Biological Trace Element Research
https://doi.org/10.1007/s12011-024-04258-6

RESEARCH

Protective effect of cholecalciferol against cobalt-induced neurotoxicity in rats: ZO-1/iFABP, ChAT/AchE and antioxidant pathways as potential therapeutic targets

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Received: 2 April 2024 / Accepted: 30 May 2024

Abstract

Cobalt (Co) toxicity has been reported to produce central nervous system and gastrointestinal abnormalities. This study assessed the therapeutic effect of cholecalciferol (Cho) supplementation against damages caused by sub-acute (14-day) cobalt chloride ($CoCl_2$) exposure in the brain and intestines. Thirty-five male Wistar rats were divided equally into five groups: Group I (control) received no treatment; Group II received oral $CoCl_2$ (100 mg/kg) only; Groups III, IV, and V received 1000, 3000 and 6000 IU/kg of cholecalciferol, respectively by oral gavage, and concurrently with $CoCl_2$. Cobalt-treated rats showed neuronal vacuolation and presence of pyknotic nuclei in the cerebral cortex and hippocampus, depletion of Purkinje cells in the cerebellum, as well as inflammation and congestion in the intestinal mucosa. Cobalt also increased brain and intestinal hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations, while simultaneously reducing glutathione (GSH) content, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities. Further, $CoCl_2$ induced increases in brain acetylcholinesterase (AchE) activity and serum zonulin (ZO-1) levels. Conversely, Cho administration suppressed $CoCl_2$-induced damages in the brain and intestines by reducing lipid peroxidation and increasing the activities of antioxidant enzymes. Remarkably, Cho produced stimulation of brain choline acetyltransferase (ChAT) and suppression of AchE activity, along with dose-dependent reduction in serum levels of ZO-1, intestinal fatty acid-binding protein (iFABP) and nitric oxide. In conclusion, the protective role of cholecalciferol against cobalt-induced toxicity occurred via modulation of cholinergic, intestinal permeability and antioxidant pathways. The results may prove significant in the context of the role of gut-brain connections in neuroprotection.

Keywords Cobalt chloride · Brain · Intestines · Cholinergic System · Zonulin · Intestinal Fatty Acid-Binding Protein

Highlights
- *Cholecalciferol is a potent regulator of both ChAT and AchE in $CoCl_2$-induced neurotoxicity*
- *Cholecalciferol preserves intestinal barrier integrity by reducing serum levels of ZO-1 and iFABP.*
- *Cholecalciferol exerted profound antioxidant activities against $CoCl_2$-induced oxidative stress.*

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Introduction

Cobalt-induced toxicity in humans and animals has become a major concern in recent years due to its renewed applications in different scenarios. From a public health perspective, environmental contamination and high human exposures to Co emanating from intense mining activities have been described in the general population [1]. The predominant pathways of human exposure include dietary intake of Co transferred into the human food chain from soil and contaminated water [2]. Furthermore, overexposure to Co from extended dosing with over-the-counter Co-containing dietary supplements and doping of athletes with Co-containing recreational items have all contributed to a considerable heightening of the risk of Co-induced neurotoxicity [3]. In the last two decades, the shift of attention to more renewable sources of energy has led to an increase in

Published online: 05 June 2024

production of rechargeable lithium-ion batteries for use in electric vehicles, smartphones, mobile phones, laptops and other electronic devices. This has also increased the risk of occupational and environmental exposures, due to the high content of Co in these batteries [4]. Besides occupational and dietary sources, patients undergoing hip arthroplasty with metal-on-metal implants also suffer from Co toxicity resulting from high levels of cobalt ions released via wear and corrosion from metallic surfaces [5].

There are substantial evidences from several clinical and pre-clinical reports implicating excess cobalt exposure in the development of neurological deficits in both the central and peripheral nervous system (e.g. cognitive decline, loss of memory, mood alterations and peripheral neuropathy) [6]. Cobalt ions are known to cross the blood-brain-barrier and bioaccumulate in the brain [7]. Gastrointestinal symptoms, such as nausea, vomiting and diarrhea have also been described in individuals exposed acutely to large amounts of cobalt salts [8]. Although reports indicate that gastrointestinal symptoms often resolve after a while, damage occurring to the epithelium may be important in the context of increased intestinal permeability and its implications for translocation of gut contents to other tissues. *In vitro* evidence from cultured human gastrointestinal epithelial cell (CaCo-2 BBe) indicated that cobalt chloride, an inducer of chemical hypoxia, was responsible for compromised barrier function [9].

Most *in vivo* studies in animal models and *in vitro* studies using neural cell lines have focused on the involvement of reactive oxygen species and expression of inflammatory, apoptotic and hypoxia markers to provide explanation of the mechanisms underlying Co neurotoxicity [10, 11]. Disturbances in the cholinergic system are also thought to account for neuronal and cognitive deficits in neurological diseases such as Alzheimer's, although the cholinergic alterations in Co-induced neurotoxicity have not been fully described. Patients with Alzheimer's disease have been shown to exhibit severe degeneration of cholinergic neurons and deficiency of acetylcholine (Ach), an excitatory neurotransmitter involved in cognition, memory and learning [12]. The amounts of Ach available in cholinergic neurons is regulated by enzymes including choline acetyl transferase (ChAT) and acetylcholinesterase (AchE) which modulate the synthesis and degradation of Ach, respectively. Consequently, these enzymes, as well as other important components of cholinergic signaling, including Ach receptors (muscarinic and nicotinic) are important biomarkers for evaluating cholinergic (dys)function [13].

The nature of cellular and molecular interactions between Co and gastrointestinal tissues are not yet well described. Approximately 25% of ingested Co is absorbed into blood circulation, although this can rise to as much as 90% depending on factors such as the amount ingested, the solubility of the cobalt salt and the individual's nutritional status, particularly in relation to iron levels [7]. It has been hypothesized that disturbances in molecular regulators of intestinal inflammation induced by ingested Co may result in loss of intestinal epithelial integrity, increased intestinal permeability and release of gut-related metabolites into the circulation with resultant systemic inflammation, which may ultimately affect the brain to cause neuroinflammation and disruption of neurotransmitter homeostasis as proposed for metals like manganese [14]. We also recently reported gut-associated responses including gut inflammation and changes in intestinal morphology, fatty acid and gut bacteria profiles following Co ingestion in rats [15]. Thus, markers of gut wall permeability including tight junction proteins such as zonulin and intestinal fatty acid-binding proteins require greater attention as potential therapeutic targets against metal-induced neurotoxicity.

Currently proposed therapeutic approaches against Co toxicity, including the use of chelation agents (e.g., EDTA), have failed to achieve desired results due to concerns about their effectiveness and safety [16]. As such, chelation therapy is yet to be accepted as a standard medical procedure for treating Co toxicity in many countries. Therefore, the identification of novel pathways involved in Co toxicity in different tissues may open the door for the discovery of novel therapeutic agents for its mitigation. Cholecalciferol (Vitamin D3) is normally classified as a fat-soluble vitamin, although it exhibits a wide range of biological functions via its active metabolite called 1, 25-dihydroxycholecalciferol, by which it regulates calcium metabolism, as well as exert its antioxidant and anti-inflammatory effects [17]. A growing body of evidence has highlighted potential links between vitamin D and gut health via its impact on gut microbiome, immunological and inflammatory processes involved in inflammatory bowel diseases [18]. Therefore, inadequate levels of vitamin D may impair normal intestinal homeostasis. By extension, vitamin D supplementation may exert neuroprotective effects via modulation of microbiota-gut-brain pathways. As vitamin D deficiency is particularly common in some populations with as much as 40% of individuals in US and Europe, and up to 80-85% of Arab people [19, 20], supplementation of diets with sources of vitamin D appears critical to prevent several associated diseases including neurodegenerative diseases [21].

Vitamin D is known to cross the blood-brain barrier and is metabolized locally in the CNS where it may directly exert neuroprotective effects via interaction with vitamin D receptors (VDRs) [22]. Also, in the intestines, vitamin D interacts with VDRs to influence bacterial colonization in the intestines, maintain intestinal tight junction architecture and exert anti-inflammatory responses [18]. As a result, it may also exhibit neuroprotective effects via the microbiota-gut-brain axis [23]. As a putative neuroprotectant, vitamin D is

believed to act through VDRs to promote neuronal health by enhancing myelination and maturation of oligodendrocytes, as well as the expression of genes that enhance survival and anti-inflammatory mechanisms in the CNS. These actions are believed to be mediated via activation of signaling pathways such as WNT and NOTCH signaling, as well as interaction with transcription factors such as NF-κβ, Nrf-2 and STATs [24].

To the best of our knowledge, no *in vivo* study has so far described the effect of cholecalciferol on Co-induced neurotoxicity and enterotoxicity. Therefore, this study was aimed at investigating the potential protective role of cholecalciferol (Cho) against neurotoxicity and enterotoxicity in a cobalt chloride ($CoCl_2$)-exposed rat model by evaluating the responses of oxidative, cholinergic, and intestinal permeability markers in male Wistar rats.

Materials and Methods

Chemicals and reagents

Cobalt chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) was a product purchased from Tianjin Kermel Chemical Reagent Co. (Xianshuigu, China). Vitamin D_3 (Cholecalciferol) was purchased as a product from Puritan's Pride® (Oakdale, NY, USA). Biochemical reagents including thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5-sulfosalicylic acid, xylenol orange, D-sorbitol, Tris Base, sodium hydroxide, reduced glutathione (GSH), 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB), 1, 2-dichloro-4-nitrobenzene (CDNB), and other major reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for Rat iFABP/ FABP2 (Intestinal Fatty acid binding protein; Catalog No. E-EL-R0572) and Rat TJP1 (Tight junction protein ZO-1; Catalog No. E-EL-RO982) were purchased from Elabscience® Biotechnology Co. Ltd (Wuhan, Hubei, China). Choline Acetyltransferase (ChAT) activity assay kit for tissue samples was also purchased from Elabscience® Biotechnology Co. Ltd (Wuhan, Hubei, China; Catalog No. E-BC-K125-S). All other chemicals used were of analytical grade in the purest form available commercially.

Animals

Thirty-five male Wistar rats, about 8 weeks old and weighing 120 ± 20 g were assigned to five groups (n = 7 per group), namely: Group I, Control; Group II, $CoCl_2$-treated (100 mg/kg); Group III ($CoCl_2$ + Cholecalciferol at 1,000 IU/kg); Group IV ($CoCl_2$ + Cholecalciferol at 3,000 IU/kg) and Group V ($CoCl_2$ + Cholecalciferol at 6,000 IU/kg). The control rats were given vehicle (distilled water) only. The $CoCl_2$-treated rats received $CoCl_2$ by oral gavage once daily for 14 consecutive days. The dosage and duration of $CoCl_2$ exposure were according to our previous results on cobalt neurotoxicity in rats [15], where 100 mg/kg was a non-lethal dosage producing neurotoxic lesions in the brain of rats. The selected doses of cholecalciferol were administered concurrently with $CoCl_2$ by oral gavage for the same number of days. On each day, dosing commenced with $CoCl_2$, followed by cholecalciferol after an interval of about three hours in order to prevent interactions that could influence Co absorption in the intestines. The dosages of cholecalciferol were carefully selected according to previous studies [25, 26]. Fedotova *et al* [27] had earlier reported that doses of vitamin D up to 200,000 IU/kg per day administered for 14 days were safe in rats.

All animals were housed in plastic cages in a well-ventilated animal house with constant temperature (22 ± 2 °C) and a photoperiod cycle of 12 h light and 12 h dark. The animals were supplied with feed and water *ad libitum* throughout the experiment, including an initial one week for acclimatization and two weeks for the actual dosing experiments. All experimental protocols and handling of animals were conducted in accordance with guidelines in the "Guide for the Care and Use of Laboratory Animals" in the NIH publication [28] as well as guidelines approved by the local institutional Animal Care and Use Ethics Committee.

Sample preparation

At the end of 14 days of dosing, the animals were fasted overnight in order to empty the small intestine, with only water provided. On the 15th day, the animals were weighed and blood was collected from all the rats via the retro-orbital plexus under slight anaesthesia into plain sample bottles. Thereafter, the animals were euthanized by cervical dislocation and the brain, and small intestines were dissected out. The duodenum and ileum were separated as the initial and terminal segments of the small intestine. Small portions of these segments (about 1 cm in length) were excised and kept in 10% buffered formalin for histopathological examination. For biochemical analyses, the remaining portions of the intestinal segments were flushed with ice-cold normal saline and the mucosa was scraped, and a 10% homogenate was made with ice-cold homogenizing buffer (50 mM Tris-HCl; pH containing 1.15% KCl). A 1:4 homogenate of the brain samples was also prepared with the same buffer. Tissue homogenization was carried out using a Teflon Homogenizer and the homogenates were then centrifuged in a refrigerated centrifuge (4 °C) at 10,000 *g* for 10 min. The supernatants were stored at – 20 °C prior to further analyses.

Analysis of oxidant and antioxidant markers

Measurement of hydrogen peroxide (H_2O_2) concentration

The brain and intestinal H_2O_2 content were measured according to the method of Wolff [29] based on ferrous ion (Fe^{2+}) oxidation (FOX) by H_2O_2 in the presence of the ferric-complexing dye, xylenol orange and sorbitol which amplified the reaction. The ferrous iron was supplied to the reaction in the form of ammonium ferrous sulfate, while the low pH required for the reaction was created by the addition of sulphuric acid. The mixture was allowed for 30 min at room temperature and the absorbance was read at a wavelength of 560 nm.

Measurement of malondialdehyde (MDA) concentration

The tissue content of MDA was used to indicate the extent of lipid peroxidation in the brain and intestines using the methods described by Varshney and Kale [30]. The homogenates in a Tris-KCl buffer (0.15 M; pH 7.4) were initially deproteinized and acidified with trichloroacetic acid (TCA, 30 % w/v), followed by the addition of thiobarbituric acid (TBA, 0.75 % in 0.1 M HCl) which formed a complex with MDA upon heating in a water bath (80 °C for 45 min) to produce a pink-coloured TBA-MDA complex. The resulting mixture was cooled on ice and centrifuged at 3, 000 rpm for 10 min. The absorbance of the clear supernatant was then measured at 532 nm. The MDA concentration was calculated with a formula using the extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Measurement of reduced glutathione (GSH) concentration

The concentration of GSH in the brain and intestines was measured according to the method of Jollow *et al* [31]. In this assay, the homogenates were first deproteinized with an equal volume of sulfosalycylic acid (4 %), followed by centrifugation at 4, 000 for 5 minutes. Thereafter, the supernatant was collected and a quantity of Ellman's reagent consisting of 5′, 5 ′-Dithiobis-(2-nitrobenzoic acid) (DTNB) prepared in phosphate buffer (0.1 M; pH 7.4) was added. The addition of the Ellman's reagent produced the development of a relatively stable yellow product indicating its reaction with non-protein sulfhydryl groups which makes the bulk of cellular non-protein sulfhydryl groups. The absorbance of the chromophoric (yellow) product (2-nitro-5-thiobenzoic acid) formed was measured at 412 nm.

Estimation of Superoxide dismutase (SOD) activity

The activity of SOD in the brain and intestinal homogenates was measured according to the method of Misra and Fridovich [32]. Briefly, the assay was based on the ability of SOD in the homogenates to inhibit the autooxidation of epinephrine in an alkaline medium provided by the carbonate buffer (0.05 M; pH 10.2). The assay involved an equilibration of the homogenate added to the carbonate buffer and freshly prepared epinephrine (0.3 mM) and the absorbance at 480 nm was monitored every 30 s for a total of 150 s. A unit of SOD activity was defined as the amount of SOD necessary to produce 50% inhibition of the oxidation of epinephrine.

Determination of Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was determined by using the method of Rotruck *et al.* [33], based on the understanding that GPx couples the oxidation of reduced glutathione to the conversion of hydrogen peroxide to water. The reaction mixture consisted of sodium azide (10 mM), GSH (4 mM), H_2O_2 (2.5 mM), TCA (10% w/v), and DTNB. The concentration of GSH consumed, measured via the absorbance at 412 nm, was used to calculate the activity of GPx.

Determination of Glutathione S-transferase (GST) activity

The activity of Glutathione S-transferase (GST) was determined according to the method of Habig *et al* [34] which measures the ability of the enzyme to catalyze the conjugation of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) over a time period of about 3 minutes with absorbance read at 340 nm in 30-seconds intervals.

Protein content

The protein content of the tissues was quantified using Biuret reagent and values were extrapolated from a standard curve prepared with bovine serum albumin [35]. The protein concentration in the homogenates was used as normalizing factor in the calculation of the activities of the various enzymes.

Measurement of intestinal permeability markers

The serum levels of intestinal fatty acid-binding protein (iFABP) and zonulin-1 (ZO-1) were measured with the aid of Enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. The serum nitrite levels were measured spectrophotometrically according to the method of Olaleye *et al.* [36], using Griess' reagent (0.1% n-(-naphthyl)-ethylene diamine dihydrochloride (NED) and sulfanilic acid). Nitric oxide concentration was then extrapolated using a sodium nitrite standard curve.

Determination of cholinergic markers

Choline acetyltransferase (ChAT) activity was determined in brain homogenates using a commercial spectrophotometric assay kit, according to the manufacturer's instructions. The assay was based on a reaction between acetyl CoA and choline under catalysis by ChAT to produce coenzyme A which was then reacted with 4, 4-dithiopyridine. The activity of ChAT was calculated indirectly by measuring the absorbance at 324 nm.

Acetylcholinesterase (AchE) activity was also evaluated spectrophotometrically according to the method described by Whittaker [37], in a reaction mixture containing the brain homogenate added to buffered Ellman's reagent (5, 5′-dithiobis-2-nitrobenzoic acid; 10 mmol/L, sodium hydrogen carbonate (17.85 mmol/L in phosphate buffer. pH 7.0) and acetylcholine iodide solution (75 mmol/L). The absorbance was measured at 30 s intervals for 3 min at 412 nm.

Histopathology

Brain and intestinal tissues were immediately fixed in 10% phosphate-buffered formalin following their harvest. The fixed tissues were processed by dehydration in graded concentrations of ethanol and embedded in paraffin, following which they were sectioned into 5 µm-thick slices and stained with haematoxylin and eosin (H&E). The slides were viewed under a light microscope (Olympus®, Japan) by a pathologist that was blinded to the treatments administered.

Statistical analysis

The data were analyzed with Graph Pad Prism software (version 8.02) and were expressed as mean ± standard deviation. The means were compared by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Significant differences were defined at $P < 0.05$; $P < 0.01$; $P < 0.001$ or $P < 0.0001$, as the case may be.

Results

The effect of cholecalciferol on body weights of $CoCl_2$-exposed rats

The changes in body weights of the animals before and after the experiment are presented in Table 1. The initial body weights ranged between 120 and 150 g with group averages that were not significantly different across the groups. All the groups registered increases in the average weights at the end of the experiment. However, the percentage weight gain of control rats was significantly higher (23.57%) than that of rats given $CoCl_2$ alone (6.27%). Treatment of rats with Cho1000 (Group III), Cho3000 (Group IV) and Cho6000 (Group V) produced slight improvement of body weight gain with animals in these groups registering percentage body weight gains of 8.26%, 11.55% and 10.53%, respectively.

The effect of cholecalciferol on oxidative stress and antioxidant markers in brain and intestines of rats exposed to $CoCl_2$

Hydrogen peroxide content

A significant ($P < 0.01$) increase in H_2O_2 was observed in the intestinal homogenate of $CoCl_2$-treated group in comparison to the control group (Fig. 1a). A similar increase in H_2O_2 content was observed in the brain homogenate of $CoCl_2$-treated rats, although the increase was not statistically significant (Fig. 1b). The administration of Cho1000, Cho3000 and Cho6000 produced dose-dependent reduction in brain and intestinal H_2O_2 contents when compared with the $CoCl_2$-treated group (Fig. 1a and b). This reduction was statistically significant with administration of Cho3000 ($P < 0.05$) and Cho6000 ($P < 0.001$) in the small intestine and with Cho6000 ($P < 0.05$) in the brain.

Reduced glutathione content

The GSH content in the brain ($P < 0.001$) and small intestines ($P < 0.05$) of $CoCl_2$-treated rats was significantly (P

Table 1 Body weight changes in the experimental animals

	Group I	Group II	Group III	Group IV	Group V
Initial body weight (g)	139.86±19.87	139.29±24.25	138.71±17.19	138.57±14.99	139.57±15.88
Final body weight (g)	183.00±27.48[a]	148.60±17.04	151.20±17.71[a]	156.67±10.89[a]	156.00±5.10[a]
Weight gain (g)	43.14	9.31	12.49	18.1	16.43
% Weight gain	23.57	6.27	8.26	11.55	10.53

Values are presented as mean ± SD; n = 7. Group I – Control; Group II – $CoCl_2$ alone; Group III – $CoCl_2$ + Cho1000; Group IV - $CoCl_2$ + Cho3000; Group V - $CoCl_2$ + Cho6000

[a]Significant (P < 0.05) as compared to the initial body weight

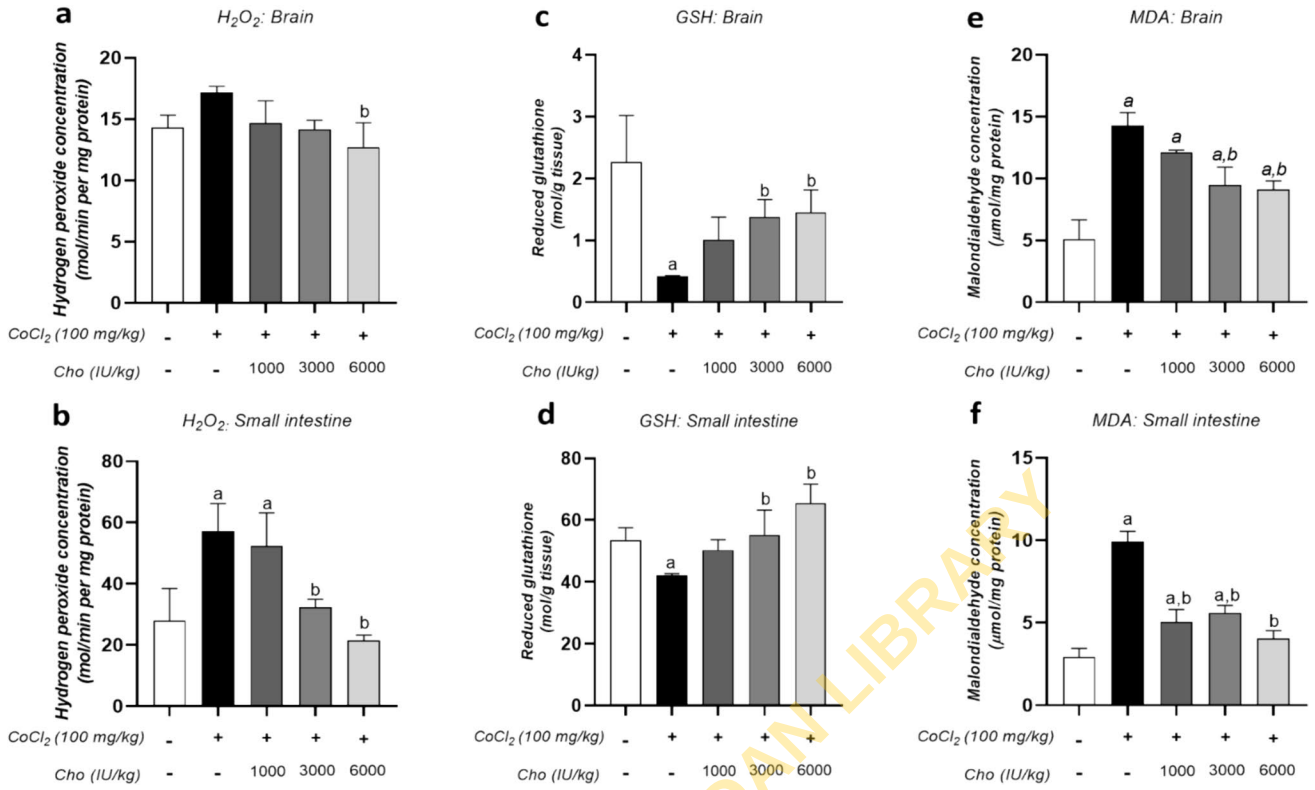

Fig. 1 Effect of cholecalciferol (Cho) on hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and reduced glutathione (GSH) levels in the brain and small intestine of cobalt chloride ($CoCl_2$)-treated rats. Data is expressed as mean ± standard deviation (n = 7). [a]indicates significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs control; [b]indicates significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs $CoCl_2$ group

< 0.05) reduced in the comparison to the control group (Fig. 1c and d). In contrast, the treatment groups showed dose-dependent increase in GSH content in the brain and intestines, which were statistically significant ($P < 0.05$) in the Cho3000 and Cho6000 groups in comparison to the $CoCl_2$-treated group.

Lipid peroxidation

As presented in Fig. 1e and f, the $CoCl_2$-group showed significant ($P < 0.0001$) increase in MDA levels as an indicator of lipid peroxidation in both the brain and small intestine in comparison with the control rats. Following sacrifice at day 15, the treatment groups showed significant reduction in the brain MDA level with Cho3000 ($P <$ 0.05) and Cho6000 (($P < 0.01$), as well as significant ($P <$ 0.0001) reduction in the intestinal MDA with administration of Cho1000, Cho3000 and Cho6000, in comparison with the $CoCl_2$-treated group.

Superoxide dismutase

The activity of SOD was significantly ($P < 0.05$) decreased in the brain of $CoCl_2$-treated rats in comparison with the control group (Fig. 2a), while the decrease observed in the small intestine was non-significant (Fig. 2b). However, in comparison to the $CoCl_2$-treated rats, the administration of Cho1000, Cho3000 and Cho6000 caused significant ($P < 0.01$) improvement of SOD activity in the brain, but not in the small intestine.

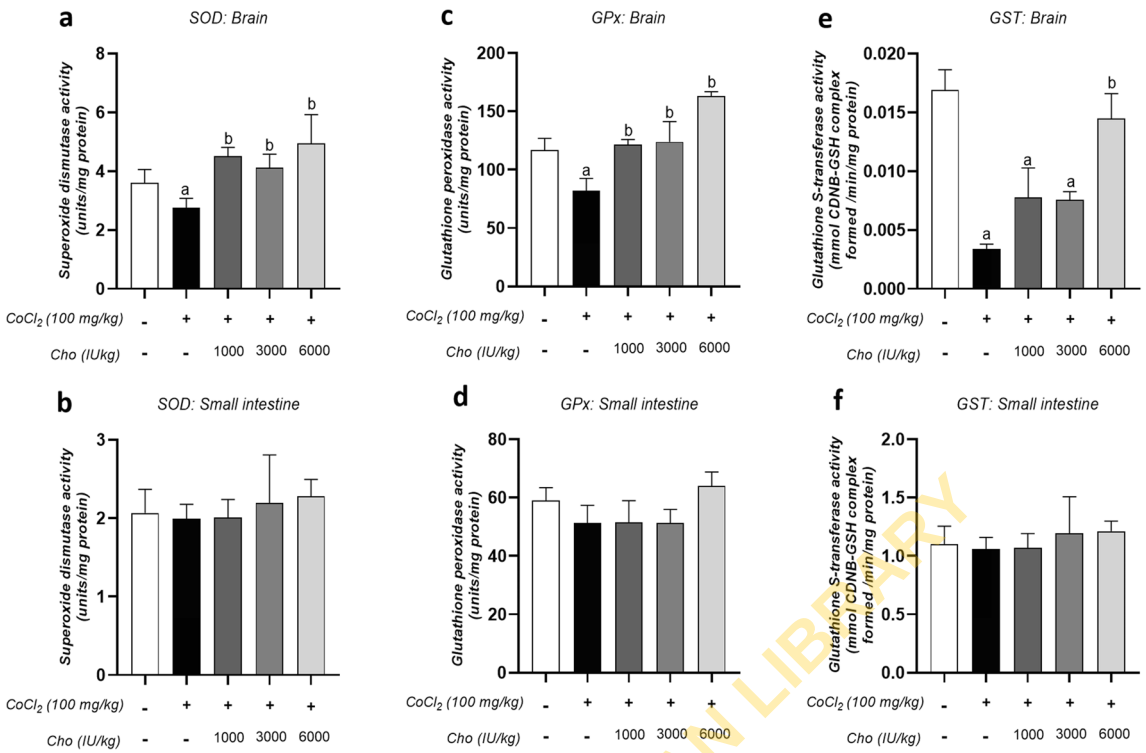

Fig. 2 Effect of cholecalciferol (Cho) on superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in the brain and small intestine of cobalt chloride ($CoCl_2$)-treated rats. Data is expressed as mean ± standard deviation (n = 7). [a]indicates significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs control; [b]indicates significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs $CoCl_2$ group

Glutathione peroxidase

The GPx activity in the brain of rats was significantly ($P < 0.05$) reduced in $CoCl_2$-treated rats, while administration of Cho1000 ($P < 0.05$), Ch03000 ($P < 0.05$) and Cho6000 ($P < 0.0001$) produced significant dose-dependent increase in the activity of GPx relative to the rats treated with $CoCl_2$ only (Fig. 2c and d). As was the case for SOD, the activity of GPx was not significantly modified in any of the treatment groups throughout the experiment. However, Cho6000 administration showed a non-significant increase in GPx activity in the intestines compared to the $CoCl_2$ group.

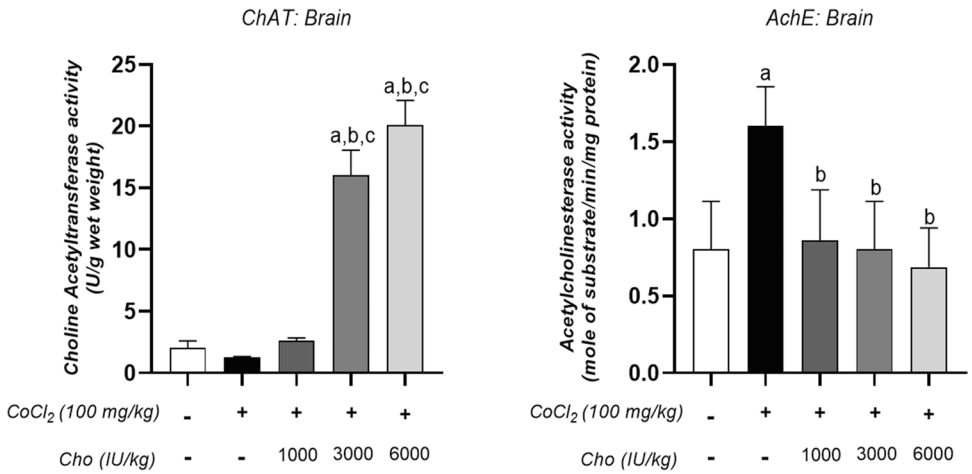

Fig. 3 Effect of cholecalciferol (Cho) on Choline acetyltransferase (ChAT) and Acetylcholinesterase (AchE) activities in the brain of cobalt chloride ($CoCl_2$)-treated rats. Data is expressed as mean ± standard deviation (n = 7). [a]indicates significant difference ($P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs control; [b]indicates significant difference ($P < 0.05$) vs $CoCl_2$ group. [c]indicates significant difference ($P < 0.01$; $P < 0.001$ or $P < 0.0001$ as appropriate; see text) vs $CoCl_2$ + Cho1000

Glutathione S-transferase

Brain GST activity was significantly ($P < 0.05$) decreased in $CoCl_2$-treated rats relative to the control (Fig. 2e), while the administration of Cho1000, Cho3000 and Cho6000 led to significant ($P < 0.05$) increase in GST activity in the brain when compared with the rats treated with $CoCl_2$ only. Similar to the other antioxidant enzymes measured, GST activity was not significantly altered in the small intestine (Fig. 3f).

The effect of cholecalciferol on cholinergic enzymes in brain of rats exposed to $CoCl_2$

Choline acetyl transferase (ChAT)

The activity of ChAT was not significantly affected in $CoCl_2$-treated rats in comparison with the control (Fig. 3). In the same vein, treatment with Cho1000 did not also affect ChAT activity. However, there was a significant (($P < 0.00001$) increase in ChAT activity when rats were administered either Cho3000 or Cho6000 along with $CoCl_2$ in comparison with control rats and those rats administered $CoCl_2$ alone.

AchE

A significant ($P < 0.01$) increase in the activity of AchE was observed in the brain of $CoCl_2$-treated rats in comparison to the control group (Fig. 3). In contrast, rats treated with Cho1000 ($P < 0.01$), Cho3000 ($P < 0.01$) and Cho6000 ($P < 0.001$) showed significant reduction in brain AchE as compared with the $CoCl_2$-treated group.

The effect of cholecalciferol on intestinal permeability markers in serum of rats exposed to $CoCl_2$

Serum ZO-1

The serum concentration of ZO-1 was significantly ($P < 0.001$) increased in the $CoCl_2$ group as compared to the control. Treatment with Cho1000 ($P < 0.05$), Cho3000 ($P < 0.001$) and Cho6000 ($P < 0.001$) showed significant decrease in serum ZO-1 levels compared to the $CoCl_2$ group (Fig. 4). The decrease in ZO-1 levels produced by Cho administration was dose-dependent.

Serum iFABP

As shown in Fig. 4, the level of iFABP in the serum of $CoCl_2$-treated rats remained similar to those of the control rats. In contrast, rats treated with Cho showed significant dose dependent reduction in serum iFABP levels at the dosages Cho1000 ($P < 0.05$), Cho3000 ($P < 0.05$) and Cho6000 ($P < 0.001$) compared to the $CoCl_2$-treated rats.

Serum Nitric oxide

Serum NO concentration showed a non-significant increase in $CoCl_2$-treated rats in comparison with the controls. Treatment of rats with Cho6000, however, produced significant ($P < 0.05$) reduction in serum NO levels when compared to the $CoCl_2$-treated rats (Fig. 4).

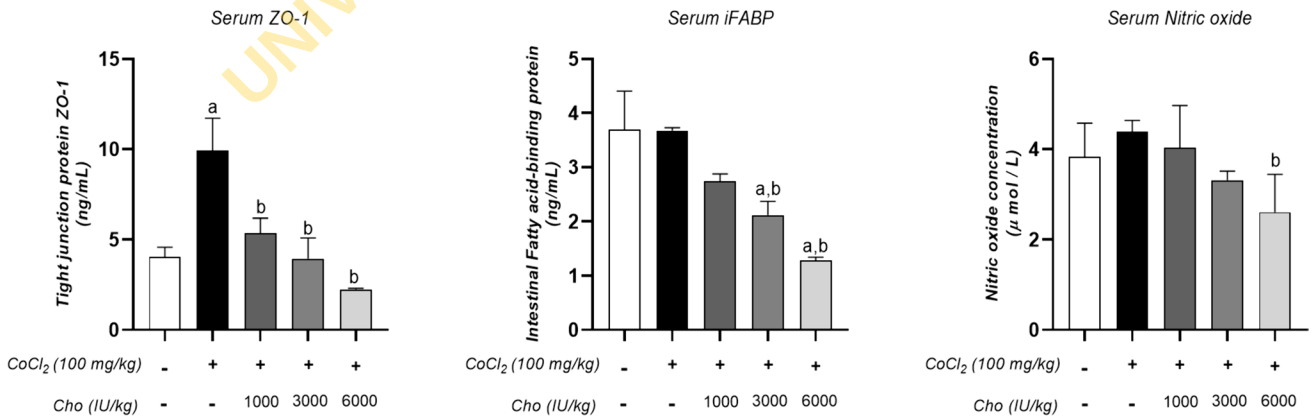

Fig. 4 Effect of cholecalciferol (Cho) on serum levels of Zonulin-1 (ZO-1), intestinal fatty acid-binding protein (iFABP) and nitric oxide in the brain of cobalt chloride ($CoCl_2$)-treated rats. Data is expressed as mean ± standard deviation (n = 7). [a]indicates significant difference ($P < 0.05$ or $P < 0.001$ as appropriate; see text) vs control; [b]indicates significant difference ($P < 0.05$ or $P < 0.001$ as appropriate; see text) vs $CoCl_2$ group

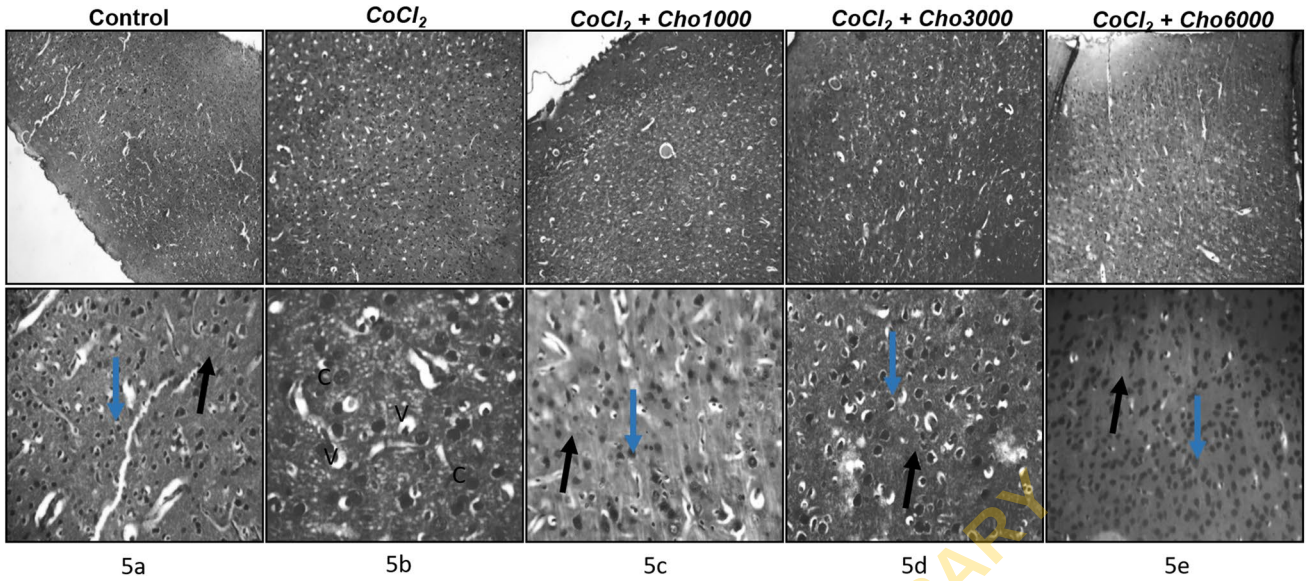

Fig. 5 Representative photomicrographs of the cerebral cortex of rats. (Upper plates: H&E, x100; Lower plates: H&E; x400). The control rats showed normal neuropil (black arrow) and intact neuronal cells (blue arrow). Rats in the $CoCl_2$ groups showed cerebral cortex with large numbers of vacuolated (V) cells as well as mild congestion (C) within the capillaries. The cerebral cortex from the $CoCl_2$ + Cho1000 and $CoCl_2$ + Cho3000 groups presented with predominantly intact neuronal cells and neuropil with reduced vascular congestion, although some vacuolated cells were still seen. The $CoCl_2$ + Cho6000 showed considerable improvement of the cytoarchitecture of the cerebral cortex with intact neurons, neuropil and capillaries

The effect of cholecalciferol on brain and intestinal histopathology

Cerebral cortex

The cerebral cortex from control rats was characterized by intact neurons which showed the typical spatial neural organizations across the layers of the cortex (Fig. 5a). In the $CoCl_2$-treated rats, the cerebral cortex showed histological changes which included large numbers of vacuolated cells along with congestion of many capillaries (Fig. 5b). Although some degree of improvement was noticed in rats treated with Cho1000 (Fig. 5c) and Cho3000 (Fig. 5d) along with $CoCl_2$ with considerable reduction in capillary congestion, the histological sections still revealed vacuolation of some neurons. However, treatment of rats with Cho6000 produced marked alleviation of the histological lesions caused by $CoCl_2$. Rats in this group presented with cerebral cortices with intact neurons, absence of vacuolation and without evidence of congestion in the capillaries, resembling those of control rats (Fig. 5e).

Hippocampus

In the control rats, the hippocampal subfields of the cornu ammonis (CA1, CA2 and CA3) were well delineated, consisting of intact, densely-packed pyramidal neurons and granule cells in the in the pyramidal and granule cell layers of the hippocampus and dentate gyrus, respectively (Fig. 6a). In rats treated with $CoCl_2$ only, CA regions of the hippocampus were still well-defined, although the pyramidal cell layer was less crowded with the cells, while the cells present were also associated with vacuolation and pyknotic nuclei (Fig. 6c). These lesions were also observed in rats treated with Cho1000 (Fig. 6c) and to a lesser extent in the Cho3000 group (Fig. 6d). However, after treatment with Cho6000, the morphology of the hippocampus was closely similar to that of the control group, with densely-packed, pyramidal cells, most of which were normal without vacuolations (Fig. 6e).

Cerebellum

The histological sections from the cerebellar cortex of control rats presented with normal histomorphology consisting of the distinct cell layers i.e. molecular cell layer, Purkinje cell layer and granular cell layer (Fig. 7a). Rats treated with $CoCl_2$ showed areas of depletion of Purkinje cells in the Purkinje cell layer, while other Purkinje cells showed chromatolysis (Fig. 7b). In contrast, histological sections from rats treated with Cho1000 (Fig. 7c), Cho3000 (Fig. 7d) and Cho6000 (Fig. 7e) along with $CoCl_2$ were devoid of the aforementioned lesions in the cerebellum, while mild degrees of cellular hyperplasia were noticed in some areas of the Purkinje cell layer.

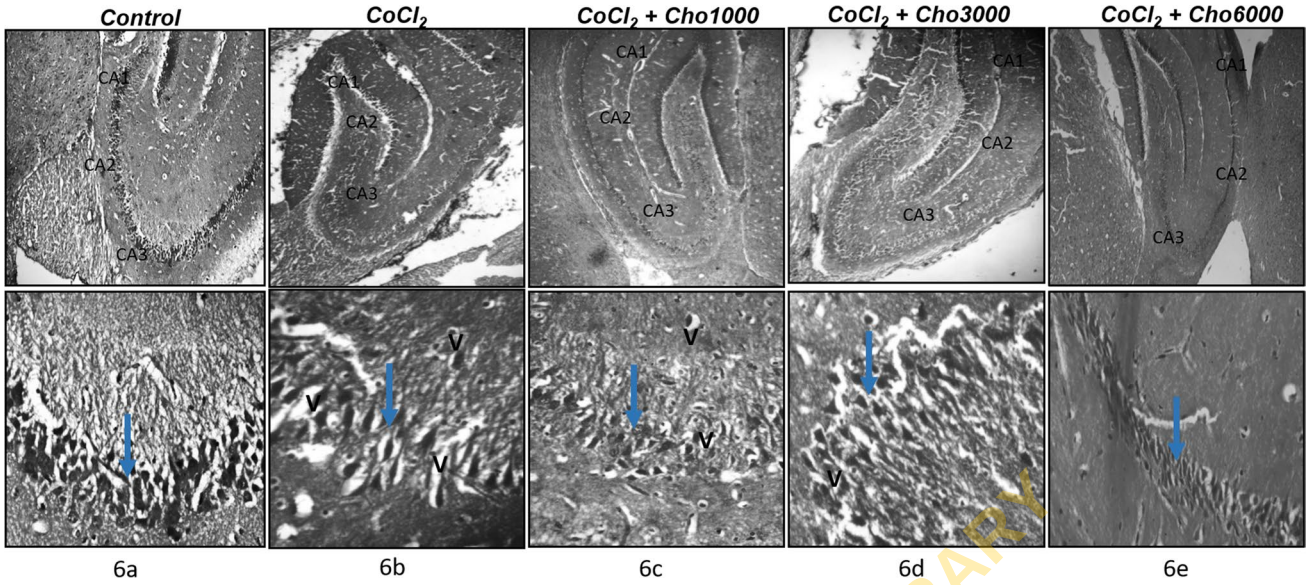

Fig. 6 Representative photomicrographs of the hippocampus of rats. Upper plates (H&E, x100) show the different subfields of the *cornu ammonis*, CA1, CA2 and CA3 across groups. Lower plates (H&E, x400) are higher magnifications showing the histomorphology of neuronal cells. The control group presented with normal cellular organization of the *cornu ammonis*, a densely-populated layer of intact pyramidal neurons are seen (blue arrow). The $CoCl_2$ group showed a sparsely-populated layer of pyramidal cells, many of which appear vacuolated (V). The $CoCl_2$ + Cho 1000 group also showed reduced cellular density and vacuolation of some cells. The $CoCl_2$ + Cho3000 group showed much improved cellular density while some degree of vacuolation were still observed. In the $CoCl_2$ + Cho6000, the histology of the hippocampus revealed tightly-packed cells and more densely-populated layer of pyramidal cells, with much reduced degree of vacuolation

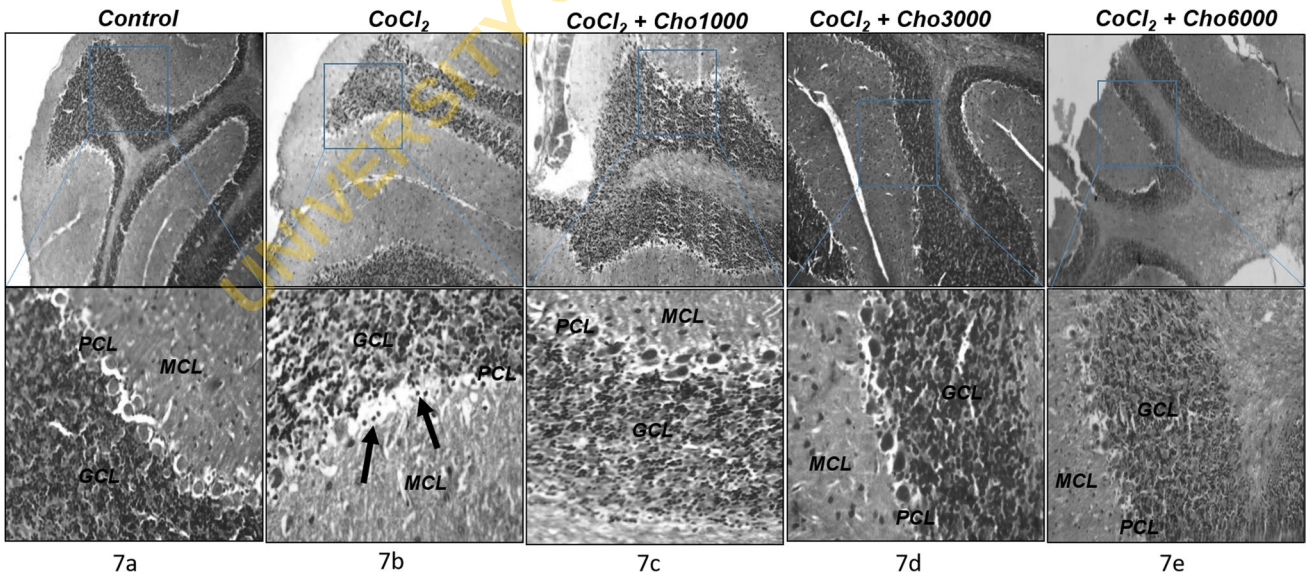

Fig. 7 Representative photomicrographs of the cerebellum in rats. Upper plates (H&E, x100) shows the general cytoarchitecture of the cerebellum. Lower plates (H&E, x400) shows greater detail of the different layers of the cerebellum, including the molecular cell layer (MCL), Purkinje cell layer (PCL) and the granular cell layer (GCL). The control group showed cerebellar cortex with normal folia with a normal hypocellular molecular layer, a monolayer of intact Purkinje cells and a dense granular layer. The $CoCl_2$ group cortex showed dying and pyknotic Purkinje cells, some of which also show chromatolysis (black arrows). The cerebellar cortex in the $CoCl_2$ + Cho1000, $CoCl_2$ + Cho3000 and $CoCl_2$ + Cho6000 groups had a similar presentation to the control with mild hyperplasia of the Purkinje cells

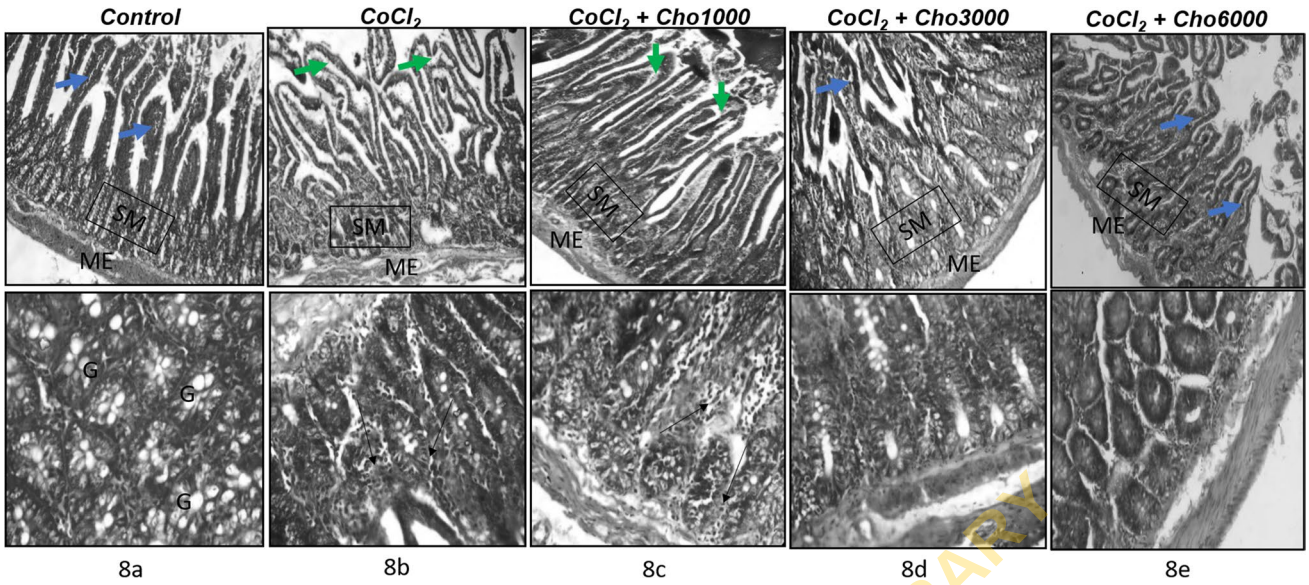

Fig. 8 Representative photomicrographs of the duodenum in rats. Upper plates (H&E, x100) show the general architecture of the different layers of the duodenum including the villi, sub-mucosa (SM) and muscularis externa (ME). Lower plates (H&E, x400) show higher magnification of the submucosa. Control rats showed well-preserved mucosal epithelium with normal villi (blue arrow). The sub mucosal layer appeared normal with well-defined submucosal (Brunner's) glands and the muscularis externa (ME) layer also appear normal. The $CoCl_2$ group showed duodenum mucosal epithelium with severely inflamed villi (green arrow) and presence of Greunhagen's space and lamina propria detached from the epithelium. The submucosa and the glands show moderate infiltration of inflammatory cells (slender arrow). The $CoCl_2$ + Cho1000 show duodenal sections moderately preserved mucosal epithelium with detached lamina propria normal villi (green arrow), mild to moderate infiltration of the submucosa by inflammatory cells, but normal muscularis layer appear normal. Rats in the $CoCl_2$ + Cho3000 and $CoCl_2$ + Cho6000 groups had duodenal sections with moderately to well-preserved mucosal epithelium, normal villi (blue arrow), very mild inflammatory cell infiltration of the lamina propria and the submucosa and normal muscularis layer

Duodenum

Control rats had well preserved villi, epithelial, mucosal and submucosal layers with normal glands and no evidence of inflammation (Fig. 8a). In contrast, duodenal tissues of the $CoCl_2$ group exhibited severe inflammation indicated by inflammatory cell infiltration into the mucosal and submucosal layers, along with the appearance of Greunhagen's spaces created by the detachment of the lamina propria from the epithelial layer (Fig. 8b). Rats treated with Cho1000 (Fig. 8c) exhibited similar pathology as those in the $CoCl_2$ group as the dose proved ineffective in alleviating tissue pathology. However, with higher doses of Cho at 3000 IU/kg (Fig. 8d) and 6000 IU/kg (Fig. 8e), there was noticeable improvement in the duodenal histomorphology as the sections showed lesser amounts of inflammatory cell infiltration, but well-preserved epithelial, mucosal and submucosal layers.

Ileum

Sections of the ileal mucosa of the $CoCl_2$ group showed poor mucosal morphology with vascular congestion and evidence of inflammatory cell infiltration (Fig. 9b). These pathologies were slightly reduced in the $CoCl_2$ + Cho1000 group (Fig. 9c). On the other hand, control rats and those supplemented with Cho at 3000 and 6000 IU/Kg showed normal histology (Fig. 9a, d and e).

Discussion

In this study, we demonstrated for the first time, the potential therapeutic effect of cholecalciferol against Co-induced neuronal and intestinal damages in rats. Previous studies have shown the beneficial effects of vitamin D against tissue injuries induced by toxic elements such as lead [38]. The present results describe, for the first time, the potential therapeutic effect of vitamin D against Co toxicity in the CNS and intestines. In this study, the administration of $CoCl_2$ was associated with reduction in the rate of body weight gain which was partially alleviated by cholecalciferol administration. This is in line with previous results where reduced body weight gain after Co exposure has been attributed to a reduction in appetite (hypophagia) [39].

Chronic Co bioaccumulation in the cerebral cortex has been associated with development of epileptic seizures, while microinjection of cobalt chloride into the lateral

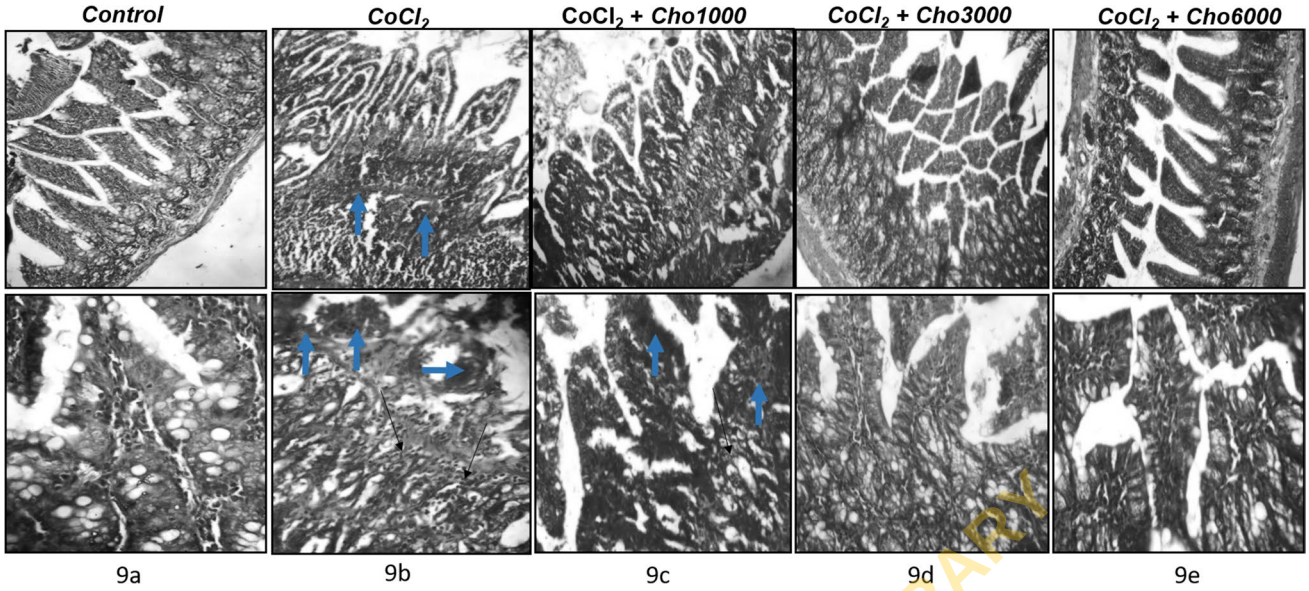

Fig. 9 Representative photomicrographs of the ileum in rats. Upper plates (H&E, x100) show the general architecture of the ileum. Lower plates (H&E, x400) show higher magnification of the ileal mucosal morphology. Control rats presented ileum sections showing well-preserved mucosal epithelium with normal villi, lamina propria and the glands show no infiltration of inflammatory cells, while the muscularis layer also appear normal. The $CoCl_2$ and $CoCl_2$ + Cho1000 groups showed ileum with poorly preserved villi, while the lamina propria, submucosa and the glands show moderate infiltration by inflammatory cells (slender arrow), along with vascular congestion in different portions of the mucosa and submucosa (blue arrow), while the muscularis layer appear normal. In the $CoCl_2$ + Cho3000 and $CoCl_2$ + Cho6000 groups, the mucosal epithelium was well preserved with normal villi and absence of inflammatory cells in the lamina propria or submucosa

cerebral ventricle of the rat has been used successfully as an animal model of epilepsy [40]. Accordingly, alterations in the activities of cholinergic enzymes have been described in chronic cobalt-induced epilepsy [41]. The cholinergic system in the CNS and PNS consists of cholinergic neurons which release acetylcholine (Ach) as the primary modulator of nervous transmission [42]. In the present study, we studied the influence of cholecalciferol on the activities of the cholinergic enzymes: choline acetyl transferase (ChAT) and acetylcholinesterase (AchE) in $CoCl_2$-treated rats. The results showed that $CoCl_2$ significantly increased AchE activity in brain homogenates, while ChAT activity was not significantly modified. Interestingly, administration of cholecalciferol produced remarkable preservation of the cholinergic system by profound stimulation of ChAT activity and dose-dependent reduction in AchE levels.

Several studies have opined that strategies for the treatment of memory dysfunction and cognitive deficits in neurodegenerative diseases should include modulation of the cholinergic system via inhibition of AchE and/or simultaneous stimulation of ChAT activity [43, 44]. Acetylcholinesterase catalyzes the hydrolysis of Ach to acetic acid and choline in cholinergic synapses in nerves and at neuromuscular junctions in muscles, thereby terminating neuronal transmission [45]. The activity of AchE is often applied as a biomarker of the neurotoxic effects of pollutant exposure due to its sensitivity, ease of measurement and its dose-dependent response to toxicant exposure [46]. Increased AchE with consequent decrease in brain acetylcholine (Ach) levels is a feature of cognitive dysfunction in neurodegenerative disorders such as Alzheimer's disease and other dementias [47]. The AchE-stimulatory activity exerted by $CoCl_2$, as obtained in the present study suggests that it may, therefore, play a role in loss of motor coordination in Co-exposed models as reported in our previous studies and elsewhere [10, 15]. In support of the above assertion, metals such as copper have been reported to cause increased specific activity and improved catalytic efficiency of AchE in brain and muscle [48]. Similarly, AchE activity was significantly increased in the brain of male Wistar rats exposed to nickel [49]. Hence, inhibitors of AchE and butyl cholinesterase (BchE), many of which have been licensed for symptomatic treatment of Alzheimer's disease, offer neuroprotective benefits by restoring synaptic levels of Ach via inhibition of its breakdown [50]. Our present results support the potential development of cholecalciferol as an inhibitor of AchE and its potential application in the modulation of neurological deficits involving alterations in Ach turnover.

Conversely, ChAT catalyzes the synthesis of Ach in a single step involving the transfer of an acetyl group from acetyl Co A to choline. The enzyme thus helps in regulating vital brain processes including cognition, learning and motor

coordination by modulating Ach levels [44]. In support of our findings, previous reports indicate significant increase in ChAT activity when rats were treated with vitamin D [51]. Interestingly, cholecalciferol supplementation has also been reported to contribute to protection of the cholinergic and antioxidant systems by normalizing "pathologically-elevated" activity of AchE which led to amelioration of Alzheimer-type memory dysfunction [52]. The current results add to the body of knowledge on new mechanisms that could be involved in the protection of the neurotoxicity of Co via modulation of the cholinergic system.

Despite some reports also confirming the ability of vitamin D to stimulate ChAT activity, the mechanisms involved are still poorly defined. Earlier studies which evaluated the mechanisms of ChAT stimulation by other compounds such as retinoic acid or sodium butyrate in human neuroblastoma cell line suggested that these compounds acted post-translationally by mechanisms such as the glycosylation of membrane proteins which eventually transduce certain signals resulting in the activation of the ChAT protein. These compounds were not found to affect DNA, RNA or protein synthesis [53]. In similar vein, a number of reports have also confirmed the inhibition of AchE by vitamin D, but the exact mechanisms are yet to be described [52, 54]. Traditionally, two modes of inhibition of choline esterases (AchE or BchE) have been identified: competitive inhibition involves the binding of the inhibitor to the active site of the enzyme, while other inhibitors bind to an aromatic gorge or peripheral anionic site in the enzyme. Traditionally, it is known that mechanisms of inhibition of AchE, especially by organophosphates and carbamate esters, involve covalent binding of the inhibitor to serine residues on the enzyme's active site [55]. As many diseases of the CNS are associated with functional abnormalities in muscarinic and nicotinic acetylcholine receptors, we recommend further studies to explore the influence of $CoCl_2$ and/or cholecalciferol on these receptors, as well as characterize the involvement of vitamin D receptors.

Our results further showed that $CoCl_2$ administration exerted significant impact on the integrity of the intestinal epithelium and intestinal permeability exemplified by increased serum levels of the tight junction protein Zonulin-1, although levels of serum iFABP and NO were not significantly affected. However, the results prove that Cho protected the intestinal epithelium from $CoCl_2$ damage by reducing the serum levels of both ZO-1 and iFABP. The iFABP is involved in intestinal fatty acid absorption and transport and its elevated level in serum level is believed to correlate with intestinal dysfunction, such that the protein is often utilized as a biomarker of intestinal hyperpermeability [56]. Similarly, increased serum or faecal levels of zonulin has been used as an indicator of increased intestinal permeability [57]. While serum level of NO is not usually regarded as a specific marker of intestinal damage, this chemical species is now increasingly recognized as a potential biomarker of inflammatory bowel disease [58], which may suggest its relevance in toxic conditions that stimulate inflammation in the intestinal mucosa. Furthermore, alterations in the homeostasis of nitric oxide have been implicated in intestinal barrier dysfunction [59]. There is a growing recognition of the involvement of Co in stimulating inflammatory responses in gastrointestinal epithelium. Increased secretion of chemokines interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) have been described in gastric and colonic epithelia exposed to Co [60]. Although, there is paucity of information on the impact of Co on gastrointestinal structures, the alterations to intestinal barrier components described in the present study may be in the context of increased intestinal permeability that predispose to bacterial and metabolite translocation to the CNS via the gut-brain axis. Our results also highlight the promise of the dual protective effects of cholecalciferol as a new possibility for treatment against toxicant-induced alterations in both the gut and brain.

The brain (due to high oxygen consumption and high content of peroxidizable lipids) and intestines (via constant xenobiotic exposure and gut inflammation) are among the most susceptible organs to oxidative stress and modifications of tissue macromolecules [61, 62]. In the present study, Co-induced oxidative stress was manifested as increased levels of brain and intestinal H_2O_2 and MDA, along with corresponding reduction in GSH levels. The activities of antioxidant enzymes, SOD, GPx and GST were significantly suppressed in the brain, although their levels remained unaltered in the intestines. Induction of oxidative stress by Co has been reported as a main mechanism of its toxicity [63]. Oxidative stress has been implicated in mediating enhanced AchE activity, while antioxidants such as vitamin E and nitric oxide synthase inhibitors were found to reverse the effect [64]. It seems plausible, therefore, that increased generation of ROS and the depletion of tissue antioxidant components could be responsible for the damage to the tissues, as observed in this study.

The morphology and structural integrity of tissues are often altered during oxidative stress [65]. Specifically, Purkinje cell depletion induced by $CoCl_2$ in the present study can be attributed to the induction of oxidative stress in line with reports by Sajdel-Sulkowska *et al*. [66]. In turn, damage to Purkinje cells has been correlated with loss of motor coordination that characterizes Co toxicity [67]. In the same vein, oxidative stress has been linked to the disruption of intestinal tight junction proteins with resultant increase in mucosal permeability and increased translocation of luminal contents into blood circulation [68]. Interestingly, the administration of cholecalciferol to rats led to dose-dependent reduction in oxidant parameters (H_2O_2 and MDA) with corresponding stimulation of antioxidant activity. This suggests that the neuroprotective and gut-protective

activities of cholecalciferol were likely mediated by its antioxidant actions as reported in previous studies [69]. Literature evidence shows that cholecalciferol promotes gut barrier integrity by preserving tight junction proteins and reduction of cellular apoptosis. As such, deficiency of vitamin D has been linked with the development of inflammatory bowel diseases [70].

Conclusions

The present study demonstrated the protective effect of cholecalciferol against $CoCl_2$-induced toxicity in the brain and intestines, highlighted by the restoration of cholinergic enzyme activities and preservation of tight junctions and gut barrier integrity. The mechanisms included the reduction of tissue ROS production and improvement of antioxidant enzyme activities. This is the first report showing the protective role of vitamin D in Co-induced neurotoxicity and enterotoxicity. We posit that more detailed studies would be needed to investigate the implications of these results on the gut-brain axis. Future studies could also be directed at investigating the involvement of muscarinic and nicotinic acetylcholine receptors in the vitamin D neuroprotection in order to fully characterize the cholinergic response. The measurement of cobalt and/or cholecalciferol concentrations in the tissues studied is recommended in future studies as they could be important in providing further insights into how their bioavailabilities or the extent of organ accumulation following their administrations, could influence on the effects obtained.

Acknowledgements The authors would like to acknowledge the support of the International Society for Neurochemistry and Committee for Aid and Education in Neurochemistry (ISN-CAEN) Category 1B grant awarded to Dr. A.S. Akinrinde

Author contributions The conception and design of the study were coordinated by Akinleye Akinrinde and approved by all authors. Preparation of materials, experimental protocols and analysis were performed by all authors. The first draft of the manuscript was written by Akinleye Akinrinde and all authors reviewed and edited the previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work received funding support from the International Society for Neurochemistry and Committee for Aid and Education in Neurochemistry (ISN-CAEN) Category 1B grant awarded to Dr. A.S. Akinrinde

Data availability The datasets generated during and/or analyzed during the current study are available within the manuscript.

Declarations

Ethical approval All experimental protocols and handling of animals were conducted in accordance with guidelines in the "Guide for the Care and Use of Laboratory Animals" in the NIH publication (PHS, 1996) and were also guided by guidelines approved by the local institutional Animal Care and Use Ethics Committee.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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