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Effect of CoO Nanoparticles on the Enzyme Activities and Neurotransmitters of the Brain of the Mice "*Mus musculus*"

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Abstract

The effects of CoO Nanoparticles (NPs) on the brain enzymes and neurotransmitters of the mice were studied. The NPs were fed orally to the mice for a period of 30 days. The chronic (30 days) exposure of mice to NPs exhibited decrease in ALP, SGPT, SGOT, glutamate dehydrogenase and acetyl cholinesterase activities and increase in ACP and glutamine synthetize activity dose dependently. Further it also caused significant dose dependent decrease in the dopamine, Serotonin, GABA, Glutamate and Acetylcholine levels. These alterations in the electrolyte and neurotransmitter concentrations as well as enzyme activities of the brain could affect the metabolism, signal transduction and change the acid base balance of the brain leading to the impairment of the brain functions.

Keywords: Brain; Cobalt Oxide; Electrolytes; Enzymes; Nanoparticle; Neurotransmitters

Introduction

Cobalt oxide NPs are generating vast interest and attention due to their outstanding properties and wide potential applications [1,2]. These, NPs are used as substrates, suitable for biomolecule immobilization owing to their easy preparation and good biocompatibility [3]. CoO nanomaterials are also used as MRI contrast agents and used for building of dextran coating as well as magnetic microspheres [4,5].

The toxicity of cobalt was reported in mouse astrocytes causing cell death via apoptosis and necrosis [6]. Cobalt NPs toxicity with reference to gene expression of mouse fibroblast cell line (BALB3T3) is well known [7]. Dose dependent cytotoxicity of cobalt NPs in macrophages where in, morphological transformation of BALB3T3 cells was observed. Busch et al. [9] reported the hypoxia effects of tungsten carbide cobalt NPs on gene expression of human keratinocytes. Syrian golden hamsters exposed to cobalt oxide dusts found no increase in tumors. However, the study was faulted for poor survival [10]. Doses of 2 and 10 mg/kg of CoO NPs given to the mixed population of rats at 2-week intervals and 4 week intervals over 2 years showed bronchi-alveolar proliferation at high rates. At low doses the rats were found with benign lung tumor and bronchi alveolar carcinoma and at high doses with adenocarcinomas and bronchi alveolar adenomas [11]. We have also reported earlier the deposition of CoO NPs in the vital regions of the body including brain causing physical and behavioral changes [12]. These NPs are also seen to affect the carbohydrate metabolism of the brain [13].

Enzymes are the biological catalysts that catalyze the chemical reaction in the body to convert the substrate into a product. All the metabolic processes in the body need enzymes for their proper functioning, so does the brain. Different Enzymes have different roles in different regions or organs of the body i.e., digestive enzymes help in breaking down food and help in digestion, liver enzymes help in liver function, similarly brain enzymes help in normal functioning of the brain. Any alteration in enzymes can disturb the metabolism and damage the brain and affect its functions. Similarly, minerals and neurotransmitters are necessary for the normal functioning of the animal cells and body at large. Neurotransmitters are the chemical s messengers that transmit signals among the neurons [14]. They connect the brain to the rest of the body by providing signals to each organ to perform their task. Any imbalance developed in these neurotransmitters can cause many neurological diseases [15]. Therefore, a proper

balance of enzymes, electrolytes and neurotransmitters is needed for the brain to perform its task..Indiscriminate use of cobalt oxide NPs has increased its possibility to enter the human body by medical applications, inhalation, ingestion and skin adsorption [12]. There is no much data on the toxicity of cobalt oxide NPs on animals and humans especially in *in vivo* condition. Brain is the control system of the body wherein, electrolytes play a major role in nerve regulation, neurotransmitters in neurotransmission, ATPases in maintaining neuronal cell membrane rigidity and enzymes in normal functioning of brain. Therefore, an attempt is made to study the effects of cobalt oxide NPs on the enzyme activities and neurotransmitters of the brain of mice.

Materials and Methods

Synthesis and Characterization of CoO Nanoparticles

The CoO NPs were prepared by chemical precipitation method using sodium carbonate as precipitating agent and water as suspending medium. The cobalt nitrate was dissolved in a solution of sodium carbonate. The precipitate obtained was rinsed with deionized water to remove impurities and dried at 100°C. Further, they were calcined at 400°C, 500°C, 600°C in muffle furnace for 1 hour at each temperature regimen with intermittent grinding.

The characterization of the nanoparticles was done using SEM (Carl-Zeiss Scanning Electron Microscope), TEM (Philips CM200 TEM electron microscope), XRD (Rigaku X-ray Diffractometer), FTIR (Perkin Elmer BX FT-IR infrared spectrometer) and Zeta sizer (Zeta sizer Ver. 6.32, serial number -MAL1037088, Malvern Instruments Ltd). The specific surface area was measured using Saunter formula [16] and density was recorded by the method recommended by [17]. The chemical properties of CoO NPs like pH and conductivity were checked using pH meter (ELiCO L1 pH Analyzer) and conduct meter (LOBAL Digital conductivity meter) respectively. The nanoparticles were also checked for their solubility in different solvents, ethers and acids by continuous and rigorous stirring.

Preparation of Nanoparticles Suspensions

The NPs were suspended in mammalian saline and dispersed by using sonicator (BRANSON Digital Sniffier) at 30% amplitude for 10 mins (12). Different concentrations (5mg/kg, 10mg/kg, 20mg/kg, 25mg/kg and 50mg/kg) were prepared for acute and chronic exposure based on the LD50 values [12].

Maintenance of Animals

The Swiss Albino Mice (*Mus musculus*) weighing 20-35 grams were kept in polypropylene cages. They were maintained at ambient laboratory conditions, at 12-hr light/dark cycle with free access to water and standard pellet diet (Hindustan Lever, Bangalore, India). Ethical approval was obtained from the Institutional Animal Ethics Committee (Ref no. 105/C-2013), based on the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines [18]. The animals were maintained at animal house facility of the Department of Zoology, Goa University.

Chronic Treatment

Animals were divided into 4 groups, each group comprising of 5 animals. The groups were divided as Control, Exp 1, Exp 2 and Exp 3. Control was fed with distilled water and experimental with NPs suspended in distilled water in concentrations of 5mg/kg, 10mg/kg and 20 mg/kg body weight of mice respectively. The animals were gavaged with NPs daily once in the morning, continuously for 30 days. After the completion of time interval, the animals were sacrificed by decapitation.

Sample Preparation

The mice were perfused with saline as reported earlier [12]. After perfusion the mice were sacrificed, brain was removed on ice slab and different parts (Cerebellum, cerebral cortex, medulla oblongata and olfactory bulb) were separated, washed thoroughly with chilled mammalian saline and homogenized in chilled distilled water [12]. The homogenates were then centrifuged at 3000 rpm for 15 mins. The supernatants thus obtained were stored in clean sterile micro centrifuge tubes at -4°C until analysis.

Enzyme Analysis

The enzyme activities were assayed using respective assay kits of Coral Crest Bio system (2015). Alkaline Phosphatase (ALP, Product no-ALP3166, Crest Bio systems, 2015), Acid Phosphatase (ACP, Product no-ACP2125A,Crest Bio systems, 2015), Lactate dehydrogenase (LDH, Product no-LDL1159, Crest Bio systems, 2015), Serum glutamic oxaloacetic transaminase (SGOT, Product no-GOT2160C,Crest Bio systems, 2015), Serum glutamic-pyruvic transaminase (SGPT, Product no-GPT2178, Crest Bio systems, 2015) were assayed using Coral Crest Bio system kits(2015) and ATPases were estimated following the method of [19]. Acetylcholinesterase was estimated using Acetylcholinesterase kit (Molecular probes, Invitrogen detection technologies, 2015), Glutamate synthetize(GS) using the protocol of [20]and Glutamate dehydrogenase(GDH) using the method of [21].

Neurotransmitter Estimations

Serotonin and Dopamine were estimated using Elisa kit from DRG Diagnostics (Product No-ELA-5061, 2015, Product No-ElA-4824, 2015) USA, with the help of Elisa plate reader (Analytical technologies Ltd). Acetylcholine was estimated using Acetyl choline kit (Molecular probes, Invitrogen detection technologies, 2015). Glutamate and GABA were estimated following the protocol of [22].

Statistical Analysis

Statistical analysis of the data was performed using Student's t-test (Graph pad Software, San Diego, CA) and ANOVA (One way and two-way analysis of variance) using XLSTAT software. The criterion for statistical significance was $p \leq 0.05$ and for highly significance was $p \leq 0.001$.

Results

Nanoparticles

The nanoparticles synthesized by chemical precipitation method were 95% pure, black in colour with specific surface area ≥23.143 m^2/g. The average size of NPs was 20-65nm with a density of 6.1g/cm^3. Under TEM and SEM they appeared semicircular and occasionally rectangular in shape with some forming clusters (Figure 1A-B). The XRD analysis of the CoO nanoparticles showed peaks at 2θ = 16.02º, 31.22º, 36.82º, 38.72º, 44.74º, 59.36º and 65.16º and FTIR spectra showed two sharp transmittance peaks at 667.37 and 582.50 cm^{-1}(Figure 2 A-B). The zeta potential of the nanoparticles was -20.4mV (Figure 3). The pH of CoO nanoparticles suspended in water is 7.2 and conductivity was 0.100 MS/cm^{-1}. The CoO NPs were insoluble in solvents including ethers, acids and water.

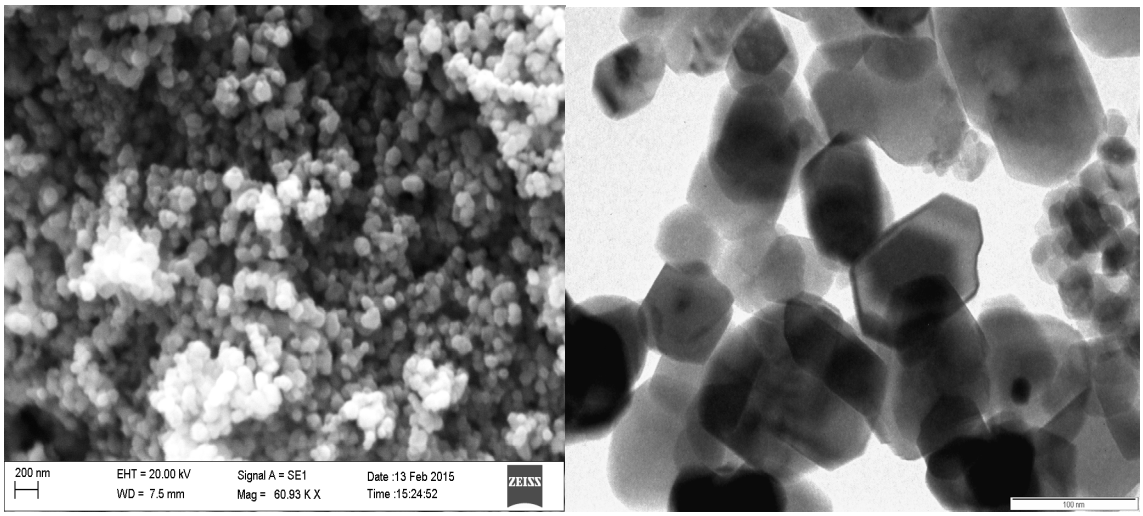

Figure 1: CoO Nanoparticles (A) SEM image (B) TEM image.

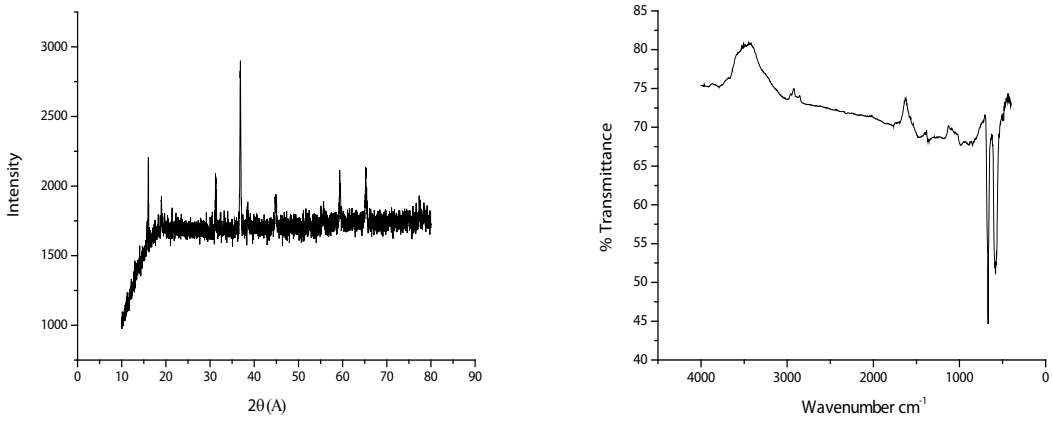

Figure 2: CoO Nanoparticles **(A)** XRD **(B)** FTIR.

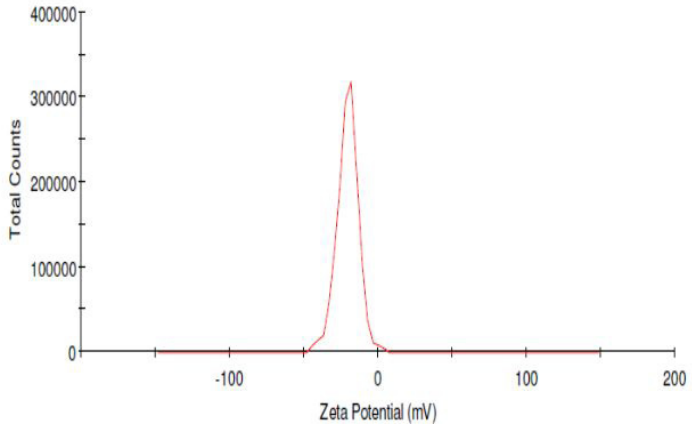

Figure 3: Zeta potential of CoO Nanoparticles.

Effect of Nanoparticles on Mice

The neuronal ALP activities significantly decreased (22-84.2%) in c, ob.(P≤0.05) cc and mo.(ns). However, ACP activities increased by 26.4% to 1.96 from all brain regions (P≤0.05) except c (Figure 4). SGOT activities dropped by12-79.9 %(P≤0.05) from all brain regions, while SGPT activities decreased by 11-57.9 % in mo. and ob. (P≤0.05) but the decrease was insignificant in c and cc (Figure 5). Mg ATPase's decreased by 11-83.4 % (P≤0.05) in all regions of brain, while Ca ATPase's decreased by11.9-48.2 % (P≤0.05) in cc and ob. in a dose dependent manner but it did not decrease significantly in "c". However, NA –K ATPases increased significantly (P≤0.05) and dose dependently (25% to 1.78fold) as compared to the controls (Figure 6). The neurotransmitter associated enzymes, glutamate synthetase, showed significant(P≤0.001) dose dependent increase (14-44.4%) in all the brain regions (Figure 7), whereas acetyl cholinesterase increased (16-78%) significantly (P≤0.001) dose dependently in brain but glutamine dehydrogenase decreased significantly and dose dependently in brain regions examined (Figure 8).

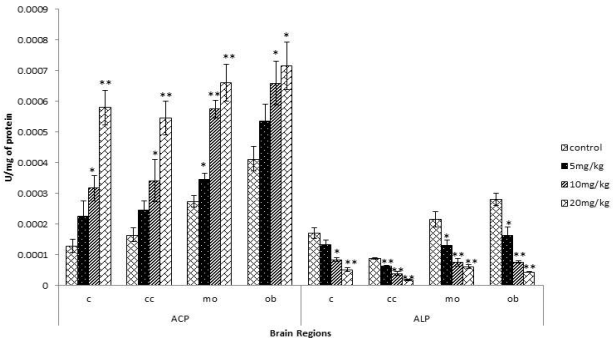

Figure 4: Chronic(30 days) effect of CoO NPs on the ACP and ALP activity of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

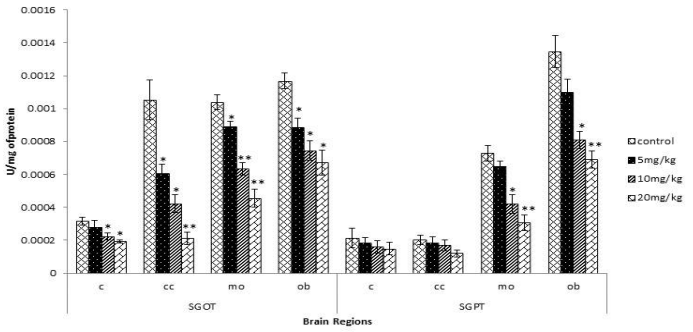

Figure 5 : Chronic(30 days) effect of CoO NPs on the SGOT and SGPT activity of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

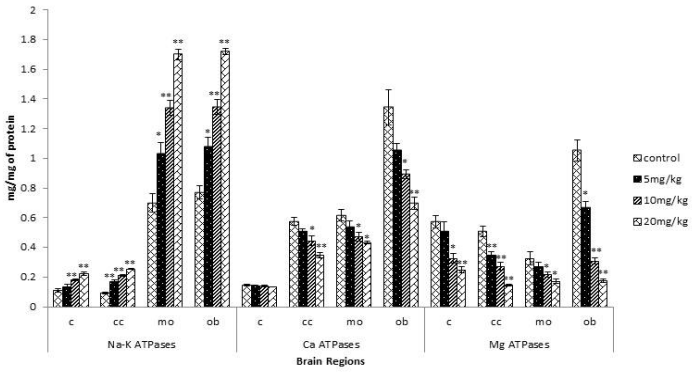

Figure 6: Chronic(30 days) effect of CoO NPs on the Na-K, Ca and Mg ATPases levels of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

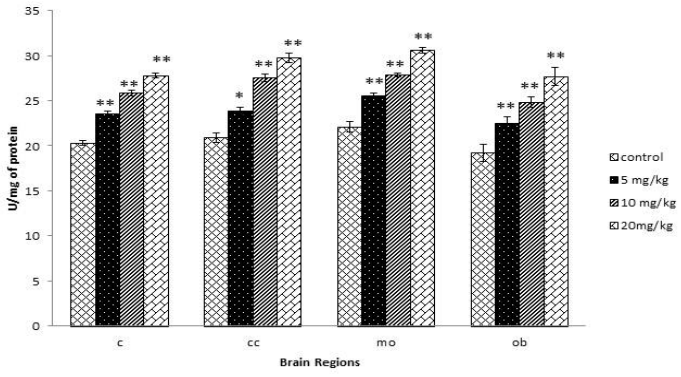

Figure 7: Chronic(30 days) effect of CoO NPs on the Glutamine synthetase activity of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

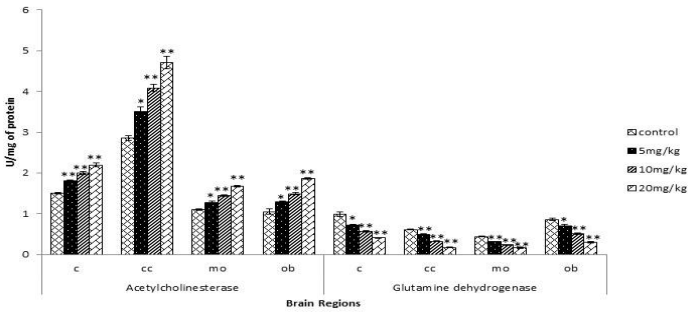

Figure 8: Chronic(30 days) effect of CoO NPs on the Acetyl cholinesterase and Glutamine dehydrogenase activity of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

The dopamine levels decreased dose dependently and significantly (P≤0.001) by 5.3- 27.6% while serotonin levels lowered by 1.7-46.4 % (P≤0.05) in all the brain regions (Figure 9). Besides, Glutamate levels increased by 6.4-72 % (P≤0.001 in all brain regions and GABA levels decreased by 22.3-90 % (P≤0.001) in all brain regions except in c (ns) (Figure 10) as compared to controls but acetylcholine decreased by 8.8-55.9 % (P≤0.05) (Figure.11).

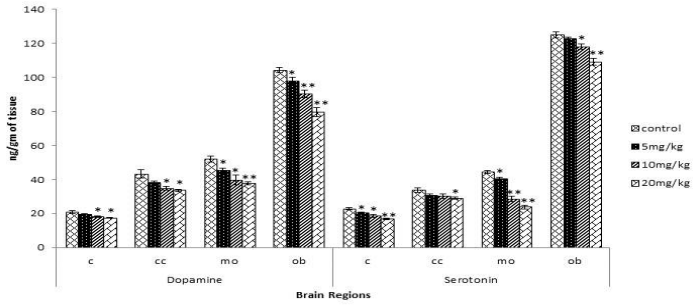

Figure 9: Chronic(30 days) effect of CoO NPs on the Dopamine and Serotonin levels of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant)

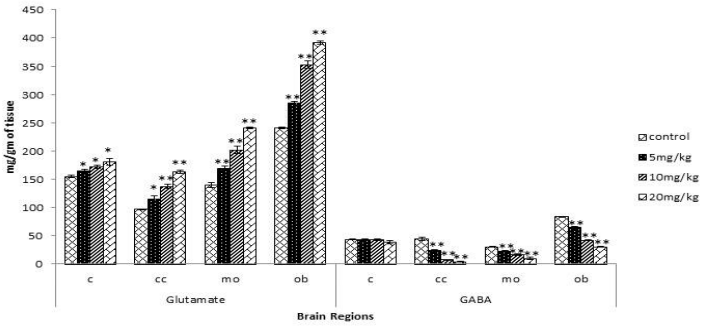

Figure 10: Chronic(30 days) effect of CoO NPs on the Glutamate and GABA levels of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

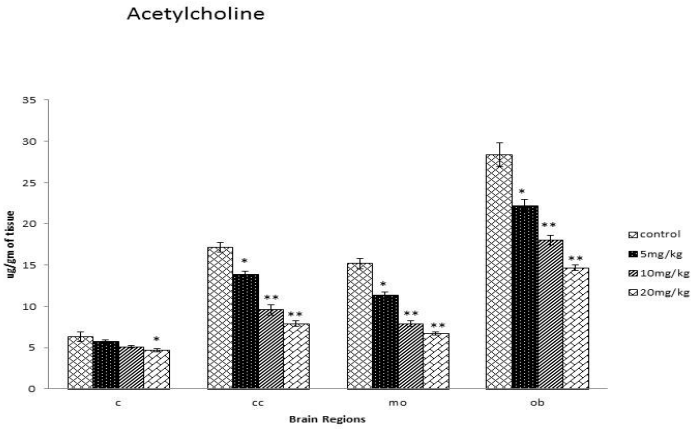

Figure 11: Chronic(30 days) effect of CoO NPs on the Acetylcholine levels of brain in mice (*-P ≤ 0.05, **- P ≤ 0.001, #- Non-significant).

Discussion

The CoO NPs synthesized were of definite shape and size. The SEM and TEM images revealed their sizes in nanometer scale. The sharp XRD peaks showed that the NPs were crystalline in nature and the peaks observed in XRD pattern matched with that in JCPDS card No: 073-1701 [23]. The two sharp peaks obtained in FTIR spectra correspond to the metal–oxygen (Co–O) stretching vibration modes of CoO [24,25]. The XRD pattern and FTIR spectra confirm its identity as CoO. The Zeta potential revealed that CoO NPs have moderate stability and strong anionic property [26]. The CoO NPs suspended in water showed neutral pH showing that these nanoparticles do not affect the pH of water but they remain suspended in all the consumable solutions and solvents and the increase in the conductivity suggests that CoO NPs promote pollution as increase in conductivity is an indicator of pollution [27,28].

Cobalt oxide nanoparticles effect on physical and behavioral changes as well as retention of nanoparticles leading to alteration in carbohydrate metabolism of brain is already reported by us in the earlier studies [12,13]. Enzymes as catalyst, electrolytes as minerals and neurotransmitters as messengers play very important role in the brain functioning.

Enzymes are very essential for the normal metabolism and proper body functioning. Chronic exposure of CoO nanoparticles led to significant damage to the brain causing increase in ACP activity, a lysosomal enzyme due to cell necrosis and lysosomal dysfunction as ACP is only released when the cells are injured[29].Increase in ACP can lead to Alzheimer's disease as any effect to the lysosomal system can lead to age related neurodegeneration[30,31]. Decline in the rate of protein synthesis

and electrolyte imbalance due to tissue dehydration could lead to decrease in the ALP activity [32]. The disturbed protein synthesis also can cause decrease in SGPT and SGOT activity suggesting that the intermediary metabolic processes are inhibited and inactive transamination and oxidative deamination has taken place [33,34]. As SGPT and SGOT are the native brain enzymes and serve as a link between carbohydrate and protein metabolism in converting α-ketoglutarate to glutamate and vice versa so decrease in both the enzyme activities shows that these inter conversions are affected [35]. Besides decrease in SGOT activity could be due to decreased transport of NADH leading to decrease in metabolic rate [36]. SGPT is involved in the synthesis of glutamate so reduction in SGPT will obstruct the glutamate synthesis causing many neurological disorders, as well as decrease in SGPT indicates reduction in protein metabolism[37,38]. Brain needs to maintain the ATPases required for cation homeostasis, impulse propagation, neurotransmitter release and to regulate electrolyte transport of cell membrane. The stress caused by CoO NPs on the ionic balance could have led to increase in Na-K ATPases causing increase in impulse transmission whereas decrease in Mg ATPases and Ca ATPases can be attributed to induction of energy crisis through disruption in oxidative phosphorylating process due to CoO NPs [39]. These changes clearly show that the CoO NPS affects the brain.

Neurotransmitter release in proper amount is essential for communication within the brain and with the rest of the body by transmitting chemical signals. Dopamine is an organic chemical which plays an important role in the brain as neurotransmitter. It plays a major role in reward system that is reward motivating behavior, motor control as well as in controlling the hormone release. On the other hand, serotonin, a neurotransmitter is synthesized by the serotonergic neurons in the brain and has various functions in mood, sleep and appetite regulation as well as some cognitive functions (memory and leaning) both are important for the brain functioning. The stress caused by continuous exposure of CoO NPs resulted in decrease of dopamine and serotonin levels, which can affect the body drastically as decrease in dopamine can lead to difficulty in movement, paralysis and even cause Parkinson's disease and decrease in serotonin levels can cause depression, anxiety, constipation [40,41].Glutamate is an excitatory neurotransmitter responsible for memory, speech, learning and cognition. The enzymes Glutamine synthetase, glutamate dehydrogenase, SGPT and SGOT are involved in glutamate metabolism. GS plays a major role in the regulation of the glutamate and detoxification of ammonia. This enzyme helps in metabolizing glutamate and ammonia into the glutamine. So decrease in the GS levels shows that the conversion process is affected which resulted in the excess of glutamate levels. On the other hand GDH is an enzyme used in converting α-ketoglutarate to glutamate and vice versa so increase in the GDH levels can also be a reason for excess of glutamate synthesis during stress created by NPs [42], as well as decrease in SGPT and SGOT activity shows that the interconversion of glutamate to α-ketoglutarate is affected. Increase in glutamate levels can lead to excitation wherein the nerves and brain cells are over stimulated leading to neurological inflammation and cell death. GABA is the so called inhibitory neurotransmitter, responsible for calming the brain and relax the person in its thoughts, processes and speech. The decrease in the GABA levels shows that the conversion of glutamate to GABA is affected as once the glutamate is built up, it gets converted to GABA with the help of an enzyme glutamate decarboxylase. So in spite of increased glutamate levels, the inhibition of its conversion to GABA shows that the Nano particles are affecting this pathway. Decrease in acetylcholine levels could be attributed to stimulation of acetylcholine esterase enzyme activity that allows rapid breakdown of acetylcholine leading to decrease in the acetylcholine levels which can cause chronic stress [43]. From these observations it's very clear that the CoO NPs affect the enzyme activities, neurotransmitter signaling and causes electrolyte alterations affecting the acid base balance of the brain and leading to neurological diseases.

Conclusion

Chronic exposure of mice to CoO NPs disturb the brain enzyme activities and neurotransmitters levels.

Acknowledgement

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Conflict of Interest

The authors declare that there is no conflict of interest for the publication of this work.

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