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Effects of Cobalt on Membrane ATPases, Oxidant, and Antioxidant Values in the Cerebrum and Cerebellum of Suckling Rats

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Abstract Chronic overexposure to cobalt (Co) may result in neurotoxic effects, but the mechanism of Co-induced neurotoxicity is not yet well established. Our study was conducted to determine whether Co is associated to the induction of central nervous system damage in pregnant rats and their progeny. Twelve pregnant female rats were randomly divided into 2 groups: group I served as controls and group II received Co (350 mg/L, orally). Treatments started from the 14th day of pregnancy until day 14 after delivery. Co concentration in plasma was higher in the treated groups than in the controls. Exposure to Co also increased the levels of MDA, PCO, H_2O_2, and AOPP, while Na^+K^+-ATPase and Mg^{2+}-ATPase, AChE, and BuChE activities decreased in the cerebrum and cerebellum of suckling pups. A smear without ladder formation on agarose gel was also shown in the cerebrum and cerebellum, indicating random DNA degradation. A reduction in GPx, SOD, CAT, GSH, NPSH, and vitamin C values was observed. The changes were confirmed by histological results. In conclusion, these data showed that the exposure of pregnant and lactating rats to Co resulted in the development of oxidative stress and the impairment of defense systems in the cerebrum and cerebellum of their suckling pups.

Keywords Cobalt chloride · Suckling rats · Cerebrum · Cerebellum · Antioxidant enzyme profiles · Histological studies

Abbreviations

AChE	Acetylcholinesterase
AOPP	Advanced oxidation protein product
Bcl-xl	B cell lymphoma-extra large
Bcl-2	B cell lymphoma 2
BuChE	Butylcholinesterase
CAT	Catalase
Co	Cobalt
DTNB	5,5′-Dithiobtis-2-nitrobenzoic acid
EGL	External granular layer
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IGL	Internal granular layer
MDA	Malondialdehyde
ML	Molecular layer
NBT	Nitro blue tetrazolium
NPSH	Nonprotein thiol
PCL	Purkinje cell layer
PCO	Protein carbonyl
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Cobalt (Co) emission into the atmospheric, aquatic, and terrestrial environments increased dramatically during the twentieth century. Stable Co may enter the environment from both natural sources and human activities. Generally, human Co

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poisoning can result from occupational, accidental, and intentional exposures [1]. Indeed, increased levels of Co were found in the urine and blood of occupationally exposed workers [2]. This heavy metal is suspected to have neurotoxic effects, as indicated by reports on memory deficit among workers exposed to hard metal both as dust powder and in mist form [3]. On the other hand, gestation impairment and its complications during pregnancy and childbirth have been reported in humans and animals exposed to Co [4]. Indeed, Co crosses the placenta and may also be found in milk, causing many disorders in newborns [5]. This heavy metal is recognized as being genotoxic and carcinogenic [6]. The action of Co ions is mediated, at least in part, by their ability to enter Fenton-type reactions [7] and trigger the production of reactive oxygen species (ROS). The latter are constantly produced and eliminated by living organisms, which results in a certain ROS steady-state level. An imbalance between ROS production and elimination in favor of the former and disturbing core and regulatory processes is called oxidative stress [8]. High ROS levels cause damage via the oxidative modification of lipids, proteins, and DNA of many cell types, including neural brain cells [9, 10]. The latter tissue is especially vulnerable to oxidative damage for a number of reasons: (1) it has a high concentration of polyunsaturated fatty acids (PUFA) compared to any other tissue in the body [11]; (2) the brain has a very high oxygen content and it consumes 20 % of the blood's oxygen, even though it makes up only 2–4 % of the body weight [12]; and (3) it undergoes a progressive accumulation of iron levels with aging [13]. Moreover, the brain uses a relatively large amount of oxygen at rather low activities of antioxidative enzymes [14]. Consequently, there is evidence of oxidative stress as an important mechanism of neuropathological disorders such as Alzheimer's disease, schizophrenia, and Parkinson's disease [15].

The brain, being a very sensitive tissue to oxidative stress, is vulnerable to damage induced by several chemical products, including metals. It exhibits distinct variations in the cellular as well as regional distribution of antioxidant defenses [16]. Thus, neural cells and/or brain regions respond differentially to metabolic rate changes associated with ROS generation [17]. Indeed, there is evidence invoking regional sensitivity to oxidative stress that is dependent on cellular and regional redox status [18]. According to some authors, the cerebellum is more susceptible to oxidative damage relative to other brain regions [19]. Although the effects of Co on the olfactory system have been investigated in adult rats [20], there are, to our knowledge, no reports about the effects of this metal on the cerebrum and cerebellum during late pregnancy and early postnatal periods. Thus, the present study was conducted to evaluate the redox state imbalance and disruption of membrane-bound ATPases and oxidative stress induced by Co in the cerebrum and cerebellum of suckling rats.

Material and Methods

Animals and Experimental Design

Wistar female rats weighing 170±10 g, obtained from the Central Pharmacy (SIPHAT, Tunisia), were housed in plastic cages in a climate-controlled facility with a constant light–dark cycle at a temperature of 22±2 °C and humidity of 40 %. Pregnant female rats were inspected daily by the presence of the vaginal plug, which indicated day 0 of pregnancy. Twelve pregnant female rats were randomly divided into 2 groups of 6 each: rats of group 1 (control group) received distilled water and served as negative controls. Those of group 2 (Co group) received, by drinking water, 350 ppm of $CoCl_2$. The Co dose represented one ninth of the LD_{50} [21]. In a pre-study, we tested different doses of Co: no toxic effects and no oxidative stress were observed in rats treated with Co at doses under 350 ppm. With this dose, oxidative stress was identified in female rats without lethal effects. But with doses over 350 mg/L, Co provoked severe signs of toxicity and mortality.

Pregnant female rats were allowed to deliver spontaneously 3 weeks after coitus. The day of birth was considered as postnatal day 0. Within 24 h after delivery, litters in both control and treated groups were culled to eight pups (four males and four females if possible) in order to maintain a similar lactation performance and to ensure maternal care [21]. Water consumption and food intake by dams were recorded daily during a lactating period.

All treatments started from day 14 of pregnancy until day 14 after delivery. $CoCl_2$ quantities ingested daily by adult rats were calculated after measuring drinking water. The experimental procedures were carried out according to the Natural Health Institute of Health Guidelines for Animal Care and approved by the Ethical Committee of Sfax Science Faculty. All animal procedures were conducted in strict conformity with the "Institute Ethical Committee Guidelines" for the care and use of laboratory animals [22]. At the end of the experimental period, mothers (n=12) and pups (n=96) were euthanized.

Blood and Brain Preparation

At the end of experiment, blood samples were collected in heparinized tubes. Plasma was separated from blood by centrifugation (3.500 rpm for 10 min). The cerebrum and cerebellum were quickly removed and cleaned from the adhering tissue. Some were used for oxidative stress markers analysis and others were minced, homogenized (10 % w/v) in an appropriate phosphate-buffered saline (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) with an Ultra Turrax homogenizer in ice-cold buffer, and centrifuged at 10.000×g for 15 min at 4 °C. The resulting supernatants were used for

various biochemical assays. Some cerebrums and cerebellums were immediately fixed in 10 % formalin solution for histological studies.

Biochemical Assays

Co Content

Co concentration in plasma was measured after acid digestion with nitric acid (65 %) using a microwave digestion system (CEM, USA, model MDS-2100). Co was estimated using a hydride vapor generation system (Perkin-Elmer model MHS-10) fitted with an atomic absorption spectrophotometer (Perkin-Elmer model Analyst100).

Protein Quantification

Cerebrum and cerebellum protein contents were measured according to the method of Lowry et al. [23] using bovine serum albumin as standard.

MDA Measurement

Malondialdehyde (MDA) concentrations, an index of lipid peroxidation, were determined spectrophotometrically according to Draper and Hadley [24]. The MDA values were calculated using 1,1,3,3-tetraethoxypropane as standard and expressed as nanomoles of MDA per milligram of protein.

Determination of AOPP Levels

Advanced oxidation protein product (AOPP) levels were determined according to the method of Kayali et al. [25]. The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as micromoles per milligram of protein.

Measurement of H_2O_2

Measurement of hydrogen peroxide (H_2O_2) was carried out by the ferrous ion oxidation xylenol orange (FOX1) method [26]. The FOX1 reagent consisted of 25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange, and 0.1 M sorbitol. Briefly, 100 μl of extract was added to 900 μl of FOX1 reagent vortexed and incubated for 30 min at room temperature. Solutions were then centrifuged at 12.000×*g* for 10 min, and the amount of H_2O_2 in the supernatant was determined using a spectrophotometer at 560 nm.

Determination of PCO Content

Protein carbonyl (PCO) was measured using the method of Reznick and Packer [27]. PCO was calculated based on the molar extinction coefficient of DNPH (ε=2.2×10^4 cm^{-1} M^{-1}) and expressed as micromoles per milligram of protein.

DNA Fragmentation Analysis

The extent of DNA fragmentation in the cerebrum and cerebellum was determined by the method described by Kanno et al. [28]. Briefly, brain tissue was homogenized in lysis buffer. The gel was observed under an ultraviolet lamp and photographed.

ATPase Assay

For the determination of ATPase activities, cerebrum and cerebellum were homogenized in Tris–HCl buffer, pH 7.4, according the method of Kawamoto et al. [29]. The resulting supernatants were immediately used for ATPase determination. Total ATPase activity was determined by Pi assay released from hydrolyzed adenosine triphosphate (ATP) forming a complex with molybdate. Enzyme activity was expressed as micromoles of Pi liberated per hour per milligram of protein.

Determination of Antioxidant Enzyme Activities

Catalase (*CAT*) activity was assayed by the method of Aebi [30]. Enzymatic reaction was initiated by adding an aliquot of 20 μl of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per milligram of protein

Superoxide dismutase (*SOD*) activity was estimated according to Beauchamp and Fridovich [31]. The reaction mixture contained tissue homogenates in potassium phosphate buffer (50 mM, pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 μM riboflavin, and 75 mM nitro blue tetrazolium (NBT). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50 % and the activity was expressed as units per milligram of protein.

Glutathione peroxidase (*GPx*) activity was measured according to Flohe and Gunzler [32]. GPx catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of reduced GSH reductase and nicotinamide adenine dinucleotide phosphate reduced form (NADPH), the oxidized reduced GSH is immediately converted to the reduced form with a concomitant oxidation of NADPH–$NADP^+$. The decrease in absorbance at 340 nm was measured. The enzyme activity was expressed as nanomoles of GSH oxidized per minute per milligram of protein.

Cerebrum and Cerebellum Total GSH Levels

Total GSH was determined by the method of Ellman [33] modified by Jollow et al. [34]. The method is based on the development of a yellow color when DTNB is added to compounds containing sulfhydryl groups. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as micrograms per milligram of protein.

Vitamin C Determination

Vitamin C determination was performed as described by Jacques-Silva et al. [35]. Protein was precipitated in 10 volumes of a cold 4 % trichloroacetic acid solution. An aliquot (300 μl) of supernatant adjusted with distilled H_2O to a final volume of 1 ml was incubated at 38 °C for 3 h, then 1 ml H_2SO_4 65 % (*v*/*v*) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenylhydrazine and $CuSO_4$ (0.075 mg/ml). The data were expressed as micromoles of ascorbic acid per milligram of protein.

Cerebrum and Cerebellum NPSH Levels

Nonprotein thiol (NPSH) levels were determined by the method of Ellman [33]. Absorbance of colorimetric reaction was measured at 412 nm. Total NPSH content was expressed as micromoles per milligram of protein.

Determination of AChE Activity

Acetylcholinesterase (AChE) activity was measured immediately in homogenates according to the method of Ellman et al. [36], using acetylthiocholine iodide as a substrate. The reaction mixture was composed as follows: phosphate buffer (0.1 M; pH 8) and 0.01 M DTNB. The hydrolysis rate of acetylthiocholine iodide is measured at 412 nm through the release of the thiol compound which, when reacted with DTNB, produces the color-forming compound TNB. The reaction was initiated by adding 0.075 M acetylthiocholine iodide. Activities were expressed as micromoles of substrate per minute per milligram of protein.

Determination of BuChE Activity

Butylcholinesterase (BuChE) activity was determined by the method of Ellman et al. [36], with some modifications. Hydrolysis rate was measured at acetylthiocholine concentrations of 0.8 mM in 1 ml assay solutions with 100 mM phosphate buffer, pH 7.5 and 1 mM DTNB. Fifty microliters of supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25 °C. All samples were run in duplicate. Activities were expressed as micromoles of substrate per minute per milligram of protein.

Histological Studies

Some cerebrum and cerebellum samples, intended for histological examination by light microscopy, were immediately fixed in 10 % of formalin and processed in a series of graded ethanol solutions. They were then embedded in paraffin, serially sectioned at 3 μm. Some sections were stained with hematoxylin–eosin. The other ones were stained with toluidine blue. Six slides were prepared from each cerebrum or cerebellum.

Statistical Analysis

The data were analyzed using the statistical package program StatView 5 Software for Windows (SAS Institute, Berkley, CA, USA). Statistical analysis was performed using one-way analysis of variance followed by Fisher's protected least significant difference test as a post hoc test for comparison between groups. All values were expressed as the means±standard deviation (SD). Differences were considered significant if $P<0.05$.

Results

The Effects of Co on the General Health of Rats

Death and abortion were not observed in Co-treated dams during the experimental period (21 days). In their suckling pups, few clinical signs such as ataxia, reduced activity, as well as tremors and nasal discharge were observed.

Concentrations of Co in Plasma

In our experimental conditions, Co concentration in plasma was higher in suckling rats of the treated groups ($P<0.01$) than in the controls (Table 1).

Cerebrum and Cerebellum AChE and BuChE Activities

$CoCl_2$ treatment of pregnant and lactating rats produced a significant reduction of AChE and BuChE activities in both cerebrum ($P<0.001$ and $P<0.05$) and cerebellum ($P<0.001$) (Table 1).

$CoCl_2$ Effects on Lipid Peroxidation and H_2O_2 Production

Our results showed that MDA contents, the major product of lipid peroxidation, increased by 55 % in the cerebrum ($P<0.001$) and by 25 % in the cerebellum ($P<0.01$) of

Table 1 Co content in the plasma of mothers and offspring and AChE and BuChE activities in the cerebrum and cerebellum of suckling pups

Parameters and treatment	Controls (n=8)	Cobalt (n=8)
Plasma cobalt content (mg/L)		
Mothers	0.093±0.0014	0.101±0.003*
Offspring	0.07±0.0010	0.09±0.0021**
AChE (μmol/min/mg protein)		
Cerebrum	0.012±0.001	0.008±0.001***
Cerebellum	0.009±0.001	0.006±0.001***
BuChE (μmol/min/mg protein)		
Cerebrum	0.077±0.015	0.049±0.015*
Cerebellum	0.146±0.020	0.077±0.018***

Number of determinations: n=8. The values are expressed as the means±SD

*P<0.05, **P<0.01, ***P<0.001; cobalt group vs control group

$CoCl_2$-treated rats when compared to the controls (Table 2). In addition, the H_2O_2 levels generated in the brain of suckling rats significantly increased, indicating oxidative stress (P<0.001).

$CoCl_2$ Effects on the Activity of ATPases

Table 2 showed that Co treatment impaired Na^+K^+-ATPase and Mg^{2+}-ATPase enzyme activities which significantly decreased in the cerebrum (P<0.001) and cerebellum (P<0.01 and P<0.001, respectively).

Markers of Protein Oxidative Damage

AOPP and PCO levels are usually used as markers of protein oxidative damage. In the Co group, a significant increase of PCO and AOPP levels in the cerebrum and cerebellum (P<0.001) of suckling rats occurred (Table 2).

Effects of Co on DNA Fragmentation

Agarose gel electrophoresis showed undetectable DNA laddering in the cerebrum and cerebellum of the control rats. The DNA intact band appeared to be condensed near the application point with no DNA smearing, suggesting no DNA fragmentation, while a smear (hallmark of necrosis) without ladder formation on agarose gels was observed in the cerebrum and cerebellum of the Co-treated rats, indicating random DNA degradation (Fig. 1).

Antioxidant Activities in the Cerebrum and Cerebellum

In $CoCl_2$-treated rats, SOD, CAT, and GPx activities decreased by 53 % (P<0.001), 35 % (P<0.05), and 21 % in the cerebrum and by 52 % (P<0.001), 71 % (P<0.001), and 39 % in the cerebellum, respectively, compared to those of the controls (Table 3). Our results also revealed a significant decrease in GSH and NPSH levels by 23 % (P<0.05) and 50 % (P<0.001) in the cerebrum and by 16 % (P<0.05) and 25 % (P<0.01) in the cerebellum, respectively (Table 4).

Table 2 Cerebrum and cerebellum MDA levels, Na^+K^+-ATPases and Mg^{2+}-ATPases activities, and H_2O_2, PCO, and AOPP levels of suckling pups controls and whose mothers were treated with Co from the 14th day of pregnancy until day 14 after delivery

Parameters and treatment	Controls (n=8)	Cobalt (n=8)
Cerebrum		
MDA (nmol MDA/mg protein)	1.039±0.31	2.32±0.68**
Na^+K^+-ATPase (μmol Pi/h/mg protein)	0.023±0.004	0.011±0.001**
Mg^{2+}-ATPase (μmol Pi/h/mg protein)	0.093±0.008	0.071±0.003**
H_2O_2 (μmol/mg protein)	0.94±0.14	2.45±0.71**
AOPP (nmol/mg protein)	0.36±0.08	0.70±0.13**
PCO (nmol/mg protein)	22.3±1.65	36.56±3.57**
Cerebellum		
MDA (nmol MDA/mg protein)	1.98±0.50	3.47±0.82*
Na^+K^+-ATPase (μmol Pi/h/mg protein)	0.037±0.005	0.025±0.003*
Mg^{2+}-ATPase (μmol Pi/h/mg protein)	0.166±0.007	0.134±0.004**
H_2O_2 (μmol/mg protein)	0.62±0.19	2.68±0.62**
AOPP (nmol/mg protein)	0.18±0.04	0.54±0.04**
PCO (nmol/mg protein)	14.38±2.00	22.55±2.27**

Number of determinations: n=8. The values are expressed as the means±SD

*P<0.01, **P<0.001; cobalt group vs control group

Vitamin C Levels

The data presented in Table 4 showed the levels of vitamin C in the cerebrum and cerebellum of the control and tested groups. The exposure of pregnant and lactating rats to Co

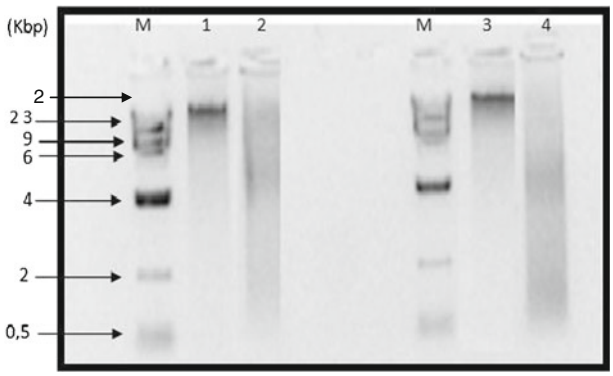

Fig. 1 Agarose gel electrophoresis of DNA fragmentation. *M* marker (phage lambda, *Hin*dIII (23, 9, 6, 4, 2, and 0.5 kbp), *lane 1* cerebrum of the control group, *lane 2* cerebrum of the Co-treated group, *lane 3* cerebellum of the control group, *lane 4* cerebellum of the Co-treated group

caused a significant decrease of vitamin C levels in the cerebrum ($P<0.001$) and in the cerebellum ($P<0.001$) of suckling pups.

Cerebrum and Cerebellum Histopathological Findings

Light microscopic examination indicated a normal structure in the cerebrum of suckling treated rats (Fig. 2b) when compared with the controls (Fig. 2a). However, there were changes on histological sections performed on the cerebellum of 14-day-old rats whose mothers were treated with $CoCl_2$. Normal histoarchitecture of the cerebellum showed four layers: an external granular layer (EGL), a molecular layer (ML), a PCL, and an internal granular layer (IGL). Purkinje cells in the PCL were well differentiated, showing elongated cellular bodies and dendritic arborization (Fig. 3a). Those of Co-treated rats were poorly differentiated with frequent pyknotic cells, and their number was reduced (Fig. 3b). Furthermore, the EGL became markedly developed (Fig. 3b).

Discussion

Co is an essential trace element required for normal growth, development, cellular homeostasis, and many enzymatic reactions [37, 38]. Although small amounts of Co are a nutritional necessity for normal brain functioning, it has been considered as neurotoxic at high doses. To our knowledge, this paper constitutes the first study evaluating cerebral and cerebellar damages induced by Co treatment in suckling rats.

Table 3 Activities of SOD, CAT, and GPx in the cerebrum and cerebellum of suckling pups controls and whose mothers were treated with Co from the 14th day of pregnancy until day 14 after delivery

Parameters and treatment	Controls (n=8)	Cobalt (n=8)
Cerebrum		
SOD (U/mg protein)	12.72±1.18	5.98±1.20**
CAT (μmol H_2O_2 degraded/mg protein)	0.15±0.05	0.097±0.058*
GPx (nmol GSH oxidized/min/mg protein)	0.14±0.03	0.11±0.03
Cerebellum		
SOD (U/mg protein)	11.90±1.53	5.69±0.82**
CAT (μmol H_2O_2 degraded/mg protein)	0.024±0.008	0.007±0.003**
GPx (nmol GSH oxidized/min/mg protein)	0.33±0.02	0.20±0.03**

Number of determinations: n=8. The values are expressed as the means±SD

*$P<0.05$, **$P<0.001$; cobalt group vs control group

Table 4 GSH, NPSH, and vitamin C levels in the cerebrum and cerebellum of suckling pups controls and whose mothers were treated with Co from the 14th day of pregnancy until day 14 after delivery

Parameters and treatment	Controls (n=8)	Cobalt (n=8)
Cerebrum		
GSH (μg/mg protein)	0.59±0.09	0.46±0.05*
NPSH (μmol GSH/mg protein)	0.006±0.001	0.003±0.0002***
Vitamin C (μmol/mg protein)	4.28±0.26	2.47±0.52***
Cerebellum		
GSH (μg/mg protein)	0.54±0.07	0.45±0.05*
NPSH (μmol GSH/mg protein)	0.004±0.0003	0.003±0.0001**
Vitamin C (μmol/mg protein)	3.40±0.82	1.56±0.40***

Number of determinations: n=8. The values are expressed as the means±SD

*$P<0.05$, **$P<0.01$, ***$P<0.001$; cobalt group vs control group

Our result demonstrated that Co concentration in the plasma of pups, whose mothers were treated by Co, was higher than in the controls. This could be due to the transfer of Co^{2+} through placenta and/or milk from mothers to their offspring. Thus, Co, when transferred to the offspring, affected cholinergic function, as demonstrated by us. Indeed, changes in AChE and BuChE activities might be an indicator of Co-induced neurotoxicity in suckling pups. This metal, which causes a depletion of neurotransmitters [39] and inhibits synaptic transmission via the presynaptic blockade of calcium channels, can block postsynaptic responses [40] and decrease exploratory behavior [41]. Moreover, neurotoxicity could be attributed to oxidative damage caused by Co treatment. One of the main manifestations of oxidative stress in the brain is MDA. The latter has been found to play an important role in the toxicity and carcinogenicity of many xenobiotics. On the other hand, it is well known that the brain is highly vulnerable to MDA due to its high rate of oxygen utilization [42], an abundant supply of PUFA, a deficient antioxidant defense [43], and a high content of transition metals like copper and iron in several regions [44]. In our findings, Co caused an increase of MDA in the cerebrum and cerebellum, suggesting its participation in generating free radicals including H_2O_2. The latter can attack PUFA in the biomembrane, leading to its dysfunction and thereby destroying the special arrangement and impairing local enzyme activities. Thus, a significant decrease in the Na^+K^+-ATPase and Mg^{2+}-ATPase levels was recorded after Co treatment. Additionally the inhibition of Mg^{2+}-ATPase could lead to a reduction in ATP production which, in turn, alters Na^+K^+ pump activity, producing neuronal dysfunction [45]. The increased free radicals generation can also lead to protein–protein cross linkages formation; protein backbones oxidation, resulting in the protein fragmentation; and modification of amino acid side chains, including sulfhydryl moieties oxidation and PCO formation, as

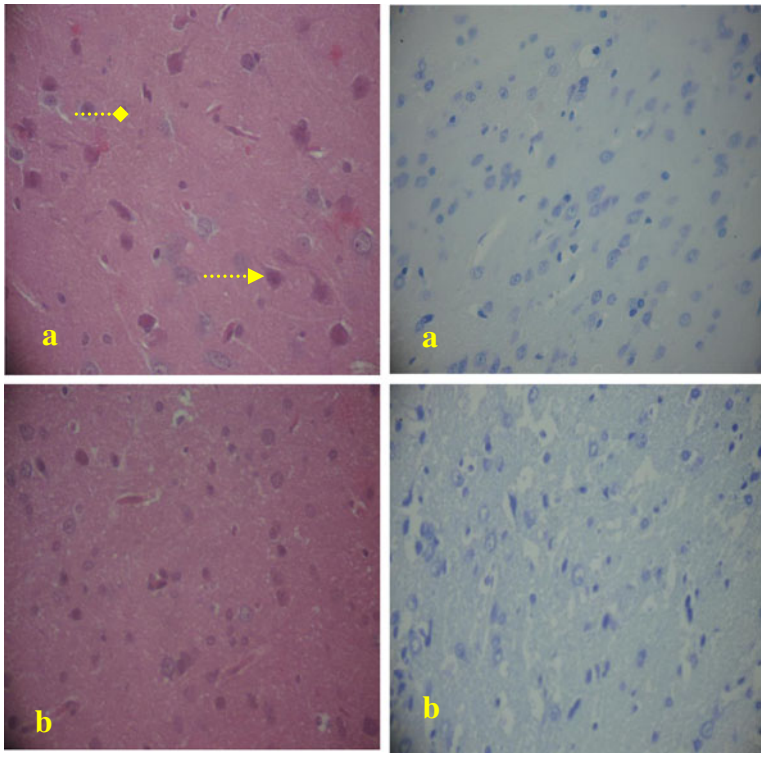

Fig. 2 Cerebrum histological sections, stained with hematoxylin–eosin and blue toluidine, of suckling pups controls (**a**) and whose mothers were treated with Co (**b**) from the 14th day of pregnancy until day 14 after delivery. Optic microscopy, HE (×400). ◆ neurons, ▶ glial cells

demonstrated by our findings. The occurrence of protein oxidative stress in the cerebrum and cerebellum of experimental rats was also confirmed by a novel marker, AOPP, which reflected an excess of free radical generation and protein oxidative damages. Free radicals attack not only proteins but also DNA bases, therefore causing mutagenic lesions. Co treatment resulted in massive DNA fragmentations with a subsequent formation of a DNA smear on agarose gel, a hallmark feature of necrosis, suggesting Co-induced brain cell damage. In fact, it has been reported that Co induces apoptosis in the neuronal PC12 cell line [46]. It triggered apoptosis in a dose-dependent and time-dependent manner as demonstrated by morphological changes and DNA fragmentation and confirmed by the decrease of the expression of Bcl-xl, a member of the Bcl-2 protein family that plays a role in the regulation of apoptosis [46].

Oxidative stress occurs when the antioxidant defense system is overwhelmed by the production of ROS. Antioxidant

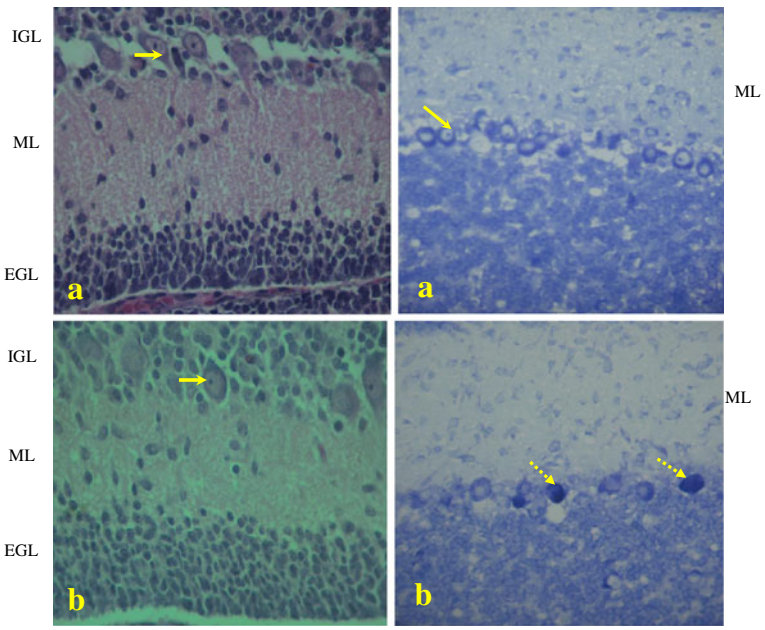

Fig. 3 Cerebellum histological sections, stained with hematoxylin–eosin and blue toluidine, of suckling pups controls (**a**) and whose mothers were treated with Co (**b**) from the 14th day of pregnancy until day 14 after delivery. Optic microscopy, HE (×400). *EGL* external granular layer, *ML* molecular layer, *IGL* internal granular layer, *solid arrows* Purkinje cells, *dashed arrows* pyknotic cells

and other cell redox state modulating enzyme systems act as the first-line defense against ROS in all cellular and extracellular compartments [47]. SOD, CAT, and GPx, the most important antioxidant enzymes, were reduced. These results indicated that the cytotoxic actions of Co in neuronal cells were mediated, at least in part, by an oxyradical mechanism involving the overproduction of ROS and downregulation of certain key antioxidant enzymes. On the other hand, nonenzymatic antioxidants like GSH and NPSH are considered as the second line of cellular defense against Co-induced oxidative damage. In our study, these parameters were greatly impaired, as indicated by a significant decrease in GSH and NPSH levels in the cerebrum and cerebellum of the Co group, reflecting their consumption through oxidative stress. Several pathways have been proposed. First, the sulfhydryl group has a high affinity for metals, forming thermodynamically stable mercaptide complexes with several metals [48]. Second, GSH may be oxidized due to the interaction with Co-induced free radicals.

It is also important to emphasize that GSH plays a critical role in regenerating vitamin C from its oxidized by-products. Our findings showed a significant decrease in the level of vitamin C after Co treatment, leading to the increased susceptibility of the brain tissue to free radical damage.

Enhanced oxidative stress in the brain after Co exposure was reflected in the significant histological changes, especially in the cerebellum of nursing pups. In fact, it is well known that the cerebellum is one of the brain parts that are strongly influenced during the early postnatal period, a critical period of life [49]. Because rat pups are born with a relatively undeveloped brain, especially an immature cerebellum, prenatal perturbation dramatically affects cerebellar development [49]. In the control group, the Purkinje cells were well differentiated, showing elongated cellular bodies and visible dendritic arborization. In contrast, the Purkinje cells of Co-treated rats were rounded, poorly differentiated, and frequently necrotic. There was also a loss of Purkinje cell numbers, as evidenced by a decreased cellularity in the PCL. Furthermore, the EGL became markedly developed in the treated group. This could be an indication of a delayed migration of granular cells towards the ML and IGL. According to Farwell et al. [50], granule cell migration depends on the recognition of extracellular neuronal guidance molecule(s) like laminin, a key guidance molecule in the developing brain. These data suggested that ingested Co was retained by the cerebellum, interfering with its physiology and inducing neurotoxicity, cell damage, and even cell death.

Conclusion

To our knowledge, this study may constitute the first attempt to evaluate the effects of Co on suckling rat brain. This metal, administered to pregnant and lactating rats at a dose of 350 ppm, caused free radical generation, increased MDA levels and protein oxidation products, and induced DNA fragmentation in the cerebrum and cerebellum of suckling rats. Co was considered as a pro-oxidant agent. It disturbed the oxidant–antioxidant balance and induced a depletion of cerebral and cerebellar antioxidant activities. Thus, the present study suggested that Co affected the structure and maturation of the brain, suggesting its neurotoxic effects.

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