in susceptible groups (3). The AChE inhibition property falls short of the neurotoxic effects of CPF via hepatic metabolism and mitochondrial dysfunction of CPF as the synthesis of reactive oxygen species (ROS) and simultaneous depletion of intrinsic antioxidants stores, e.g., superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (4). This oxidative load provides a cellular condition that is conducive to the emergence of macromolecular impairment (lipid peroxidation, protein oxidation and nucleic acid oxidation).

The cholinergic disruption of the hippocampus is particularly susceptible due to its overabundance of cholinergic innervation by the septohippocampal cholinergic projection of the medial septum, as well as the diagonal band of Broca. Acetylcholine, a derivative of acetyl-CoA and choline generated by the choline acetyltransferase (ChAT), is involved in cognitive functions involving long-term potentiation (LTP), spatial learning and memory consolidation via the stimulation of nicotinic and muscarinic receptor types (5). Among these, the muscarinic M1 receptor (CHRM1) is highly expressed in pyramidal neurons of the hippocampus and is coupled to Gq/11 proteins to stimulate phospholipase C and mobilise intracellular calcium; events that are crucial for neural plasticity and encoding of memories. Importantly, pesticide-induced oxidative stress leads to down-regulation of CHRM1 by several mechanisms, including direct oxidative damage of the receptor, altered transcriptional regulation modulated by nuclear factor kappa B (NF-κB) signalling and impaired receptor trafficking to the cell surface (6). Consequently, CHRM1 mRNA abundance is a sensitive molecular biomarker to assess pesticide-induced neurotoxicity and therapeutic intervention.

Cerium oxide nanoparticles ($CeO_2$ NPs) have proven to be effective as an interesting therapeutic platform due to their remarkable catalytic activity. Unlike conventional antioxidants, which suffer from stoichiometric inactivation after single cycles of reactions, $CeO_2$ NPs demonstrate inherent SOD- and CAT-mimetic activities and are therefore classified as nanozymes (7). These properties stem from the reversible $Ce^{3+}/Ce^{4+}$ redox cycling occurring at the surface of these nanoparticles, which allows for continuous ROS neutralisation by multiple catalytic cycles. After systemic administration, the $CeO_2$ NPs can traverse the blood–brain barrier (BBB) attributable to the transcellular transport and transient disruption of the BBB, and then accumulate in the hippocampal neurons (8). Previous neurotoxicological studies have shown that $CeO_2$ NPs pre-treatment reduces ROS-dependent neuroinflammation, prevents mitochondrial dysfunction and excitotoxicity in multiple neurotoxic paradigms (9). However, mechanistic studies investigating the role of $CeO_2$ NPs as modulating pesticide-induced changes in cholinergic gene expression are rather limited.

Accordingly, this paper sought to explore the neuroprotective influences of $CeO_2$ NPs against CPF-induced cholinergic dysfunction in the rat hippocampal formation by assessing the expression of cholinergic genes (AChE and CHRM1) and important oxidative stress indicators, such as lipid peroxidation and antioxidant enzyme activities

## Materials and Methods

### *Experimental Animals and Housing*

Forty adult male Wistar rats (Rattus norvegicus), weighing 200–250 g (approximately 8 weeks of age), were procured from the institutional breeding colony. Animals were acclimatised to laboratory conditions for seven days before experimental manipulation. Throughout the acclimatisation and experimental periods, rats were housed in polycarbonate cages (four animals per cage) in a regulated environment with a 12-h light/dark cycle (lights on at 06:00), a surrounding temperature of 22 ± 2°C, and a relative humidity of 50 ± 5%. Standard rodent chow and dechlorinated drinking water were provided ad libitum. All experimental procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

### *Experimental Design and Grouping*

Following acclimatisation, rats were allocated randomly to 4 treatment cohorts (each cohort

comprised 10 rats) using a randomisation table generated by GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The treatment protocol was conducted over 28 consecutive days:
Group 1 (Control; G1): Animals administered vehicle only (corn oil; 0.5mL/kg body weight) given by oral gavage daily.
Group 2 (CPF Only; G2): Chlorpyrifos (5 mg /kg body weight; 99% purity, CAS No. 2921-88-2; Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil and administered daily via oral gavage to animals.
Group 3 ($CeO_2$ NPs Only; G3): (Wien et al., 2017) Animals were administered cerium oxide nanoparticles (0.5 mg/kg body weight) in normal saline by intraperitoneal injection for 10 days daily. Nanoparticles had particle sizes of a mean of 20 + -5 nm as determined by transmission electron microscopy (TEM; JEOL JEM-2100, Tokyo, Japan).
Group 4 (CPF + $CeO_2$ NPs; G4): In this group, $CeO_2$ NPs (0.5 mg/kg, i.p.) were given 30 min before CPF (5 mg/kg, oral gavage) daily.
The dosage of CPF was chosen from preliminary studies showing evidence of sub-lethal exposure with quantifiable neurochemical changes without becoming overtly toxic through systemic toxicity. The dosage of CEO_2 NPs was based on applications in neuroprotection studies in which efficacy without adverse effects was determined (8). Body weights were recorded weekly during the experimental period to monitor systemic health status.

*Tissue Collection and Processing*
Following the treatment period (28 days), the rats were euthanised by carbon dioxide inhalation and cervical dislocation. Brains were quickly removed, and hippocampal formations were dissected bilaterally on a chilled stainless-steel surface at a temperature of 4 °C. Hippocampal samples of tissue (approx. 100-150 mg animals-1) were immediately cryopreserved in liquid nitrogen and preserved at −80°C before utilisation in subsequent molecular and biochemical analyses.

*RNA Extraction and Quality Assessment*
Whole RNA was isolated from hippocampal tissue specimens through the RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines. In brief, frozen tissue samples were homogenised in QIAzol lysis solution employing a TissueLyser II (Qiagen) operated at 25 Hz for 2 × 2 min. Following chloroform addition and phase separation by centrifugation (12,000 × g, 15 minutes, 4°C), the aqueous phase containing RNA was recovered and combined with 70% ethanol. The mixture was loaded onto RNeasy spin columns, and after sequential washing steps, total RNA was eluted in nuclease-free water.
RNA quality and level were determined spectrophotometrically with a NanoDrop 2000 system (Thermo Fisher Scientific, Waltham, MA, USA). The samples having an absorbance ratio (OD260/280) of 1.8 to 2.0 were said to be pure enough. The RNA integrity was measured employing a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the RNA integrity number (RIN) was 7.5 or above to use downstream.

*Reverse Transcription and Complementary DNA Synthesis*
First-strand complementary DNA (cDNA) was generated from 1 µg of total RNA employing the SuperScript IV First-Strand cDNA preparation Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocols. The reaction mixture consisted of 1 µg RNA, 50 uM oligo(dT)20 primers, 10 mM deoxynucleotide triphosphate (dNTP) mix and nuclease-free water. After initial denaturation at 65 °C for 5 minutes, the mixture was combined with 5X SuperScript IV buffer, dithiothreitol (DTT) 100 mM, RNase inhibitor, and SuperScript IV reverse transcriptase. The reaction was conducted at 55°C for 10 min; subsequently, the enzyme was rendered inactive at 80°C for 10 min. Synthesised cDNA was stored at -20 °C before analysis by qRT-PCR

*Primer Design and Validation*
Rattus norvegicus specific sequences from the National Centre for Biotechnology Information (NCBI) GenBank database were used for designing gene-specific primers. Primer specificity was validated using the Basic Local Alignment Search Tool (BLAST) investigation with the rat genome. Primers sequences used include the following:
Acetylcholinesterase (AChE; Gene ID: 83817):
Forward: 5'-GCCATCAATGATGGCGATGTC-3'
Reverse: 5'-TCCTCGGTGATGCCATTGAAG-3'
Product length: 156 base pairs
Melting temperature (Tm): 60°C
Muscarinic Acetylcholine Receptor M1 (CHRM1; Gene ID: 25240):
Forward: 5'-GGCAACCTGGATCTCATCGG-3'
Reverse: 5'-CACGAACACGTAGCTGAGCTC-3'
Product length: 142 base pairs
Melting temperature (Tm): 60°C
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH; Gene ID: 24383):
Forward: 5'-ACATGGTCTGTGAGGGCGAG-3'
Reverse: 5'-GATGGCAACAATCTCCACTTTG-3'
Product length: 165 base pairs
Melting temperature (Tm): 60°C

Primer validation included the generation of standard curves using serially diluted cDNA (10-fold dilutions; $10^3$ to $10^8$ copies). The gradients obtained from the calibration curves were used to determine amplification efficiency values, and acceptable efficiencies were 90-110%. Analysis of the melt curves after each run was carried out to ensure single product amplification was conducted by single peaks at the desired melting temperatures.

*Quantitative Real-Time Polymerase Chain Reaction.*
*qRT-PCR was carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) that was equipped with fluorescence detection by SYBR Green.* Each 20 µL reaction comprised 10 µL SYBR Green Master Mix (Applied Biosystems), 2 µL template cDNA (50 ng/µL), 1 µL forward primer (10 pmol), 1 µL reverse primer (10 pmol), and 6 µL nuclease-free water. All reactions were conducted in duplicate using 96-well optical plates sealed with adhesive optical covers.
Thermal cycling parameters comprised initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 15s denaturation at 95°C and 1 min annealing/extension at 60°C. Fluorescence was measured while the annealing/extension phase was performed during the cycle. Following amplification, melt curve analysis was carried out by a gradual increase of the temperature from 60 to 95°C and continuous monitoring of the fluorescence.

*Relative Quantification of Gene Expression*
Relative gene expression was obtained utilising a relative quantification method of threshold cycle ($2^{-}\Delta\Delta$Ct) (10). The threshold cycle (Ct) was considered to be the cycle number in which the fluorescence level was higher than the background levels. ΔCt values were computed as the variation between Ct of the target gene and Ct of the reference gene (GAPDH). The results of the ΔΔCt value were then calculated as the variation between the ΔCt of the intervention group and the ΔCt of the control group. Relative gene expression levels were given as fold expression relative to the control group (calibrator), where control expression was normalised as 1.0 (100%).

*Assessment of Oxidative Stress Markers*
Lipid Peroxidation
The concentration of Malondialdehyde (MDA) was analysed with Lipid Peroxidation MDA Assay Kit (Solarbio Life Sciences, Beijing, China; Catalogue No. BC0025) based on the manufacturer's protocol. The principle of the test is the reaction of MDA with thiobarbituric acid (TBA) in an acidic environment at 95°C, which gives a pink chromogen with a maximum absorbance at 532 nm. Hippocampal tissue homogenates (10% w/v) in ice-cold phosphate-buffered saline (PBS; 50 mM, pH 7.4) at a concentration of 0.01% of butylated hydroxytoluene (BHT) were used to prevent artificial oxidation. After centrifuging at 10,000 × g for 10 min at 4°C, supernatants were collected for evaluation. MDA gave values employing 1,1,3,3-tetraethoxypropane from a standard curve and were given as nmol MDA/mg of protein.
Superoxide Dismutase Activity: The entire SOD activity was assessed employing the Superoxide Dismutase Assay Kit (Solarbio Life Sciences; Catalogue No. BC0175) through the xanthine-xanthine oxidase system. This technique is used to monitor the production of the superoxide radicals through the reduction of the nitroblue tetrazolium (NBT). The amount of enzyme that inhibited NBT reduction by 50 per cent was considered to be one unit of SOD activity. The homogenates of the hippocampus were done in cold PBS, centrifuged at 12 000 × g for 15 minutes at 4°C, and the supernatants assayed as per the protocol of the kit. The changes in absorbance were observed at 560 nm, and SOD activity was given as units per milligram protein.

Catalase Activity
Catalase Assay Kit (Solarbio Life Sciences; Catalogue No. BC0205) was used to measure the CAT activity on the basis of $H_2O_2$ breakdown. The assay is a determination of the reduction in absorbance at 240 nm due to the breakdown of $H_2O_2$. In brief, 10 mM $H_2O_2$ in 50 mM phosphate buffer (pH 7.0) was incubated with hippocampal supernatants, and the change in absorbance was followed over 3 minutes at 25 °C. A unit of One unit of CAT activity was characterised as the breakdown of 1 µmol $H_2O_2$ per minute of enzyme decomposing within the defined assay parameters. Findings were reported as the µmol $H_2O_2$ used/min/mg protein.

Glutathione Peroxidase Activity
GPx activity was assessed employing the Glutathione Peroxidase Assay Kit (Solarbio Life Sciences; Catalogue No. BC1195) through an indirect technique, in which it is an oxidative reaction with NADPH. The assay is based on the reduction of cumene hydroperoxide by GPx-mediated reduction and oxidation of NADPH by glutathione reductase. The reduction of absorbance at 340 nm, which is proportional to NADPH consumption, is proportional to GPx activity. Supernatants of hippocampal cells were incubated with lower glutathione, glutathione reductase, and NADPH. Cumene hydroperoxide

was introduced to initiate the reaction; subsequent determination was performed of absorbance over 5 minutes at 340 nm. The amount of enzyme oxidising 1 nmol NADPH per minute was defined as one unit of GPx activity. The results were given in the form of nmol NADPH oxidised/min/milligram protein.

Protein Determination: The protein concentrations in the hippocampal homogenates were measured using the Bradford assay (11) employing bovine serum albumin (BSA) as a standard. In short, a 10 µL sample was appropriately diluted, followed by mixing 200 µL Coomassie Brilliant Blue G-250 reagent and measuring the absorbance at 595 nm with 5 minutes of incubation. The values of oxidative stress markers were normalised using the protein concentrations obtained with a standard BSA curve (0-1000 µg/mL).

*Statistical Analysis*

Data were statistically analysed employing SPSS (v26.0; IBM Corporation, Armonk, NY, USA) and reported as mean ± SE. Normality of distribution was measured employing the Shapiro-Wilk test, whereas variance homogeneity was measured employing Levene's test. Data satisfying both normality and homogeneity criteria were analysed employing one-way analysis of variance (ANOVA), after which Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons. For data not conforming to parametric assumptions, the Kruskal-Wallis non-parametric test was used, after which Dunn's post-hoc test.

Effect sizes were expressed as partial eta-squared ($\eta^2$) values derived from partial eta-squared ($\eta^2$) values. The Pearson correlation test was carried out to evaluate the associations among oxidative stress indicators and cholinergic gene expression levels. Statistical significance was set at $\alpha = 0.05$ (two-tailed).

## Results

*General Observations and Body Weight Changes*

All animals tolerated the experimental protocols well throughout the 28-day study period. None of the treatment groups showed any mortality, seizures, or overt signs of distress. Behavioural observations indicated no visible variations in locomotion, grooming or feeding activity among groups.

Body weight was measured each week and given as the final values in Table 1, indicating a gradual rise in all groups. At the end of the study, the mean body weights were similar in terms of control (G1): 287.22 ± 15.08 g; CPF-only (G2): 281.36 ±18.15 g; $CeO_2$ NPs-only (G3): 289.95 +16.08 g and CPF + $CeO_2$ NPs (G4): 286.24 +14.19 g. The ANOVA showed that no significant difference was observed among them ($F_{3,36}$ = 0.42, p = 0.74), which means that pesticides and nanoparticle treatments do not affect the systemic growth or overall health condition significantly.

**Table 1: Final Body Weight Measurements Across Experimental Groups**

| Group | Treatment | Final Body Weight (g) (Mean ± SEM) |
|---|---|---|
| G1 | Control | 287.22 ± 15.08 |
| G2 | CPF Only | 281.36 ± 18.15 |
| G3 | $CeO_2$ NPs Only | 289.95 ± 16.08 |
| G4 | CPF + $CeO_2$ NPs | 286.24 ± 14.19 |

*One-way ANOVA: $F_{3,36}$ = 0.42, p = 0.74. No significant differences among groups*

*Acetylcholinesterase (AChE) mRNA Expression*

The quantitative real-time PCR profile indicated that changes were significantly different in the hippocampal AChE mRNA expression between the treatment groups, as indicated in Table 2. The main influence of treatment was significant employing one-way ANOVA ($F_{3,36}$ = 8.94, p < 0.001), with treatment assignment explaining approximately 42% of the variance in AChE expression ($\eta^2$ = 0.42).

In the CPF-only group (G2), AChE mRNA expression was markedly reduced to 42 ± 8% of control levels (p < 0.001 versus G1). This high level of inhibition suggests that exposure to CPF not only prevents the AChE enzyme activity but also silences AChE gene expression, impairing the ability of the cell to produce compensatory levels of enzymes.

The AChE mRNA of the $CeO_2$ NPs-only group (G3) did not differ significantly from controls (101 ± 12 of control; p = 0.98 versus G1), indicating that treatment with nanoparticles alone does not have a significant independent effect on AChE gene expression. This discovery validates the biocompatibility of $CeO_2$ NPs at the dosage that was tested regarding to the regulation of cholinergic genes.

Notably, the CPF + $CeO_2$ NPs co-treatment group (G4) exhibited significantly recovered AChE mRNA expression, to the point of 76 per cent of control levels. This is a large increase over the CPF-only group (p < 0.01 versus G2), which is roughly 80% of the transcriptional

suppression caused by pesticides. Although AChE mRNA in G4 did not completely restore to the control level, the protective effect was very high, and it supports the hypothesis that $CeO_2$ NPs alleviate the transcriptional effects of organophosphate exposure.

**Table 2: Relative AChE mRNA Expression Levels**

| Group | AChE mRNA Expression (% of Control) | Statistical Comparison |
|---|---|---|
| G1 (Control) | 100% (baseline) | --- |
| G2 (CPF Only) | 42 ± 8% | p < 0.001 vs. G1 |
| G3 ($CeO_2$ NPs Only) | 101 ± 12% | p = 0.98 vs. G1 (NS) |
| G4 (CPF + $CeO_2$ NPs) | 76 ± 11% | p < 0.01 vs. G2 |

*Data revealed as mean ± SEM. NS: Not significant. One-way ANOVA: $F_{3,36}$ = 8.94, p < 0.001; $\eta^2$ = 0.42.*

*The Muscarinic M1 Receptor (CHRM1) mRNA Expression.*

The level of CHRM1 mRNA expression was likewise analyzed and showed even greater dysfunction in response to pesticide treatment than AChE, as shown in Table 3. The treatment effect of one-way ANOVA was significant ($F_{3,36}$ = 12.67, p 0.001), and the treatment explained about 51 per cent of the variance ( 2 = 0.51). Exposure of group G2 to CPF decreased CHRM1 mRNA to 31 ± 7% of the control levels (p < 0.001 as relative to G1), which was a 69 per cent downregulation. This significantly higher inhibition than AChE indicates that CHRM1 can be especially susceptible to CPF-induced oxidative stress, or post-synaptic cholinergic factors can be especially sensitive to pesticide injury.

In line with AChE results, these nanoparticle biocompatibility results showed that $CeO_2$ NPs alone (G3) did not affect the expression of CHRM1 (103 ± 9% of control; p = 0.95 versus G1).

Surprisingly, the pretreatment with $CeO_2$ NPs in the co-treatment group (G4) was very effective in preventing CHRM1 downregulation and the mRNA levels were kept at 71 ± 12% of control (p < 0.01 versus G2). This was about 130% of the CPF-induced suppression - in itself enough to maintain functional capacity of acetylcholine signalling at post-synaptic sites. Enhanced CHRM1 expression to almost 70 per cent of control values indicates that $CeO_2$ NPs are able to provide adequate protection to this essential receptor against oxidative transcriptional repression.

**Table 3: Relative CHRM1 mRNA Expression Levels**

| Group | CHRM1 mRNA Expression (% of Control) | Statistical Comparison |
|---|---|---|
| G1 (Control) | 100% (baseline) | --- |
| G2 (CPF Only) | 31 ± 7% | p < 0.001 vs. G1 |
| G3 ($CeO_2$ NPs Only) | 103 ± 9% | p = 0.95 vs. G1 (NS) |
| G4 (CPF + $CeO_2$ NPs) | 71 ± 12% | p < 0.01 vs. G2 |

*Data revealed as mean ± SEM. NS: Not significant. One-way ANOVA: $F_{3,36}$ = 12.67, p < 0.001; $\eta^2$ = 0.51.*

*Oxidative Stress Markers*

Lipid Peroxidation (Malondialdehyde)

The concentration of malondialdehyde and oxidative deterioration of membranes showed a significant difference among groups of treatment (F 3, 6 = 10.18, p = 0.001; 0.46), which is available in Table 4.

Control hippocampi (G1) were found to have a baseline of 98 + 11 nmol/mg protein of MDA. The maximum increase in the MDA of group G2, which was exposed to CPF, was 356 ± 42 nmo/mg protein (p < 0.001 compared to G1), a 3.6-fold rise. This high increase is a sign of extreme oxidative membrane damage, and polyunsaturated fatty acids in the hippocampal neuronal membranes have gone through extensive peroxidation.

The $CeO_2$ NPs-only group (G3) did not differ significantly from controls (105 13 nmol/mg protein; p = 0.87 versus G1), which proved that oxidative membrane damage was not caused by treatment with nanoparticles alone.

It is striking how the co-treatment of $CeO_2$ NPs in group G4 reduced the MDA accumulation significantly to 187 +-28 nmol/mg protein. This is a 48 per cent lower reduction when compared to the CPF-only group (p < 0.001 versus G2). The fact that MDA amounts in G4 did not decrease significantly to reach control values (about 1.9-fold), but rather the significant decrease in products of lipid peroxidation, favours the model that $CeO_2$ NPs are effective free radical scavengers, and they prevent the degradation of the hippocampal membrane.

Superoxide Dismutase Activity

Analysis of SOD activity revealed significant treatment effects ($F_{3,36}$ = 9.76, p < 0.001; η² = 0.45), as presented in Table 4. Control hippocampi (G1) exhibited SOD activity of 42.3 ± 3.8 U/mg protein. The group G2 that was exposed to CPF inhibited the SOD activity to 24.1 + 4.2 U/mg protein (p < 0.001 compared to G1), which is equivalent to 43.1%. The fall is also indicative of oxidative inactivation of the enzyme as well as possible transcriptional down-regulation.

$CeO_2$ NPs alone (G3) demonstrated no independent effect on SOD activity (41.7 ± 3.5 U/mg protein; p = 0.96 versus G1). The G4 treatment (protective treatment) exhibited the highest SOD activity of 36.8 ± 4.1 U/mg protein (p < 0.01 compared to G2), which was about 87 per cent of the pesticide-induced enzyme loss. This partial, but important restoration suggests that the $CeO_2$ NPs not only complement endogenous SOD activity by their own, intrinsic SOD-mimetic activity, but also maintain the endogenous enzyme against oxidative inactivation.

Catalase Activity

The pattern of catalase activity was similar to SOD, with a significant treatment effect ($F_{3,36}$ = 8.43, p < 0.001; η 2 = 0.41). Control catalase activity measured 15.2 ± 1.6 µmol $H_2O_2$/min/mg protein (G1). CPF-exposed animals (G2) demonstrated significantly reduced activity at 8.7 ± 1.4 µmol $H_2O_2$/min/mg protein (p < 0.001 versus G1), a 43% decrease.

$CeO_2$ NP-only treatment (G3) showed no significant effect (15.1 ± 1.5 µmol $H_2O_2$/min/mg protein; p = 0.99 versus G1). CPF + $CeO_2$ NPs co-treatment (G4) effectively restored catalase activity to 13.1 ± 1.7 µmol $H_2O_2$/min/mg protein (p < 0.01 versus G2), representing 74% functional recovery. The catalase activity should be preserved to break down hydrogen peroxide, which is formed during cell metabolism and during the catalytic activity of the nanoparticles in catalysis.

Glutathione Peroxidase Activity

Glutathione peroxidase activity exhibited significant treatment-related variation ($F_{3,36}$ = 7.92, p < 0.001; η² = 0.39). Control animals (G1) showed GPx activity of 8.4 ± 0.8 µmol NADPH/min/mg protein, which declined sharply in CPF-treated animals (G2) to 4.6 ± 0.9 µmol NADPH/min/mg protein (p < 0.001 versus G1), a 45% reduction.

$CeO_2$ NPs alone (G3) exerted no independent effect (8.3 ± 0.9 µmol NADPH/min/mg protein; p = 0.97 versus G1). In the protective treatment group (G4), GPx activity has been recovered to the level of 7.20 ± 0.9 81% of the pesticide-inhibited activity of 7.2 0.9 NADPH/min/mg protein (p < 0.01 compared to G2). This recovery trend is consistent with the idea that $CeO_2$ NPs prevent oxidative inactivation of thiol-dependent antioxidant pathways.

**Table 4: Effects of Experimental Treatments on Hippocampal Oxidative Stress Markers and Antioxidant Enzyme Activities**

| Group | MDA (nmol/mg protein) | SOD Activity (U/mg protein) | CAT Activity (µmol $H_2O_2$/min/mg protein) | GPx Activity (µmol NADPH/min/mg protein) |
|---|---|---|---|---|
| G1 (Control) | 98 ± 11 | 42.3 ± 3.8 | 15.2 ± 1.6 | 8.4 ± 0.8 |
| G2 (CPF Only) | 356 ± 42*** | 24.1 ± 4.2*** | 8.7 ± 1.4*** | 4.6 ± 0.9*** |
| G3 ($CeO_2$ NPs Only) | 105 ± 13ns | 41.7 ± 3.5ns | 15.1 ± 1.5ns | 8.3 ± 0.9ns |
| G4 (CPF + $CeO_2$ NPs) | 187 ± 28***## | 36.8 ± 4.1***## | 13.1 ± 1.7***## | 7.2 ± 0.9***## |

*Data revealed as mean ± SEM. ***p < 0.001 vs. G1; ##p < 0.01 vs. G2; ns: not significant vs. G1 (p > 0.05). MDA; SOD; CAT; GPx*

**Discussion**

The existing study suggests that cerium oxide nanoparticles play an important role in counteracting chlorpyrifos-induced cholinergic dysfunction in the rat hippocampus by maintaining AChE and CHRM1 gene expression and restoring redox homeostasis. These data offer insights into the mechanistic basis of the neuroprotective properties of $CeO_2$ NPs and position them as agents that can be used in countering organophosphate neurotoxicity as a therapeutic intervention.

*Pesticide-Induced Cholinergic Dysregulation*

Such enormous loss of hippocampal AChE mRNA and CHRM1 mRNA after CPF exposure (42 and 31 per cent of control, respectively) is in line with recent mechanistic definitions of organophosphate neurotoxicity (12, 13, 14). Although acute CPF toxicity is mostly associated with irreversible AChE inhibition at the protein stage, the transcriptional downregulation in this case shows another, more complex pathway of cholinergic dysfunction. It is not limited to the classical concept of OP toxicity or the view that exposure to pesticides impairs the ability of the neuron to maintain compensatory levels of enzyme synthesis, which further contributes to

the cholinergic deficiencies that persist after the acute inhibition of enzymes (15).

The fact that CHRM1 was more suppressed than AChE (69% and 58% decrease, respectively) points to the possibility of post-synaptic cholinergic components being more susceptible to oxidative stress induced by CPF. This selective oxidation of receptors may also indicate involvement of several convergent pathways: direct oxidative alteration of receptor proteins, changes in transcriptional regulation by redox-sensitive transcription factors and defective receptor transport to the synaptic membrane (16). The latest studies also found that NF- kB and activator protein-1 (AP-1) are the key mediators of muscarinic receptor gene expression, which is both oxidatively modified in situations of ROS generation by the pesticide.

*Mechanisms of $CeO_2$ Nanoparticle Neuroprotection*

It is anticipated that the neuroprotective properties of $CeO_2$ NPs characterised in the current study are achievable via various synergistic mechanisms. The SOD-mimetic and CAT-mimetic functions of the $CeO_2$ NPs are intrinsic and are based on reversible $Ce^{3+}/Ce^{4+}$ redox cycling at the surface of the nanoparticles, which allows the nanoparticles to continuously scavenge superoxide anions and hydrogen peroxide produced in CPF metabolism (17). This catalytic effect has also been used to explain the drastic decrease in the level of MDA (48% reduction) and maintenance of endogenous antioxidant enzyme activities (74-87% rescue) in the co-treatment group.

Notably, $CeO_2$ NPs are not only replacements of lost endogenous antioxidants but also seem to prevent the oxidative inactivation of natural enzymatic systems. The fact that SOD, CAT, and GPx functions are conserved to near-control levels indicates that $CeO_2$ NPs help to preserve the cellular redox environment to support the optimal functioning of the enzymes. This opposing system helps to avoid the vicious circle according to which the oxidative stress inactivates antioxidant enzymes, and the subsequent increase in the amount of ROS and the increased damage to the cells ensues (18).

Of particular interest is the effect of $CeO_2$ NPs co-treatment in restoring AChE and CHRM1 gene expression (to 76% and 71% of control, respectively). The inverse relationship between the markers of oxidative stress and the cholinergic gene expression (r = -0.78 between MDA and CHRM1) indicates that oxidative stress inhibits transcription of the cholinergic genes directly, probably by oxidising the transcription factors like cAMP response element binding protein (CREB) and specificity protein 1 (Sp1), which participate in the regulation of the cholinergic genes. $CeO_2$ NPs alleviate the intracellular load of ROS, thereby providing transcriptional conditions that allow AChE and CHRM1 to express, thereby maintaining the molecular machinery that promotes cholinergic neurotransmission.

Recent findings indicate further that $CeO_2$ NPs can directly regulate cellular signalling cascades by interacting with kinase pathways. Cerium oxide nanoparticles have surface-reactive sites that may interact with the signalling pathways of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt, which is very important in neuronal survival and receptor regulation (19). These interactions may also help in the maintenance of CHRM1 expression that is seen in this study, but it would take a thorough proteomic and phosphoproteomic study to conclusively determine these interactions.

*Oxidative Stress and Lipid Peroxidation Dynamics*

The changes in the pattern of oxidative stress (MDA (3.6-fold) and SOD (43%), CAT (43%) and GPx (45%) activities) in the CPF-exposed animals show that the broad-spectrum antioxidants are depleted and not the activities of selective enzymes. Such an overall oxidative disruption is suggestive of the multifactoriality of CPF-induced production of ROS, which entails the dysfunction of the mitochondrial electron transport chain and the cytochrome P450-mediated metabolism, as well as the activation of the inflammatory NADPH oxidase (20).

Such disproportional increase of MDA in comparison with the reduction of particular antioxidant enzymes might suggest amplification of oxidative damages by autocatalytic lipid peroxidative cascades. Oxidation of all polyunsaturated fatty acids generates an increasing amount of free radicals, and it is self-reinforcing, thus continuing to form more damage even after the initial generation of ROS (21). The specified mechanism explains why a relatively small level of oxidative stress can induce severe effects on membranes and transcriptional dysfunction.

It is also interesting to note that the oxidative parameters of $CeO_2$ NPs (48% MDA decrease, 74-87% enzyme recovery) are partially but significantly normalised, and in the given instance, it is unlikely that total elimination of ROS will be necessary to provide neuroprotection cover. Rather, it might appear that it is possible to minimise oxidative load to a level where it is not dangerous to cause damage to a critical level to preserve the functioning of the cell and gene expression. This therapeutic consequence of this threshold effect is

fundamental since it suggests that $CeO_2$ NPs do not need full normalisation of redox status to open clinical benefit.

*Comparison with the Past Neuroprotection Studies.*

The described neuroprotective effect of the $CeO_2$ NPs is consistent with an emerging body of literature on an improved therapeutic potential of the same under a variety of neurological paradigms. $CeO_2$ NPs were also reported to protect ischemic stroke injury in the past, in which infarct size and scores of neurological deficits were reduced through the inhibition of ROS and preservation of blood-brain barrier integrity (22). $CeO_2$ NPs have also been shown to be effective in models of Alzheimer's, where they suppress the neuroinflammation and the synaptic malfunction induced by 25 2 -amyloid, or in models of Parkinson's, where they protect the dopaminergic neurons from 6-hydroxydopamine toxicity (23, 24).

The wide range of pathological processes that facilitate the neuroprotective activity of the $CeO_2$ NPs suggests that the catalytic capacity of ROS-scavenging of $CeO_2$ NPs is a universalised treatment therapy applicable in numerous neurodegenerative disorders. This is a broad-spectrum action unlike the conventional antioxidants, which are generally specific to one form of ROS and are typically inactivated during single cycles. The regenerative catalytic process of $CeO_2$ NPs allows the sustained protection, whereas the traditional pharmacological method cannot achieve this.

*Clinical Implications and Translational Considerations*

Clinical implications of the CHRM1 preservation by $CeO_2$ NPs have a specific clinical impact. The dysfunctional M1 receptor has been detected as the critical determinant in the cognitive impairment of various neurodegenerative diseases, and M1 receptor positive allosteric modulators have been demonstrated to have therapeutic benefit in clinical trials with Alzheimer's disease (25). $CeO_2$ NPs have the potential to offer cognitive protection, complementing pharmacological treatment that addresses receptor functionality by keeping CHRM1 active in individuals exposed to pesticides.

Nonetheless, the way $CeO_2$ NPs may pass important translational hurdles before becoming clinically feasible is yet to be achieved. Cerium oxide nanoparticles have not been studied in detail on long-term biocompatibility and tissue clearance kinetics in human neural tissue. Perivascular compartment accumulation or glial cell accumulation not related to acute toxicity in this 28-day experiment may cause chronic inflammatory processes with long-term exposure (26). Moreover, the regulatory principles that govern nanomedicines which target the central nervous system continue to be extremely high, and it is essential that the nanoparticle pharmacokinetics, biodistribution, and possible off-target effects should be thoroughly characterised (27).

*Study Limitations and Future Directions*

There are a number of weaknesses to the current study that must be noted. To start with, the 28-day sub-chronic exposure regimen, although suitable for the study of oxidative and transcriptional alterations, does not allow the research to be conducted on the nature of acute cholinergic toxicity and prolonged neurodegenerative effects. The future research needs to consider both the acute (24-72 hour) and extended (3-6 month) exposure windows in order to comprehensively describe the nature of the time-dependent CPF-induced dysfunction and the $CeO_2$ NPs-based protection.

Second, the dosing dose of single $CeO_2$ NPs (0.5 mg/kg) does not give a full dose-response characterisation. Neuroprotective effectiveness that depends on concentration is yet to be determined, and the best parameters of therapeutic doses have not been elaborated. Follow-up research must also be done to compare the doses of various nanoparticles (0.1-2.0 mg/kg) to determine dose response curves and possible high dose toxicities.

Third, the CPF dose used (5 mg/kg), although it is sub-lethal and gives measurable neurochemical effects without systemic toxicity, is not necessarily related to human environmental exposure conditions. The populations that are occupationally exposed generally undergo chronic low-level exposure that levels to nanomolar levels in serum. These findings would need the use of physiologically based pharmacokinetic modelling to extrapolate such findings to human-equivalent exposure conditions.

Fourth, the whole hippocampal tissue homogenates were analysed by gene expression, which ruled out the option of characterising cell-type-specific molecular responses. The pyramidal neurons, GABAergic interneurons, as well as glial populations, are rather prone to oxidative injury and nanoparticle interactions. Further investigations that use laser capture microdissection or single-cell RNA sequencing would offer cellular resolution data and would help determine vulnerable neuronal subpopulations.

Fifth, the analysis concentrated on the transcriptional changes and oxidative stress

markers without direct evaluation of the protein expression and cholinergic activity. Although the mRNA levels are usually in accordance with the protein levels, post-transcriptional control mechanisms may affect the functional outcomes. Mechanistic conclusions would be enhanced by complementary examinations of AChE enzymatic activity, CHRM1 protein expression and cholinergic neurotransmission.

Lastly, though this research shows a neuroprotective effect in healthy animals, agricultural workers who are exposed to pesticides usually present with comorbidities, including nutritional deficiencies, co-exposure to various agrochemicals, and underlying pathologies. Future research ought to determine $CeO_2$ NPs efficiency on more complex models that factor in these real-life variables.

## Conclusion

The current research indicates that cerium oxide nanoparticles have important neuroprotective effects on the cholinergic dysfunction induced by chlorpyrifos in the rat hippocampus via two mechanisms: maintenance of cholinergic gene expression and recovery of redox homeostasis. Exposure to CPF only resulted in significant inhibition of the hippocampal AChE and CHRM1 mRNA, with intense oxidative damage, high levels of lipid peroxidation, and loss of antioxidant enzyme functions. $CeO_2$ NPs co-treatment induced a significant decline in these effects, restoring cholinergic gene expression to 71-76% control, decreasing lipid peroxidation by 48% and restoring antioxidant enzyme activities by 74–87%. Notably, the induction of any negative effect on any parameter measured with the use of $CeO_2$ NPs solely did not occur, which proves that this type of nanoparticle is biocompatible with the administered dosage.

These results identify $CeO_2$ nanoparticles as therapeutic prospects in reducing the neurotoxicity of organophosphates, which provides synergistic effects in suppressing mitochondrial cholinergic signalling degradation and neurotrophic oxidative damage in the brain tissue. The maintenance of muscarinic M1 receptor expression has its own clinical implications, and this implies that it may be used in other areas other than pesticide neurotoxicity, including the management of other neurodegenerative diseases, which involve cholinergic dysfunction. Future research ought to focus on these unanswered questions on the best dose, safety in the long-term, effects cell-type-specifically and transfer to human exposures.

## List of Abbreviations

AChE: Acetylcholinesterase
ANOVA: Analysis of variance
AP-1: Activator protein-1
BBB: Blood-brain barrier
BHT: Butylated hydroxytoluene
BLAST: Basic local alignment search tool
BSA: Bovine serum albumin
CAT: Catalase
cDNA: Complementary DNA
$CeO_2$ NPs: Cerium oxide nanoparticles
ChAT: Choline acetyltransferase
CHRM1: Muscarinic acetylcholine receptor M1
CPF: Chlorpyrifos
CREB: cAMP response element binding protein
Ct: Threshold cycle
dNTP: Deoxynucleotide triphosphate
DTT: Dithiothreitol
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GPx: Glutathione peroxidase
$H_2O_2$: Hydrogen peroxide
HSD: Honestly significant difference
IACUC: Institutional Animal Care and Use Committee
LTP: Long-term potentiation
MAPK: Mitogen-activated protein kinase
MDA: Malondialdehyde
MeSH: Medical Subject Headings
mRNA: Messenger RNA
NADPH: Nicotinamide adenine dinucleotide phosphate
NBT: Nitroblue tetrazolium
NCBI: National Centre for Biotechnology Information
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
OP: Organophosphate
OPIDN: Organophosphate-Induced Delayed Neurotoxicity
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PI3K: Phosphoinositide 3-kinase
qRT-PCR: Quantitative real-time polymerase chain reaction
RIN RNA Integrity Number
ROS: Reactive oxygen species
SEM: Standard error of the mean
SOD: Superoxide dismutase
Sp1: Specificity protein 1
TBA: Thiobarbituric acid
TEM: Transmission electron microscopy

## Declarations

*Ethics Approval and Consent to Participate*

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Al-Qadisiyah (Protocol Number: BIO-2023-042) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

*Consent for Publication*
All authors approved the publication of this work under the Creative Commons Attribution-NonCommercial 4.0 license.

*Data Availability*
The datasets generated and analysed in the present study are available from the corresponding author upon reasonable request.

*Competing Interests*
The authors declare no competing interests.

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*Author Contributions*
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