ANALYSING THE SHORT- TERM EFFECTS OF CADMIUM OXIDE NANOPARTICLES ON BRAIN OF *MUS MUSCULUS*

Dissertation submitted

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In partial fulfilment of the degree of Masters in Science in Zoology

By

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, **"Analyzing the short-term effects of Cadmium Oxide Nanoparticles on Brain of *Mus musculus*"** is based on the results of investigations carried out by me in the **Zoology Discipline** at the **School of Biological Sciences and Biotechnology**, Goa University under the Supervision **Dr. Shamshad Bi M. Shaikh** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Analysing the short-term effects of Cadmium Oxide Nanoparticles on Brain of *Mus musculus*"** is a bonafide work carried out by **Ms. Melvita Alvares** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in Zoology Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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DEDICATION

Dedicated to the ones who gave me life.

To my MOM who is my strength and to my heavenly DAD who showers his blessings upon me.

TABLE OF CONTENT

ACKNOWLEDGEMENT

"Gratitude can transform common days into thanksgivings, turn routine jobs into joy, and change ordinary opportunities into blessings."

- *William Arthur Ward*

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Melvita Alvares

24th April 2023

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

µl- microliter

µm- micrometer

µmole- micromole

AAS- Atomic Absorption Spectrometry

AgNPs- Silver Nanoparticles

ALP- Alkaline Phosphatase

ALT- Alanine transaminase

ANOVA- Analysis of Variance

AST- Aspartate transaminase

C- Cerebellum

CAT- Catalase activity

CC- Cerebral Cortex

Cd- Cadmium

CdNP- Cadmium Nanoparticle

CdO- Cadmium Oxide

CdO NPs- Cadmium Oxide Nanoparticles

CdS- Cadmium Sulphate

CdTe- Cadmium telluride

Cu- Copper

$CuSO_4$- Copper Sulphate

dl- Deciliter

DNA- Deoxyribose Nucliec Acid

DNPH- 2,4-Dinitrophenylhydrazine

DTNB- 5, 5'- dithiobis, 2-nitrobenzoic acid

DW- Distilled Water

Exp.- Experiment

Fig.- Figure

FSH- Follicle Stimulating Hormone

g- Gram

GABA- Gamma-aminobutyric acid

GI- Gastrointestinal

GPx- Glutathione Peroxidase

GSH- Reduced Glutathione

H_2O- Water

H_2O_2- Hydrogen Peroxide

H_2SO_4- Sulphuric Acid

HCl- Hydrochloric Acid

HP- Hippocampus

Kg- Kilogram

LD_{50}- Lethal Dose

LH- Luteinizing Hormone

M- Molarity

mg- milligram

min- Minutes

ml- Milliliters

mm- Millimetre

MO- Medulla Oblongata

MO NPs- Metal oxide nanoparticles

N- Normality

NaOH- Sodium Hydroxide

nm- Nanometer

NP- Nanoparticles

NS- Non-significant

O_2- Oxygen

OD- Optical Density

OECD- The Organization for Economic Cooperation and Development

pH- Potential of hydrogen

ROS- Reactive Oxygen Species

SOD- Superoxide dismutases

TBA- Thiobarbituric acid

TCA- Trichloroacetic Acid

ZnO- Zinc oxide

PREFACE

This thesis is submitted in fulfilment of the requirement for the degree of Masters in Zoology and comprises research work carried out by the author under the guidance of Dr. Shamshad Shaikh Assistance Professor of Zoology, Goa University from 2022 to 2023.

Cadmium Oxide Nanoparticles (CdO NPs) are growing in their applicative dynamism. Simultaneously, their exposure to humans and the environment is also rapidly increasing during manufacturing, handling, or disposal. Over and above all, their minute size, smaller than cells and cellular organelles allows them to penetrate the basic biological structures, disrupting their normal function and inducing toxic effects like tissue inflammation and altered cellular redox balance toward oxidation, causing abnormal functions or cell death. Thus, investigations of the CdO NP's effect on *Mus musculus* will provide data about its toxicity that can be further used to generate preventive, measures to reduce CdO NPs exposure in humans.

There are very few studies, undertaken to analyze the toxicity of CdO NPs via oral gavaging. Almost no reports on their toxicity to the Brain functioning in animals exist. This thesis is contributing to the knowledge of the determination of LD_{50} value, investigating their toxicity in the behaviour, anatomy, and brain functioning of *Mus musculus*.

The thesis is divided into five main chapters. The first chapter deals with an introduction giving applications of nanoparticles, background, and sources of cadmium, cadmium absorption into the body (Gastrointestinal tract, Respiratory

system, and Dermal), information on the Central Nervous System (Brain) and *Mus musculus* as an animal model. The 2nd chapter includes a survey of the literature and the aims and objectives of the work.

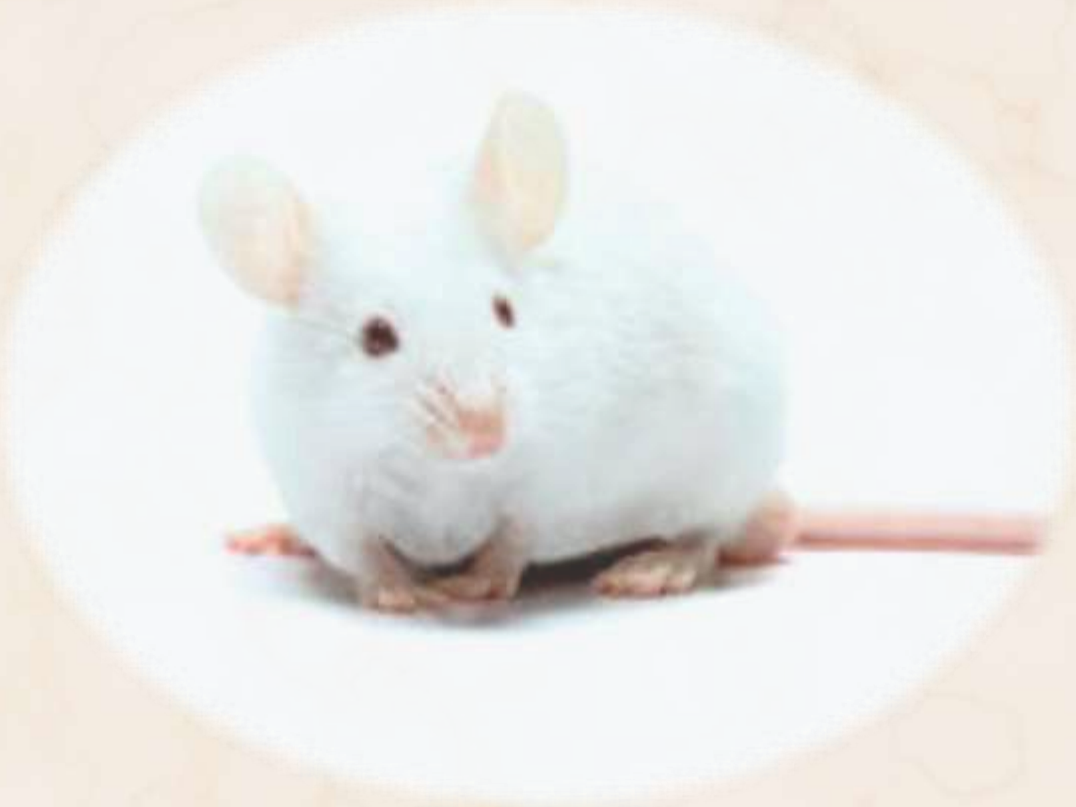
Chapter 3 gives the material and methods used for the study. The exposure and maintenance techniques, histochemical analysis along with different biochemical estimations.

Chapter 4 represents the results embodying observations of animal behaviour, deposition of CdO NPs in the brain, and histological and biochemical changes occurring in the brain regions of *Mus musculus*.

The last chapter, i.e. Chapter 5 gives elaborate discussions. The reasons and the effects of changes occurring in the brain of mice under the influence of CdO NPs are discussed. Conclusion with a summary, future work references, and contributions from the thesis follows chapter 5.

CHAPTER -1

INTRODUCTION



1. INTRODUCTION

Environmental conditions constitute one of the four major determinants of human health, and the air is the medium causing the most direct exposure to harmful substances (Balmuri *et al.*, 2017). Airborne particulate matter can be classified as sedimenting dust which is>10 µm in size, and suspended or fine dust which is 100 nm-10 µm in size and is often called PM10 (Particulate Matter). "Nano-objects" are defined as substances that have one or more external dimensions in the nanoscale (1–100 nm)(Horie & Fujita, 2011). Whereas, a nanoparticle (NP) is defined as a small entity that acts as an entire unit concerning its properties (Srivastava *et al.,* 2015). Currently, research in this area is of intense scientific interest due to its wide potential applications. Many NPs are synthesized in today's world. These NPs are not new to the environment but already exist since the beginning of the Earth (Garcia-Arenas *et al*., 1999).

The entry of NPs into the environment may be anthropogenic or natural. Anthropogenic sources for the entry of NPs into the environment are primarily ore exploitation and secondarily from industrial activities, transport activities, and energy production whereas, natural sources for nanoparticles are forest fires, volcanoes, gas-to-particle conversion, photochemical reactions, soil erosion, plants and animals e.g. shed skin and hair (Hewakuruppu *et al.*, 2013; R. Taylor *et al*., 2013). The chemical composition, type, particle size, coating, charge, and concentration of nanoparticles may all influence their ultimate effect on humans and animals (Dumkova *et al.*, 2016; Sax, 1975). Due to their novel physicochemical properties, they are widely utilized in diverse areas of industry,

including textiles, cosmetics, sunscreens, dietary supplements, medical devices, prescription drugs, and electronics. Since the demand for nanoparticles is increasing, they are being introduced into the environment causing humans to be exposed to NPs at an increasing rate. It is due to these reasons that nanotoxicology is being developed as a novel field to study the potential risk of NPs and their mechanisms of action (Demir *et al*., 2020).

1.1. Applications of Nanoparticles

The most emerging applications of nanotechnology are: Nanomedicine, which includes medical applications of nanomaterials and biological devices, nanoelectronics biosensors, and possible future uses of molecular nanotechnology such as biological machines. Nanobiotechnology means the connection of nanotechnology with biology. It involves developing nanotools for the relevant biological problems e.g. peptide nanosheets (Anajwala *et al*., 2010; Lalwani *et al*., 2013; Zhang *et al*., 2013). Green nanotechnology refers to producing NPs without affecting or harming the environment or human health and producing nano-products to take care of environmental problems. Industries associated with food products and consumer goods like cosmetics, sports products, textiles, surfaces, and coatings could do wonders. Nanoparticles can also be useful for gene and drug delivery in cancer therapy because of their distinct properties (Horie & Fujita, 2011).

1.2. Metal oxide nanoparticles

Metal oxide nanoparticles are produced industrially in large volumes and are an important material in the fields of ecology, and medicine. These NPs have shown fascinating physical and chemical properties i.e., good sensitivity,

catalytic and selective activity, unusual adsorptive behaviour, and superparamagnetic state (D'Amora *et al.*, 2022). In addition to their industrial application, such as catalysts, these are also widely used in the production of sunscreens, cosmetics, Food additives, pharmaceutical products, fabrication of microelectronic circuits, sensors, piezoelectric devices, fuel cells, and coatings for the passivation of surfaces against corrosion (Horie & Fujita, 2011).

Metal oxide nanoparticles present some toxic defects as they enter into the cells and interact with DNA, proteins, and organelles wherein they induce the formation of reactive oxidative species (ROS) and interfere with the antioxidant mechanisms (Institóris *et al.*, 2002; Karami *et al.*, 2008). The increase in the production and accumulation of ROS in cells and tissues leads to oxidative stress and consequently to lipid peroxidation, DNA damage, inflammation, and cell death (D'Amora *et al*., 2022).

1.3. Background and sources of Cadmium

Cadmium (Cd) is a non-essential transition metal (Cd; atomic number 48, atomic weight 112.41) belonging to group XII of the periodic table of chemical elements possessing health risks for both humans and animals. It burns in the air and produces various compounds, such as cadmium oxide, whereas hydrochloric, sulfuric, and nitric acids dissolve cadmium and produce, respectively, cadmium chloride, cadmium sulphate, and cadmium nitrate (Genchi *et al*., 2020).

Recently, Cadmium compounds have also been found to possess qualities as the starting material for manufacturing quantum dots, which are being extensively used in both medical diagnostic imaging and targeted therapeutics (Blum *et al*.,

2015) As a result of the increased production and application of Cadmium NPs, the likelihood of its exposure to humans has increased considerably (Demir *et al.*, 2020). The main environmental outdoor sources of Cd-containing NPs for the general population could be traffic roads, industry, power plants, and other combustion sources while the indoor sources include wall painting, consumer products, and tobacco smoke (Tulinska *et al.*, 2020). Cd is absorbed in high concentrations from water, food, and air contamination. Depending on the level of soil contamination, food that is derived from plants generally contains a higher concentration of Cd than meat, egg, milk, and dairy product (Buzea *et al*., 2007; Oberdörster *et al*., 2005; Peralta-Videa *et al*., 2011). Cd that has been used for electroplating and is also found in industrial paints is hazardous when sprayed. The atmospheric deposition of such airborne cadmium, mining, and use of certain sources of fertilizers that contain cadmium and sewage sludge on farms may lead to soil contamination and increased absorption by crops and vegetables which are then consumed by humans (Genchi *et al*., 2020).

1.4. Cadmium Absorption into the body naturally (through Gastrointestinal tract, Respiratory tract, and Dermis)

The absorption of Cd into the body is mainly via the respiratory tract and to a smaller extent via the gastrointestinal tract, while absorption through the skin is found to be very rare. Once Cd enters the body, it is transported to the bloodstream via erythrocytes and albumin and is then later accumulated in the kidneys liver, and gut (Satarug, 2018; Tinkov *et al*., 2018). Unlike metals like copper (Cu) or zinc (Zn) which are essential micronutrients, Cd has no known biological function in higher organisms and can damage numerous biochemical

pathways, even at low concentrations. It is an extremely toxic element of ongoing concern due to its worldwide anthropogenic mobilization, its diverse toxic effects, extremely long biological half-life (approximately 20–30 years in humans) (Dumkova *et al.*, 2016) low rate of excretion from the body via kidneys, urine, saliva, milk (during lactation)(Tinkov *et al.*, 2018), and storage predominantly in soft tissues such as liver and kidneys (Dumkova *et al*., 2016).

Amounts of Cd consumed by humans are absorbed by the digestive system at a rate of about 5%. Gastrointestinal absorption of Cd in the diet is determined by the content of essential elements (zinc, magnesium, selenium, calcium, and iron), vitamins, polyphenols, antioxidants, and other active biomolecules. Enhanced intake of these elements may prevent the absorption and toxic effects of Cd while deficiency of the same may increase gastrointestinal absorption and accumulation of Cd in the body (Genchi *et al.*, 2020). Furthermore, an increase in a diet containing high fibre can lead to an increase in dietary cadmium intake (Berglund *et al*., 1998). The most important metabolic parameter for the uptake of Cd is lack of iron as it has been seen that people with low iron supplies showed a 6% higher uptake of iron as compared to those with a balanced iron stock (Flanagan *et al*., 1978, Godt *et al.*, 2006).

About 2-50% of Cd in the airborne forms is absorbed from the respiratory tract and this depends primarily on particle size (Papp *et al.*, 2012). The major source of Cd intoxication via inhalation is considered to be cigarette smoke. The human lung reabsorbs about 40-60% of Cd in tobacco smoke (Godt *et al.*, 2006), and smokers are found to have 4-5 times higher levels of Cd in their bloodstream as compared to non-smokers (Berglund *et al*., 1998). Workers that are exposed to

Cadmium-containing fumes develop acute respiratory distress syndromes (ARDS) (Papp *et al*., 2012).

The human skin is exposed to soil and water during daily activities where chemicals from both water and soil, including metals can get into the skin and have the potential to be absorbed into the body (Wester *et al.,* 1992). But there has been little research done on the dermal absorption of cadmium (Godt *et al*., 2006). Two mechanisms facilitate Cd absorption via skin i.e. in epidermal keratins, either free Cd ion binds to cysteine sulfhydryl radicals or Cd promotes and combines with metallothionein.(Godt *et al.,* 2006). Like Cd, its different forms (CdS, CdCl, CdTe, CdO, etc) also gets absorbed in the body via different routes, and one of its important widely used form is CdO (Jr., 1999).

1.5. Application of cadmium oxide NPs

One important metal oxide nanoparticle (NP) widely used in industries is cadmium oxide (CdO). They have been used in the manufacture of batteries, dyes, fire retardants, solar sensors, biosensors, photodiodes, phototransistors, photovoltaic cells, transparent electrodes, liquid crystal displays, infra-red-detectors, nanostructured films, anti-reflection coats, antibacterial age, and as a major ingredient for electroplating baths and pigments (Blum *et al.*, 2015; Genchi *et al.*, 2020; Tulinska *et al.*, 2020). Since, CdO NPs are growing in their applicative dynamism, their exposure to humans and the environment is also increasing rapidly thus effecting different systems of the body like (Respiratory System, Gastrointestinal System, Reproductive System, Circulatory System, etc) including Central Nervous System (Advance, 2010).

1.6. Central Nervous System (Brain)

In all animals, the brain serves as the centre of the nervous system. It is the most complex part of the animal's body (Grose *et al.*, 1987). It has centralized control over the other organs of the body that allow muscle activity, secretion of hormones as well as coordinate responses to changes in the environment (Anderson *et al.*, 2008). Neurotoxicity occurs when neurotoxins (natural or artificial toxic substances) alter the normal activity or functioning of the nervous system causing injury to nervous tissue which can eventually damage, disrupt or kill the neurons that help in transmitting neuronal signals (P. Taylor & Goldman, 2008). Neurotoxicity can occur due to exposure to different substances used during radiation treatment, chemotherapy, certain drug abuse, organ transplants, drug therapies, exposure to heavy metals, certain foods and pesticides, some naturally occurring chemicals, cosmetics, industrial and cleaning solvents, and food additives (Hadley *et al.*, 1979). The symptoms/effects of these exposures may appear immediately or may be delayed (Kakkar & Kaur, 2011; Wu *et al.*, 2013). Similar effects/symptoms of NPs exposure are observed in the brain of humans and mice, because of which mice can be used as an animal model in studies.

1.7. *Mus musculus* as an animal model

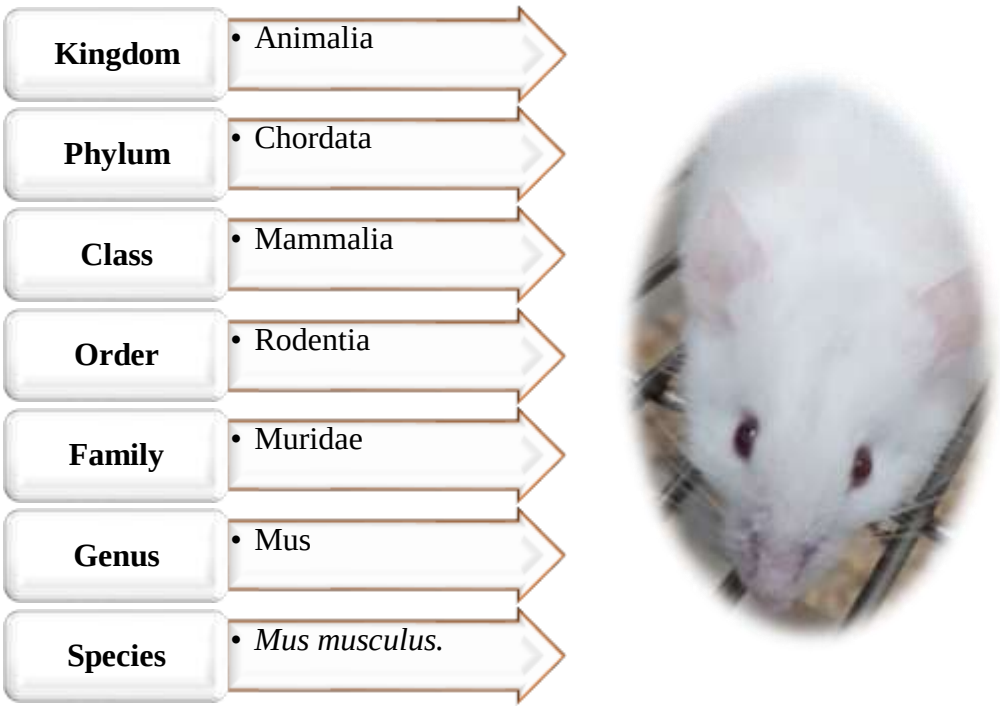
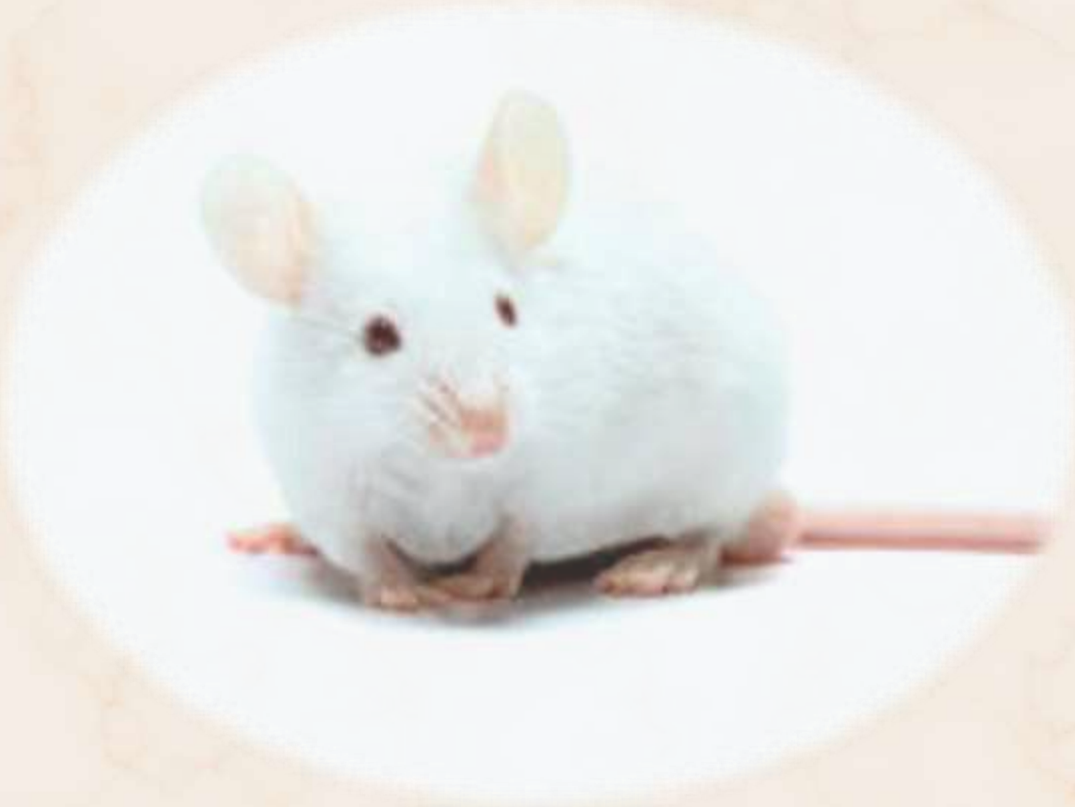


Fig 1. Classification of *Mus musculus*

The mice share many features with humans at the anatomical, cellular, biochemical, and molecular level. Anxiety, hunger, circadian rhythm, aggression, memory, sexual behaviour, and other emotional reactions are additional tasks that the mice brain shares with human brain. In addition, mice are relatively cheap, easily accommodated, and easy to handle, they breed in captivity and the generation time is relatively short; they reach adulthood in approx. 3 months and typically live for about 2 years (Meer, 2005). Because of all the above reasons many studies use mice models to approximate human behavioural responses under physiological and pathological conditions.

CHAPTER 2-

LITERATURE REVIEW



2. LITERATURE REVIEW

A non-essential metal called cadmium has no recognized biological use. When burned in the air, it produces a variety of chemicals, such as cadmium oxide. Cadmium is dissolved in sulfuric, nitric, and hydrochloric acids, forming cadmium chloride, cadmium sulphate, and cadmium nitrate, respectively (Genchi *et al.*, 2020). Ronald, et al (1985) estimated that cadmium has adverse effects on fishes, wildlife, and invertebrates.

2.1. Effect of CdNPs on Aquatic organisms

In a study conducted by Verma et al., (2020), the effect of CdSNPs was studied in the gills of a freshwater fish *Channa punctatus* by exposing the fish to CdSNPs for 15 and 30 days. At the same dosages and exposure times, CdSNPs were found to cause a higher level of lipid peroxidation as determined by thiobarbituric acid reactive chemicals than the CdS bulk particles (Verma *et al*., 2020). Sangeetha et al., (2017) research revealed that CdNP exposure caused parenchymal cells to arc, hepatocytes to degenerate, intrahepatic spaces to enlarge, unique vacuoles to appear, pyknosis and cytoplasmolysis, necrosis in the liver tissues of *C. catla,* while kidney tissues showed severe disorganization of tubules, reduction in the glomerulus, dilation of Bowman's space, degeneration of epithelial cells lining the renal tubules and focal necrosis with haemorrhage among the renal parenchyma .

In a 2014 study, Ladhar et al. investigated the effects of cadmium sulphide nanoparticles (CdSNPs) on Danio rerio zebrafish (Ladhar *et al.*, 2014). The results showed genomic alteration, selected stress response genes were either

repressed or upregulated in tissues. Cadmium accumulation in fish tissues (brain and muscles) was also observed after 60 days of exposure.

2.2. Effect of CdNPs toxicity on Terrestrial organisms

Nguyen et al., (2019) study examined how cadmium telluride quantum dots (CdTe-QDs) were distributed throughout the body and their potential toxicity in male BALB/c mice for up to a week following a single intravenous injection. These CdTe-QDs were detected mostly in the liver, followed by the spleen and kidney. Histological analysis of the tissues showed hepatic hemorrhage and necrotic areas in the spleen when exposed to high doses. While a significant increase in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin levels, as well as a reduction in albumin, was observed (Nguyen *et al.*, 2019).

The main routes of CdO NPs absorption are the respiratory and gastrointestinal (GI) tract and little CdO penetrates through the skin. Usually, the greatest industrial hazard for humans and animals is from pulmonary absorption of CdO. About 10 to 40% of inhaled Cd is retained with the exact amount influenced mainly by mucociliary and alveolar clearance in the lungs.

2.3. Toxicity of CdO NPs administered via Various routes

2.3.1. Intratracheal route

Intratracheal instillation of CdO in the rat showed induction of lung metallothionein (MT) and a slight increase in reduced GSH concentration as well as the activity of glucose-Q phosphate dehydrogenase (GCIPDH). Consequently, the activity of SOD decreased but that of GPx and GR remained

unchanged. These observations suggested that metallothionine played an important role in the detoxification of instilled CdO, whereas, the antioxidant enzymes had a minimal role (Hirano *et al.,* 1990). Cadmium exposure has also been shown to cause behavioural and neurological disorders (Institó Ris *et al.,* 2002). A study conducted by Papp et al., (2012) in which Cd-containing fumes were exposed to Wister rats by subacute intratracheal instillation. Their study showed the spectrum of spontaneous cortical electrical activity shifted to higher frequencies, the latency of sensory-evoked potentials lengthened, and the frequency following ability of the somatosensory evoked potential was impaired even without any detectable Cd deposition in the brain demonstrating the role of Cd NP in causing nervous system damage and showing the possibility of modelling human neurotoxic damage in rats (Papp *et al.,* 2012).

2.3.2. Intraperitoneal route

Only study done on outbred male rats which were repeatedly injected intraperitoneally with either a combination or separately with lead oxide and/or cadmium oxide nanoparticles to study its effects on the contractile characteristics of isolated right ventricle trabeculae and papillary muscles showed that these NPs reduced the mechanical work produced by both types of myocardial muscles (Klinova *et al*., 2021).

2.3.3. Inhalation route

A multitude of research has been done on the toxicity of Cadmium oxide mostly administered through the inhalation route. When metal compounds are inhaled, the penetration into the lung is determined by the particle size and chemical form. In another experimental study on rats, exposure to CdO dust for 90 mins

caused a decrease in the number of macrophages. While a 15-minute exposure to CdO and aluminium microparticles caused the absolute number of alveolar macrophages to decrease at first and later increased (Bouley *et al*., 2021). In 2016, Lebedová et al. documented the gradual uptake and distribution of short and longer-term exposure to CdO NP in mice through inhalation and found a greater portion of NP retained in the lungs while longer exposure led to the redistribution of Cd to kidneys, spleen, liver and at low levels to the brain (Lebedová *et al*., 2016). A study by Takenaka et al., (2004), indicated that inhalation of ultrafine CdO particles resulted in an efficient deposition in rat lungs, and the adverse effects of ultrafine CdO and fine CdO appear to be comparable. In addition, the CdO that reached the deep lung showed to stimulate an inflammatory response where neutrophils and monocytes enter the lungs to ingest inhaled material (Takenaka *et al*., 2004).

A study by Dumkova et al., (2016), reported that inhaled CdO NP in the lungs caused significant alterations in parenchyma tissue including hyperaemia, enlarged pulmonary septa, congested capillaries, alveolar emphysema and small areas of atelectasis. The inhaled NP was shown to cause localised hepatic necrosis and periportal inflammation in the liver, but only mild alterations in the kidney, such as diffusely thickened filtration membrane with intramembranous electron-dense deposits, were seen (Dumkova *et al*., 2016). Furthermore, Tulinska et al., (2020) reported that kidneys from CdO NPs treated mice showed an increase in pro-fibrotic factors TGF-β2 (transforming growth factor-β2), α-SMA (α-smooth muscle actin), and collagen I, but not in TGF-β1 or CTGF (connective tissue growth factor). All these factors are involved in the formation

of renal tubular interstitial fibrosis which can lead to chronic renal failure (Tulinska *et al*., 2020).

Cd also has the potential to affect reproduction and development in many different ways (Thompson *et al*.,2008). Blum et al., (2012), demonstrated that inhalation of CdO during pregnancy decreased the incidence of pregnancy, altered placental weight, and showed a significantly slower body weight gain in neonates exposed to CdO NP (Blum *et al*., 2012). Another study by Blum et al., (2015), also revealed that inhaled CdO NP increased Kim 1 mRNA expression in the kidneys of directly exposed pregnant rats and their new born offspring (Blum *et al*., 2015). In a study by Baraikski (1984), female rats were exposed to CdO aerosols (0.02 and 0.16 mg Cd/m3) which showed a reduction of exploratory motor activity in 3-month-old pups from the 0.16 mg Cd/m^3 group and male offspring from 0.02 mg Cd/m^3 group. The offspring of the female mice exposed to low concentrations of CdO also showed central nervous system dysfunction (Baraikski, 1984). In another study, the effects of aerosols of cadmium chloride and cadmium oxide on pulmonary biochemical function were compared in rats and rabbits. Both compounds after 72h exposure have been shown to cause multifocal, interstitial pneumonitis but the CdO lesion was found to be more severe (Grose *et al*., 1987).

A study done by Hadley et al., (1979) exposed rats to an aerosol of cadmium oxide for a period of one year. The rats have been found to exhibit a significant increase in blood pressure, blood glucose levels, urinary protein, and seminiferous tubule degeneration. Lung tumour was observed among 34 of the rats exposed to CdO (Hadley *et al*., 1979). In a study by Boisset et al., (1978), Young male rats were exposed to CdO-containing particles and sacrificed at

different times in a three-month exposure period. For this period, the growth of the lungs was damaged showing some degree of permanent damage to pulmonary lobes whereas the liver was found to be affected to a lesser extent followed by no effects seen on the growth of kidneys. A total of 12% of the inhaled cadmium was found to be deposited in the lungs and the clearance of pulmonary cadmium was slow, exponential, and monophasic. A slight accumulation of Cd was seen in the liver and kidney as well (Boisset *et al*., 1978).

2.3.4. Oral route

In a study, the toxic effect of metal nanoparticles such as Ag, Cu, ZnO, CdO, and Sn NPs was investigated on the reproductive system in adult male rats. Results have shown a significant elevation of luteinizing hormone by most nanoparticles except AgNPs. While all showed to cause a rise in the follicular stimulating hormone but increase in testosterone was caused only by Cu and Sn NPs (Naser *et al.*, 2020). In another study, done on Wister rats, which were fed with three concentrations of CdO NPs for a period of 10 days via oral gavaging and the results indicated that the enzyme concentration (creatine phosphate kinase, Alanine aminotransferase, and Aspartate transaminase) in the blood increased with increased concentrations of NPs (Masoumeh *et al*., 2017).

From the above literature review, it can be clearly seen that most studies evaluate the effects of CdO NPs administered through inhalation, intratracheal and intraperitoneal routes. While there are limited studies done to evaluate the effects of CdO NPs administered via oral route, which becomes the lacunae that needs to be studied.

2.4. Lacunae

CdO NPs have gained huge popularity in recent times due to their versatility but contradictory toxicity effects are also being researched due to the increase in emission of these nanoparticles in the environment and their exposure to humans. Hence it is essential to gain an in-depth understanding of its effect and consequences. The animal research studies of CdO NPs are far too few concerning intraperitoneal, intratracheal, or inhalation/intranasal exposure, while just a few studies have focussed specifically on oral administration of CdO NPs restricting their work on reproductive toxicity, antioxidant enzymes, and effects on hepatocytes. Much less data are available on both acute and chronic effects of CdO NPs on mice which turns out to be the gap that needs to be explored (Rana *et al*., 2021).

In the present study Cadmium oxide nanoparticles (CdO NP) will be administered in different doses through the oral gavaging exposure method to study the acute effects of CdO NP on mice. Oral gavaging turns out to be one important route of exposure due to contamination of nanoparticles in food or succeeding swallowing or because of hand-to-mouth contact or accidental intake during the manufacture of CdO NP or related products.

In the present study, after administering different doses of CdO NPs through the oral route for 14 days. The effects of CdO NPs will be evaluated in *Mus musculus* to understand and analyze the degree of toxicity caused by these nanoparticles.

2.5. Hypothesis

This study hypotheses that CdO NPs can induce adverse toxic effects on the behaviour, anatomy, and physiology of brain functioning and it will, in turn, affect the normal functioning of the body as a whole.

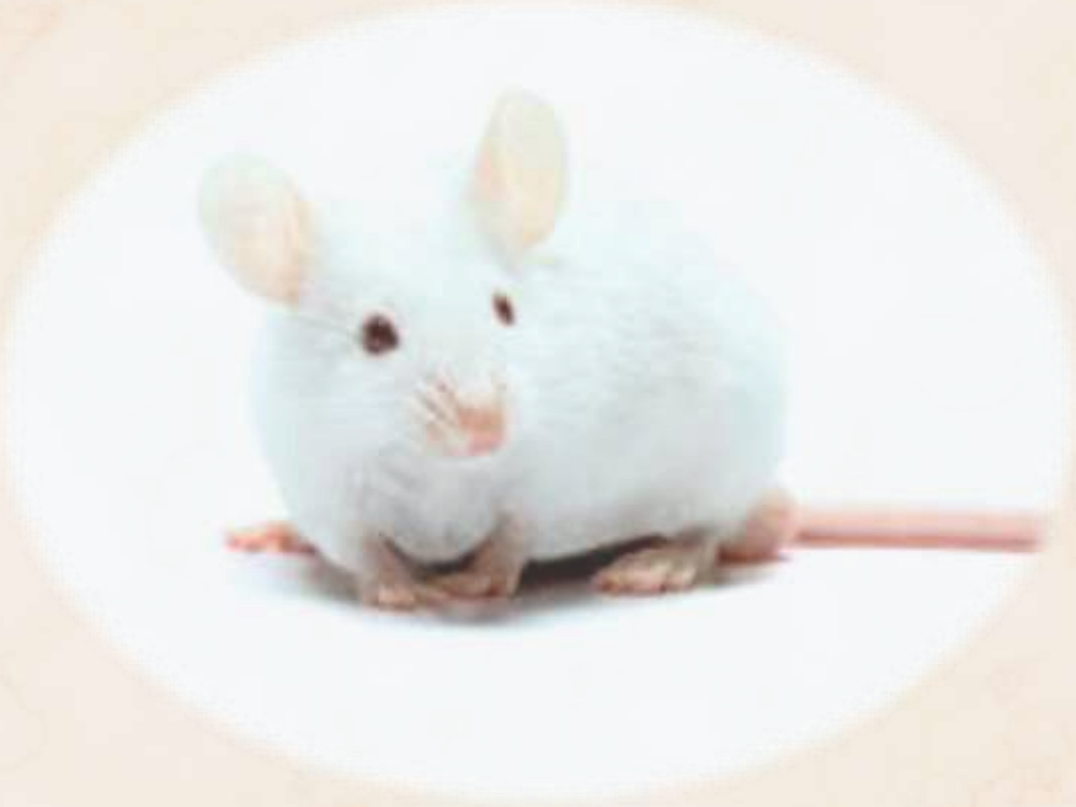
2.6. Objectives of the study

Based on the Lacunae stated, the objectives of the study are:

- To determine the LD_{50} of CdO NPs in *Mus musculus* via oral gavaging.
- Estimating the accumulation/distribution of CdO NPs in the brain regions.
- To analyze the acute effects of CdO on the behavior of *Mus musculus.*
- To evaluate the structural changes in the different regions of the brain.
- To evaluate its effect on brain functioning.

CHAPTER 3-

MATERIALS AND METHOD



3. MATERIALS AND METHODS

3.1. Apparatus and instruments

General laboratory glassware including volumetric flask, measuring cylinders, beakers, pestle and mortar, glass rods, test tubes, plastic and glass reagent bottles, centrifuge tubes, pipette stands, micropipette tips, Eppendorf tubes, and micropipettes were used. All glassware was soaked in 4% chromic acid overnight. All the glassware was rinsed with distilled water and sterilized by keeping it in a hot air oven before use.

Weighing balances, water bath, pH meter, Vortex, Refrigerator, Deep freezer (-20^{o}C), Centrifuge, hot air oven, and Colorimetric spectrophotometer, used in the study are available in the Department of Zoology, Goa University. Microtome for histology was utilized from Ashwini Pathology Lab and Atomic Absorption Spectroscopy was utilized from Ita Lab.

3.2. Chemicals and Reagents

The chemicals used were of Analytical Grade from Thermo-fisher, Sigma-Aldrich, Himedia, etc.

3.3. Procurement of the nanoparticles

The cadmium oxide nanoparticles (CdO NPs) with a molecular weight of 128.41g/mol were obtained from Nanoshel LLC (Product code: NS6130-03-379) and their specifications are as follows:

Appearance-Brown, Red-brown, or Rust

Purity-99.7+%

Grain size (nm)=<100nm

Specific surface area (m2/g)=>90

Loss of weight in drying (%)=<0.3

Loss of weight in burning (%)=<0.2

Trace of metal analysis=≤ 5000 ppm (meets the requirement)

% Cadmium- 85.8-89.3 Wt %

3.4. Biological materials- Animal model (*Mus Musculus)*

Mice are widely used as experimental animals in studies because they share a high degree of anatomical, physiological, and genetic similarity with humans. Furthermore, they are also considered because of their small size, short reproductive cycle and lifespan, low cost, and easy availability (Hickman *et al.*, 2017).

3.5. Procurement of animals

A total of 26 healthy male Swiss Albino Mice (*Mus musculus*), 6-8 weeks of age having an average body weight of 20-28g, were obtained from the National Institute of Biosciences, Pune, India. The necessary ethical approval was acquired from the Animal Ethics Committee of Goa University (Ref no. GUZ/IAEC/22-23/N4) for the use of these animals before the start of the experimentation.

3.6. Maintenance of animals

All 26 Male Swiss Albino Mice (*Mus musculus*), were kept and maintained at the animal house facility of the Department of Zoology, Goa University by

strictly following the CPCSEA guidelines throughout the study period. After the initial acclimatization for a period of one week, the animals were used for experimentation. The mice were housed in polypropylene cages with stainless steel lids, having provision for food and water. They were given standard bedding containing sawdust which was replaced twice a week to maintain hygiene. The animals were maintained at ambient laboratory conditions (Temp = 21±0.6°C, 63±5% humidity, and 12h dark-light cycle) and fed with a commercial diet and tap water provided ad libitum (Tulinska *et al.*, 2020).

3.7. LD_{50} measurement for acute toxicity

Requirements: Male Swiss Albino mice (*Mus musculus*), CdO Nanoparticles, distilled water, syringe, and feeding tubes.

To obtain the LD_{50} for CdO NPs, the experiments along with its intervals were designed according to the method put forth by the Organization for Economic Cooperation and Development (OECD). According to OECD guidelines, the first animal will receive a dose one step below the assumed estimate of the $LD_{50.}$ If the animal survives, the second animal will receive a higher dose. If the first animal dies, the second animal will receive a lower dose (Shaikh *et al.*, 2015). For estimating $LD_{50,}$ a total of 6 mice were used of which 2 were first administered with the initial dose of 50mg/kg, and monitored for their behaviour, motor impairment, and survival. Depending on whether the mice die or survive, the dose administered was either decreased or increased respectively and the LD_{50} was calculated.

Groups	No. of individuals in each group	Doses (mg/kg)	Vehicle of administration
Control	5	0	Distilled water
Experimental 1	5	4.5mg/kg	Distilled water
Experimental 2	5	9mg/kg	Distilled water
Experimental 3	5	18mg/kg	Distilled water
Total	20		

Table 1. Experimental Set Up

3.8. Experimental setup

Once the LD_{50} is estimated, the mice were randomly assigned to 4 groups, each comprising of 7 individuals i.e., 3 experimental and 1 control group. CdO NPs of different concentrations were fed once daily to the mice of the experimental groups by suspending the NPs in distilled water for 14 days (Naser et al., 2020) while the mice in the control group were fed only with distilled water.

3.9. General examination

For a period of 14 days, the mice were routinely examined for their survival, evident behaviour, or motor impairments.

3.9.1. Behaviour

Animals were observed daily for behavioural changes such as rolling of the tail, hyperactivity, drowsiness, overfeeding, isolation, or any other distinct behavioural changes during the period of 14 days.

3.10. Euthanasia

After 14 days of exposure, all the animals were sacrificed by cervical dislocation. The procedure involves the dislocation of the spinal column from the neck/brain by applying pressure.

3.11. Collection of tissue

After sacrificing and dissecting the mice, the brain was removed and washed with physiological saline, and weighed quickly. Followed by separating the brain regions i.e. CC, C, MO, and HP. The tissues used for biochemical estimations were stored at -20^{0}C until further use.

3.12. Histology

Brain regions (CC, C, MO, and HP) for histological examinations were washed with physiological saline, weighed, and stored in 10% formalin. After dehydration with 70% alcohol, the samples were embedded in paraffin wax, and six-micron thick sections were thus prepared and stained with haematoxylin and eosin by Ashwini Pathology Lab. The prepared slides were further analysed under a light microscope.

3.13. Retention of CdO NPs in the Brain

Cadmium concentration in the brain was analysed using atomic absorption spectrophotometry (AAS). After decapitation of mice, the brain tissue was removed and rinsed with mammalian saline and stored at - 4°C until use. The tissue sample were then dried in a clean oven completely at 60- 70° C. Dried tissue was digested using 30% hydrogen peroxide to white ash at 50-60°C, further digested with concentrated 0.1 ml of nitric acid (ultra-pure and trace

metal free). The white ash obtained was then dissolved in 0.25 N nitric acid (1-4 ml) based on the digested tissue weight. Digested sample was then diluted appropriately (1:2-1:50 v/v, depending on the amount and type of tissue) using 0.25 N ultra-pure nitric acid prior to analysis of cadmium using atomic absorption spectrophotometry (Lasagna-Reeves *et al.*, 2010) and then metal concentrations were calculated using the formula as below

% retention = Retention of NPs in tissue/ Retention of NPs in all tissues * 100

3.14. Extraction and estimations of biomolecules

Brain regions were collected and used for biochemical estimations.

3.14.1. Assays for metabolites

3.14.1.1. Total carbohydrates and Free sugars,

10% tissue (brain regions) homogenate in ice-cold water was prepared. The homogenate was deproteinized by adding equal amounts of 0.3 N barium hydroxide and 5% zinc sulphate. After centrifugation at 800 x g for 15 minutes the supernatant was stored for estimation of total carbohydrates and free sugars (Roy *et al*.,)

A. Total carbohydrate*:*

Carbohydrates are dehydrated by conc. H2SO4 to form furfural. Furfural condenses with anthrone to form a blue-coloured complex, which can be measured colorimetrically at 620nm.

0.1m1 of deproteinized aliquot was diluted up to 1.0m1 with distilled water. To this, 4m1 of anthrone reagent was added and incubated for 10 minutes in a boiling water bath. The intensity of the colour developed was measured at

620nm against a suitable blank (Carroll, 1956). Quantification of the total carbohydrate content was done with the help of a standard curve of total carbohydrates (100pg of glucose/ml).

- **Anthrone reagent:** 0.2gm of anthrone was dissolved in 100m1 of concentrated sulphuric acid.

B. Free sugars:

When sugars are heated with alkaline copper reagent it forms a cuprous oxide, which gives blue coloured complex with arsenomolybdate reagent, the intensity of which can be measured at 540 nm.

To 1.0 ml deproteinized sample, 1.0 ml alkaline copper reagent was added and incubated in the boiling water bath for 20 minutes. After cooling to room temperature lml of arsenic-molybdate colour reagent was added. The mixture was diluted with 7 ml of distilled water. The intensity of the colour was read at 540 nm against a suitable blank (Nelson, 1944). Quantification of tissue-free sugar concentration was calculated with the help of a glucose standard curve, prepared by using 200 µg/ml-glucose as a standard solution.

- **Alkaline copper reagent:** A] a)- 12.0g anhydrous sodium carbonate and 6.0g sodium potassium tartarate were dissolved in 125 ml of distilled water. b)- 2.0 g of copper sulphate was dissolved in 25m1 distilled water. Both solutions a and b were mixed, and 8.0g of sodium bicarbonate was added to it by stirring to prepare solution A.

  B] 90.0g of anhydrous sodium sulphate was dissolved in 250 ml of distilled water. Boiled to expel air and then cooled to room temperature to prepare

solution B. Now both the solutions A and B were mixed and the volume was made up to 500m1 with distilled water.

- **Arsenomolybdate colour reagent:** 25.0 g of ammonium molybdate was dissolved in 450 ml of distilled water. 21m1 of concentrated sulphuric acid was added slowly while mixing. To this, 3.0 g disodium hydrogen arsenate (already dissolved in 25m1 of water) was added. Mixed well, and stored in an amber coloured bottle at 37°C for 48 hours.

3.14.1.2. Total Protein, Albumin, and Globulins

Extraction 10% tissue homogenate was prepared using equal amounts of ice-cold distilled water and 10% PCA and centrifuged at 800 x g for 15 minutes. The residue was re-extracted with chloroform: methanol (2:1) followed by diethyl ether. The supernatants were collected after centrifugations at 800 x g for 15 minutes and were pooled together. The pooled supernatant was washed with salt by adding a sufficient 5% sodium chloride solution, keeping it overnight for complete phase separation at refrigerated temperature (Folch et al., 1957). The residue was again treated with 10% perchloric acid at 90°C for 30 minutes and centrifuged. The supernatant was discarded and the residue was dissolved in 1N sodium hydroxide solution which was stored for estimation of protein (Roy et al., 1991).

A. Total Protein:

Total protein Copper from alkaline copper reagent reacts with protein to form a protein complex. Amino acids from the complex react with tungstic acid from the Folin Cio-Calteau reagent, to give a blue colour. The intensity of the blue

colour is directly proportional to the amount of tyrosine and tryptophan present and can be measured at 690nm.

- **Lowry's reagent**-To 98.0m1 of 4% sodium carbonate, l ml of each 2% copper sulphate, and 4% sodium—potassium tartrate was added to make the volume up to 100 ml.

0.1m1 of the tissue extract for protein was diluted up to 0.5 ml with distilled water to which 5 ml of Lowry's reagent was added and incubated for 15 minutes at room temperature. Then 0.5m1 of Folin Cio-Calteau (1:2 dilution) reagent was added and kept for a further incubation period of 30 minutes. The blue-coloured complex intensity was measured against a suitable blank at 690nm (Lowry et al., 1951). Quantification of the protein content of the sample was done with the help of a standard curve of bovine serum albumin (100µg/ml BSA in 1N NaOH).

B. Albumin and Globulins:

At pH 4.1, the Albumin present in the sample binds specifically with bromocresol green to form green coloured complex, the intensity of which was measured colorimetrically by using 640nm.

- **Albumin reagent:** 8.85g succinic acid, 0.108g of bromocresol green, 0.1g of sodium azide, and 4.0 ml of Triton-X 100 were dissolved in 900m1 of distilled water. The pH of this solution was adjusted to 4.1 by using 1N sodium hydroxide. The final volume was made up to l00 ml by using distilled water.

50ul of tissue protein extract was mixed with 5ml of albumin reagent. The mixture was incubated at room temperature for 10 minutes. The intensity of the colour was measured at 640nm against a suitable blank (Godkar, 1994). Albumin present in the samples was quantified with the help of the albumin standard curve (4.0g/dl prepared in 0.1g/dl sodium azide). The calculation of Globulins present in the samples is done by subtracting the albumin values from total proteins.

3.14.2. Assays for antioxidants

3.14.2.1. Thiobarbituric acid reactive substances (TBARS):

Malondialdehyde forms a 1:2 adduct with thiobarbituric acid to produce a coloured complex. The intensity of the colour was measured spectrophotometrically at 535 nm. Other lipoproteins are precipitated out by trichloroacetic acid and avoided from interfering in the reaction. Only water-soluble malondialdehyde reacts with thiobarbituric acid and produces a coloured complex.

- **TBA-TCA-HC1 Reagent:** 0.37% Thiobarbituric acid, 15% Trichloroacetic acid and 0.25(N) Hydrochloric acid were mixed in a 1:1:1 ratio to prepare this reagent.

The brain tissue was homogenized in Tris HCl buffer pH 7.0 to prepare a 2% homogenate. 0.1 ml of tissue homogenate or 10 µl serum was treated with 2m1 of TBA-TCA-HC1 reagent and placed in a boiling water bath for 15 minutes, cool, ed and centrifuged at room temperature for 10 minutes at 500 x g. The absorbance of clear supernatant was measured against a suitable blank at 535 nm (Niehaus and Samuelsson, 1968). Thiobarbituric acid reactive substance

concentration was estimated with the help of a standard curve of malondialdehyde (109mole/m1).

3.14.2.2. Reduced glutathione (GSH):

GSH reacts with 5, 5'- dithiols, and 2-nitrobenzoic acid to produce a yellow-coloured compound. The intensity of the colour can be measured spectrophotometrically at 412nm.

- **5, 5'- dithiols, 2-nitrobenzoic acid (DTNB) reagent:** 19.8mg of 5, 5'- dithiols, 2-nitrobenzoic acid was dissolved in 100m1 of 0.1%sodium nitrate to prepare this reagent.

Tissue was homogenized with 5% TCA to prepare a 2% homogenate and centrifuged at 500 x g for 5 minutes to remove the precipitate. To 1.0 ml of diluted tissue extract or serum, 2 ml of 5, 5'- dithiols, 2-nitrobenzoic acid (DTNB) reagent was added to make the final volume 3.0 nil (Moron et. al., 1979). Absorbance was read at 412nm against a suitable blank. The reduced glutathione content of the samples was quantified with the help of a standard curve of reduced glutathione (0.2mnole/m1 in 5% TCA).

3.14.3. Assays for enzymes

3.14.3.1. Catalase:

The reaction of catalase is the decomposition of hydrogen peroxide into water and molecular oxygen.

2 H202 ----------> 2 H20 + 02

The remaining H202 in the samples was not decomposed by the enzyme. reacts with dichromate to give a blue precipitate of perchromic acid. This unstable

precipitate is then decomposed by heating to give a green-colored stable compound. The intensity of the green color can be measured at 620nm.

Reagents

- **Dichromate acetic acid reagent-** 5% potassium dichromate and glacial acetic acid were taken in a 1: 3 ratios to prepare this reagent.

1.5ml of phosphate buffer (0.01 M, pH 7.0) and 0.4 ml of the substrate (0.2 M H202) were taken and incubated at 37°C for 5 minutes. 0.1 ml phosphate buffer was added to prepare the enzyme blank and 0.1 ml serum or tissue homogenate was added to assay the enzyme activity. This reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 2.0 m1 of dichromate acetic acid reagent. The solution was then heated in a boiling water bath for 10 minutes, mixed well and the intensity of the color was measured against a reference blank at 620 nm (Sinha, 1962). Protein content in the enzyme was estimated as described earlier. The enzyme activity was quantified with the help of a reference curve of hydrogen peroxide (2 µmole/ ml) and expressed as moles of H202 consumed/min/mg protein.

3.14.3.2. Alkaline phosphatase (ALP)

Paranitrophenyl phosphate is colorless in which the enzyme ALP splits off the phosphate group from it to form p-nitrophenol, which is also colorless in the acid medium. Under alkaline conditions, this is converted to p-nitrophenoxide ions, which exhibit yellow color whose intensity is directly proportional to the enzyme present in the sample and can be measured at 405nm.

4-nitrophenyl phosphate + H2O---------- ► Phosphate + 4-Nitrophenolate

Reagents

- **ALP substrate —** 680 mg of p-nitrophenyl phosphate was added to 8.0 mg of MgCl2 solution which was prepared by adding 30mg of MgCl2 to 10 ml glycine buffer.

2.7 ml of glycine buffer was added with 0.2 ml of the substrate (freshly prepared) and incubated at 37°C for 5 minutes. To this 0.1 ml of 5% tissue, the homogenate was added to assay the enzyme activity. An enzyme blank was prepared by mixing 2.7 ml buffer, 0.2 ml substrate, and 0.1m1 of distilled water simultaneously. The reaction mixture was incubated at 37°C for 15 minutes and the reaction was stopped by adding 5 ml of 0.25 N NaOH. The intensity of the products of this reaction (p-nitrophenol) was measured at 405 nm (King and Armstrong, 1934). The amount of the p-nitrophenol released by alkaline phosphatase was quantified via the p-nitrophenol standard curve (250 ug/ml). Protein content in the enzyme was estimated as described earlier. The enzyme activity was expressed as (IU/mg protein). The unit can be defined as the quantity of alkaline phosphatase that liberates 1 mg of phosphate ion from glycerol 2-phosphate in 1 hour under standard conditions.

3.14.4. Neurotransmitters assays

Sample preparation

The brain regions were thawed and homogenized in ice cold water for Glutamate and GABA assay. The homogenization was done in a cold chamber and the samples were stored at -4° C until used for the assay.

3.14.4.1 Glutamate

Reagents:

- Butanol: Acetic acid: Water- 12:3:5
- Ninhydrin reagent: 0.25%
- Copper sulfate solution: 0.005% in 75% ethanol
- Standard glutamate: 0.1g of glutamate in 10ml distilled water

Take two rectangular pieces of approximately 8mm X 4mm dimensions of whatman No 1 chromatography paper. On both paper strips make a line impression approximately 2 cm from one edge of the paper and on the other edge make a rolling fold to hang the paper on the glass rod. Now take one strip and on the impression, line load the first spot with 1µl of brain homogenate sample, also on other, load the spot of standard glutamate solution (1µl). Now hang the strip to a glass rod either by fixing it with cello tape or by rolling the paper. Similarly, do for all. Place the paper strips separately in the beaker containing butanol: acetic acid: water (12:3:5 v/v) as solvent. Remove the strips once the solvent reaches the top of the peper and dry them. After drying paper strips, spray them with ninhydrin reagent and place in an oven at 100^{o}C for 4 minutes. The portions which carry glutamate are cut and eluted with 0.005% $CuSO_4$ (prepared in 75% ethanol). Their absorbance is read against blank at 515 nm in a spectrophotometer.

The concentration of glutamate is calculated by using the following formula

OD (Sample)/ OD (Standard) * Known concentration of standard

3.14.4.2. GABA (Gamma Aminobutyric Acid)

Reagents:

Preparation of stock standard solution of GABA:

- 10mg/ml GABA std: Dissolve 0.1g of GABA in 10ml DW
- Phenol (6%) prepared by dissolving 6ml phenol in 100ml DW
- Sodium hypochlorite (9%) was prepared by adding 9g sodium hypochlorite into 100ml DW.

  Preparation of 0.2M borate buffer pH- 9

- Dissolve 12.37g of boric acid (H_3BO_3) and 14.91g of potassium chloride (KCl) in water and dilute with water to 1000ml
- Prepare a 0.2M NaOH solution: dilute approximately in 1M NaOH solution

  To prepare borate buffer of Ph 9.0, take 20.8ml of 0.2M NaOH and 50ml of 0.2M Boric acid, mix well and use.

  Reaction mixture: 1ml of sample + 1ml of 0.2M borate buffer + 2ml of 6% Phenol reagent + 0.8ml of 9% Sodium hypochlorite, mix the contents and boil for 10 minutes, then cool for 20 minutes or until blue colour appears. The same reaction mixture may be added to the series of standard and 1ml of DW for representing standard and blank tubes. Read the absorbance of the solutions present in the sample and standards on the spectrophotometer at 645nm using blank (Das & Roy, 1997).

CHAPTER 4-

RESULTS

4. RESULTS

4.1. LD_{50}

The LD_{50} of CdO NPs for acute toxicity for a period of 14 days was found to be 90 mg/kg body weight of mice.

4.2. Retention of CdO NPs in the Brain

The mice gavaged with CdO NPs showed a significant ($P<0.0001$) increase in retention of Cd in the brain. The retention was found to be 1.8116±0.1157 mg/kg. The percentage of retention of Cd in the given tissue was found to be 0.5-1% (Table 2).

4.3. Behavioural changes in mice

The animals showed various behavioural changes during 14 days of the CdO NPs exposure period which are as follows

Drowsiness- During the quarantine period all mice were active without any slight sign of drowsiness and this behaviour was constant for 14 days. At the commencement of the exposure period, the control group was active and didn't show drowsiness. The experimental groups showed drowsiness, after 6^{th} day of exposure the Exp. 3 group animals showed moderate drowsiness which constantly increased over the exposure period. Apparently, Exp. Groups 1 and 2 did not show drowsiness in the initial days of exposure but minute drowsiness was observed after the 10^{th} day of exposure. There were no other drastic behavioural changes observed during the exposure period as mentioned in the Table 3.

Cadmium Accumulation	Control	Experimental group (III)
Total Cd intake at the end of 14 days exposure period (mg/kg)	0	252
Total cadmium retention in the tissue- Brain (mg/kg)	0.0081± 0.0007	1.8116±0.1157
Total percentage retention (%)	-	0.5- 1

Table 2: Retention of CdO NPs in the brain

All values are expressed as mean ± SEM, (P<0.0001)

Behavioural Parameters	Control	Experimental 1	Experimental 2	Experimental 3
Rolling of tail	Absent	Absent	Absent	Absent
Hyperactivity	Absent	Absent	Absent	Absent
Drowsiness	Absent	Minute	Minute	Moderate
Overfeeding	Absent	Absent	Absent	Absent
Isolation	Absent	Absent	Absent	Absent

Table 3. Acute effects of CdO NPs on the behaviour of *Mus musculus* at the end of 14 days of exposure.

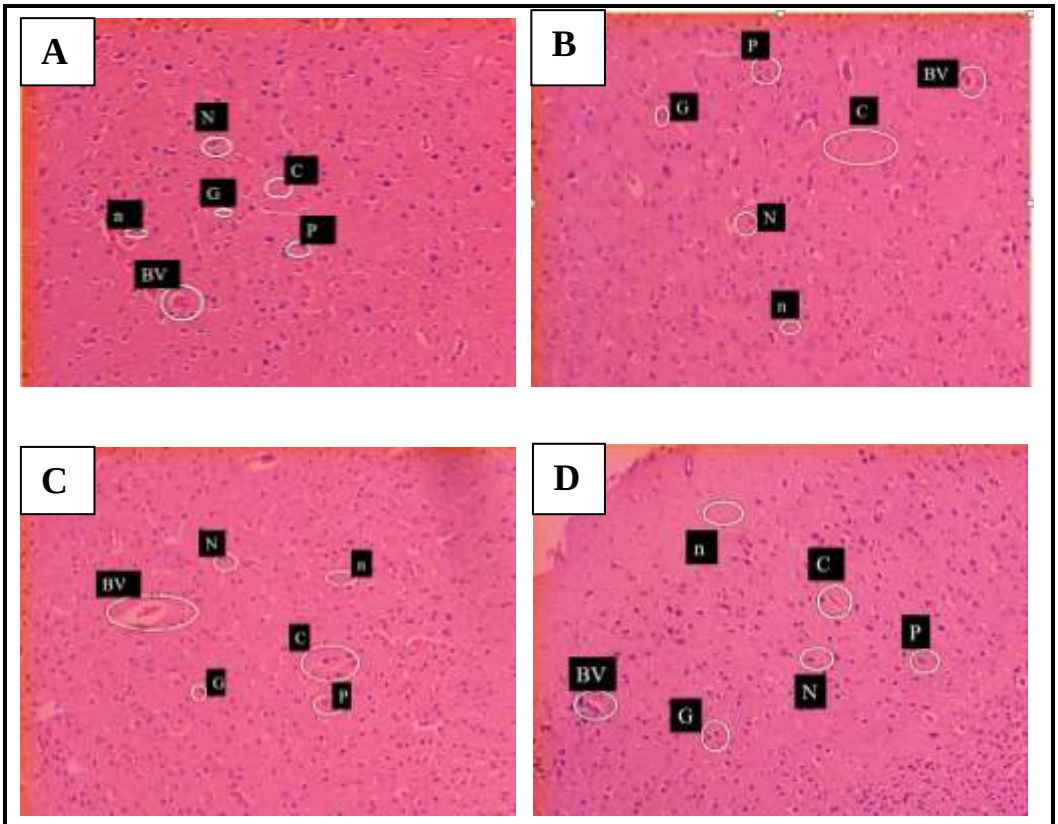


Fig 2- Acute effects CdO NPs on Cerebral cortex **(A)-** Control showing glial cells (G), blood vessel (BV), capillaries (C), Neurons (N), and Pyramidal cells (P). **(B)-** Exp.1- showed degeneration of glial cells (G) and pyramidal cells (P) **(C) –** Exp 2- showed degeneration of glial cells (G), degeneration of pyramidal cells (P), and inflammation of capillaries (C) **(D)-** Exp 3- showed decrease and degeneration of glial cells (G) and pyramidal cells (P), necrosis of neurons (N), degeneration of blood vessels (BV) and inflammation in capillaries (C).

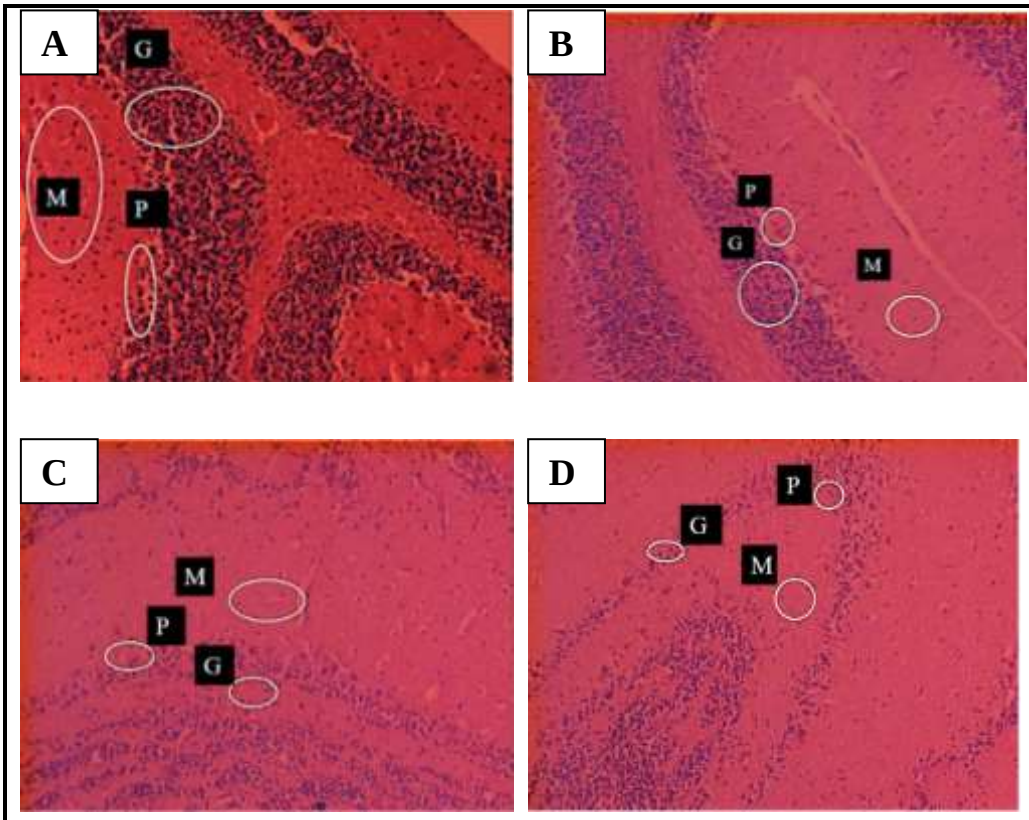


Fig 3- Acute effects of CdO NPs on Cerebellum **(A)-** Control showing granular layer (G), molecular layer (M), and Purkinje cell layer (P) **(B)-** Exp 1- showed a slight decrease in the number of granular cells (G) **(C)-** Exp.2- showed a decrease in the number of granular cells (G) and degeneration and decrease in the number of Purkinje (P) **(D)-** Exp.3- showed degeneration and decrease in the number of molecular cells (M), granular cells (G), and Purkinje cells (P).

4.4. Effect of CdO NPs on the anatomy of Brain regions (Cerebral Cortex, Cerebellum, Medulla Oblongata, and Hippocampus) of *Mus musculus.*

4.4.1. Cerebral Cortex

Under the light microscope, the normal architecture of the cerebral cortex with glial cells, blood vessels, capillaries, and pyramidal cells was observed in control group mice (Fig. 2A). The mice treated with a 4.5mg/kg dose of CdO NPs showed degeneration of glial cells and pyramidal cells (Fig. 2B). The CC of the mice treated with a 9mg/kg dose of CdO NPs showed degeneration of glial cells, degeneration of pyramidal cells, and inflammation of capillaries (Fig. 2C). The CC of the mice treated with an 18mg/kg dose of CdO NPs showed a decrease and degeneration of glial cells and pyramidal cells, necrosis of neurons, degeneration of blood vessels, and inflammation in capillaries. (Fig. 2D)

4.4.2. Cerebellum

Under the light microscope, the normal architecture of mice cerebellum with granular, molecular, and Purkinje layers was observed in control (Fig. 3A). The mice exposed to a 4.5mg/kg dose of CdO NPs showed a slight decrease in the number of granular cells (Fig. 3B). The mice exposed to a 9mg/kg dose of CdO NPs showed a decrease in the number of granular cells and degeneration and a decrease in the number of Purkinje (Fig. 3C). The mice treated with an 18mg/kg dose of CdO NPs showed degeneration and a decrease in the number of molecular cells, granular cells, and Purkinje cells in the C region of the brain (Fig. 3D).

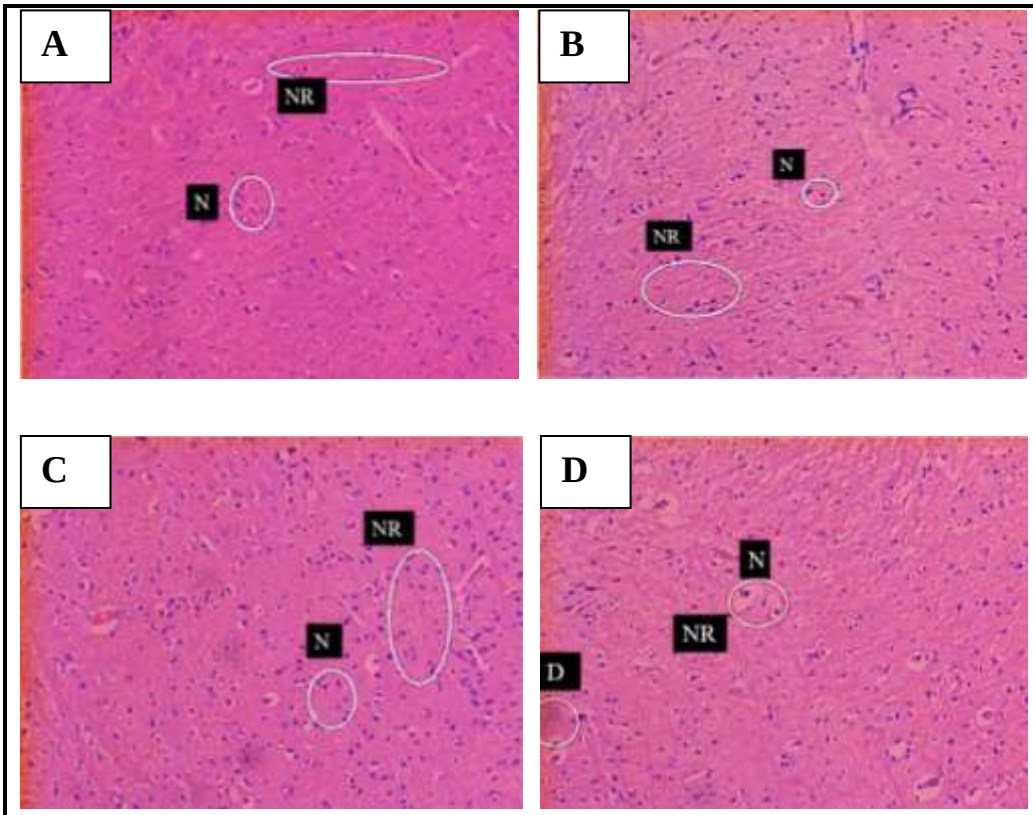


Fig 4- Acute effects of CdO NPs on Medulla oblongata (A)- Control showing nerve rootlets (NR) and nerve cells (N) (B)- Exp. 1- showed degeneration of nerve cells (N), (C)- Exp. 2- showed distortion in the nerve rootlets (NR) and degeneration of nerve cells (N) (D)- Exp. 3- showed distortion in the nerve rootlets (NR), deposition of NPs (D), and necrosis of nerve cells (N).

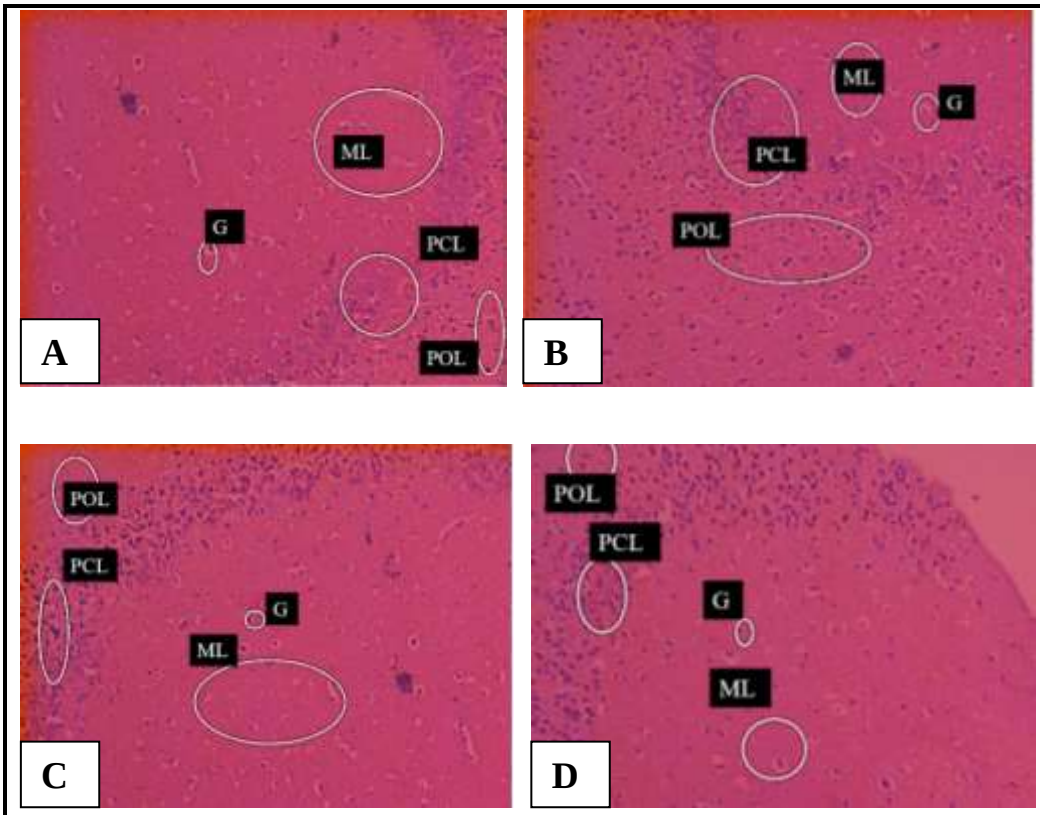


Fig 5- Acute effects of CdO NPs on Hippocampus (A)- Control showing polymorphic layer (POL), pyramidal cell layer (PCL), molecular layer (ML), and glial cells (G) (B)- Exp. 1- showed Disintegration in pyramidal cell layer (PCL), (C)- Exp. 2- showed distortion in the pyramidal cell layer (PCL) and degeneration of glial cells (G) in POL and ML. (D)- Exp. 3- showed distortion in the pyramidal cell layer (PCL), and shrunken glial cells with pyknotic nuclei (G).

4.4.3. Medulla Oblongata

Under the light microscope, the Medulla oblongata of the control group mice with nerve rootlets and nerve cells was observed (Fig 4A). The mice gavaged with a 4.5mg/kg dose of CdO NPs showed degeneration of nerve cells (Fig. 4B). The mice gavaged with a 9mg/kg dose of CdO NPs showed distortion in the nerve rootlets and degeneration of nerve cells (Fig. 4C). The MO of mice exposed to 18mg/kg dose of CdO NPs showed distortion in the nerve rootlets, deposition of NPs, and necrosis of nerve cells (Fig. 4D).

4.4.4. Hippocampus

Under the light microscope, the Hippocampus of the Control group mice with polymorphic layer, pyramidal cell layer, molecular layer, and glial cells was observed (Fig. 5A). The mice exposed to a 4.5mg/kg dose of CdO NPs showed disintegration in the pyramidal cell layer (Fig. 5B), The mice exposed to a 9mg/kg dose of CdO NPs showed distortion in the pyramidal cell layer and degeneration glial cells in POL and ML (Fig. 5C). The HP of mice exposed to an 18mg/kg dose of CdO NPs showed distortion in the pyramidal cell layer and shrunken glial cells with pyknotic nuclei (Fig. 5D).

4.5. Effects of CdO NPs on brain metabolites and Enzymes

The changes in the concentration of different biomolecules and enzymes in mice exposed to CdO NPs for 14 days are revealed in Figures 6 to 13.

The daily oral gavaging of CdO NPs in the mice for 14 days promoted a significant dose-dependent increase in carbohydrates levels in CC, C, MO, and HP ($F= 6.5- 7.5$, $P\leq0.01$) while the most prominent increase was observed in MO (Fig 6). The dosage of CdO NPs brought a significant alteration in the concentration of free sugars in CC, C, MO, and HP ($F=4- 6$, $P\leq0.05$). The variation of free sugar concentration is minor or non-significant in Exp. 1, however, a significant increase is observed in Exp. 3 of all regions due to the higher dose of CdO NPs comparatively (Fig 7).

It is noted that the total protein concentration was significantly decreased in the CC, C, MO, and HP in the CdO NPs exposed mice ($F=6.5- 9.5$, $P\leq 0.05$). The concentration significantly decreased in Exp. 3 compared to the control, although the decline was indistinguishable in both Exp. 2 and 3 of all regions (Fig 8). Similarly, a significant variation was present in albumin levels. The concentration of albumin levels declined significantly in all four regions ($F= 5-10$, $P\leq0.01$) however, a more prominent decrease was observed in CC compared to other regions (Fig 9).

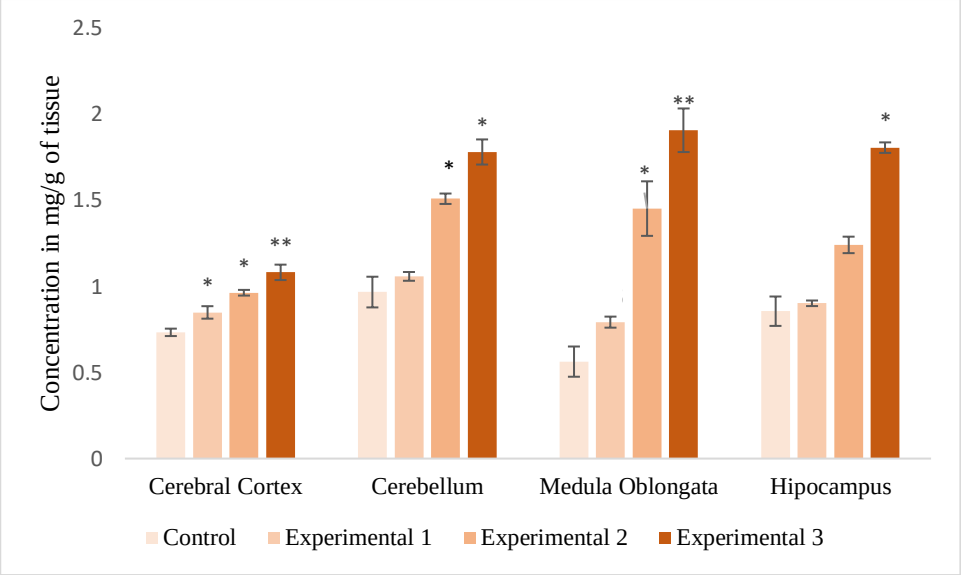


Figure 6- Effects of acute exposure to CdO NPs on the Carbohydrate content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant)

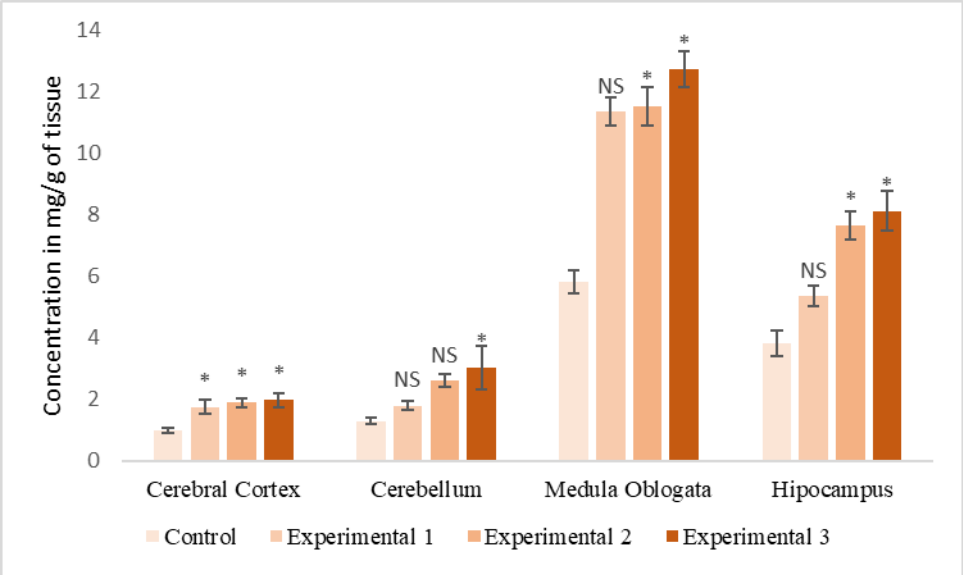


Figure 7- Effects of acute exposure to CdO NPs on the free sugar content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant)

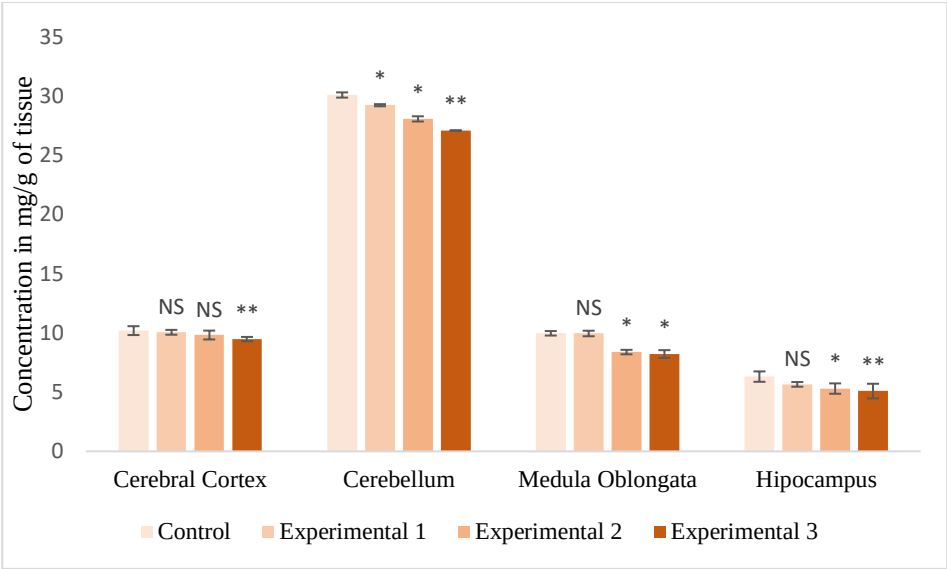


Figure 8- Effects of acute exposure to CdO NPs on the Protein content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant)

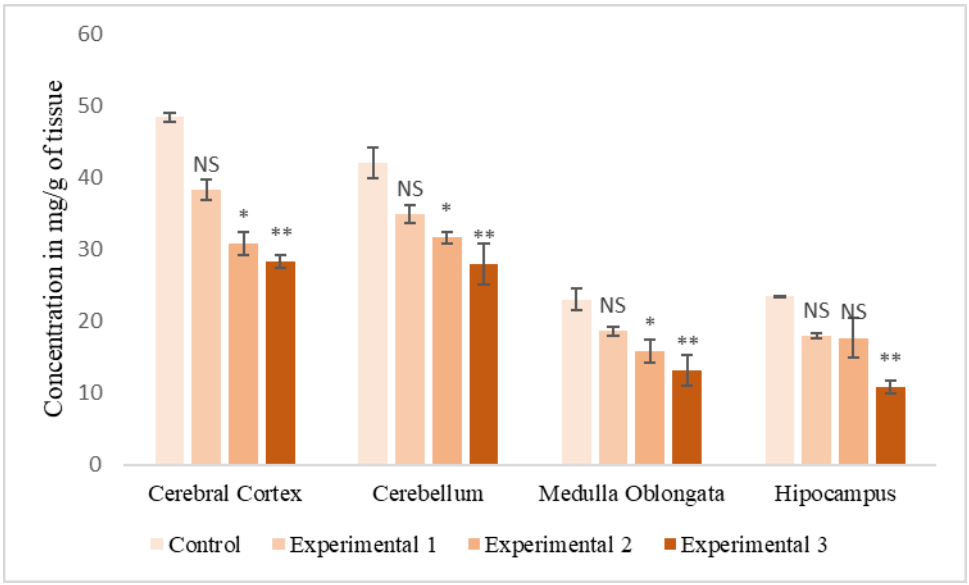


Figure 9- Effects of acute exposure to CdO NPs on the Albumin content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant)

Upon exposure to CdO NPs, the concentration of reduced glutathione significantly decreased in the experimental group (F=6.5- 15, P≤ 0.001). The induction of reduced levels of reduced glutathione was gradual in all the 3 experimental groups of all four regions (Fig 10). Whereas, the concentration of TBARS showed an elevation in the experimental groups compared to the control ones (F=5-11, P≤0.01). However, the increase is indistinguishable in Exp 1 and 2 of all regions (Fig 11).

Catalase activity showed notable changes in the brain regions of mice from different experimental groups. A significant increase (F= 4.5- 12.5, P≤ 0.001) was observed due to the effect of CdO NPs in the exposed mice. The catalase activity showed a significant difference in the Exp. 3 of CC, C, MO, and HP however, moderate differences were observed in all the brain regions of the 3rd Exp group (Fig 12). Whereas ALP activity was noted to be significantly decreasing in CC, C, MO, and HP (F= 5- 9, P<0.01). However, a prominent decline was observed in the Exp 3 of all the brain regions (Fig 13).

4.6. Effects of CdO NPs on brain Neurotransmitters

The mice daily orally administered with CdO NPs for a period of 14 days showed dose-dependent and significant increases in GABA levels in C, CC, MO, and HP (F= 5- 15, P<0.01) (Fig 14). Whereas, the Glutamate levels dropped significantly in C, CC, MO, and HP (F= 3.5- 5.5, P≤ 0.05)(Fig 15).

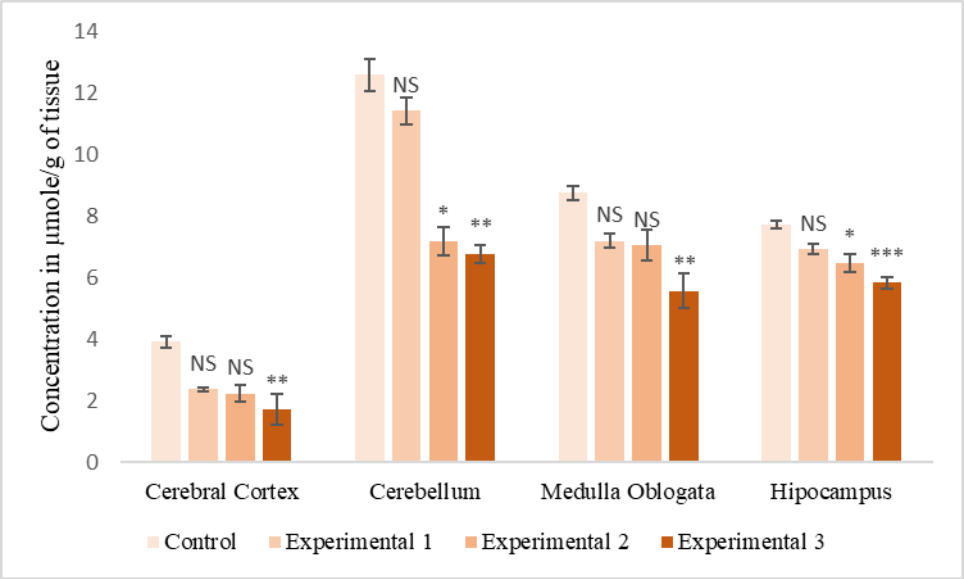


Figure 10- Effects of acute exposure to CdO NPs on the Reduced Glutathione content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant, *** -P≤ 0.001, very highly significant)

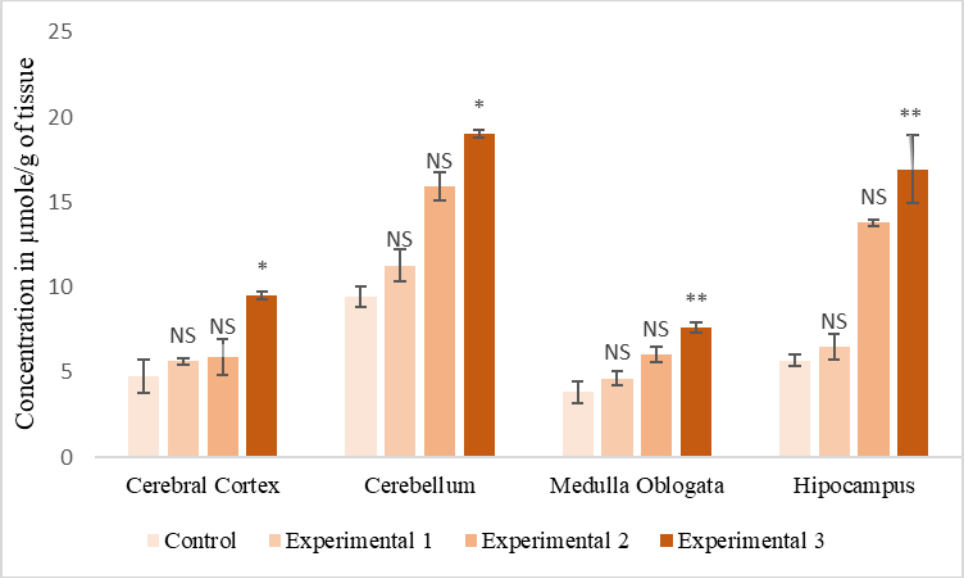


Figure 11- Effects of acute exposure to CdO NPs on the TBARS content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant)

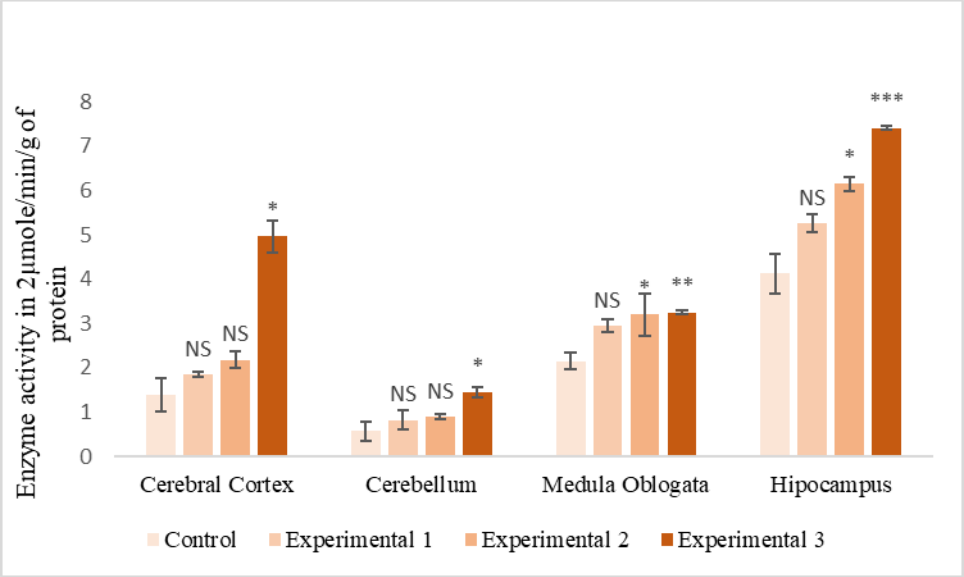


Figure 12- Effects of acute exposure to CdO NPs on the Catalase content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant, *** -P≤ 0.001, very highly significant)

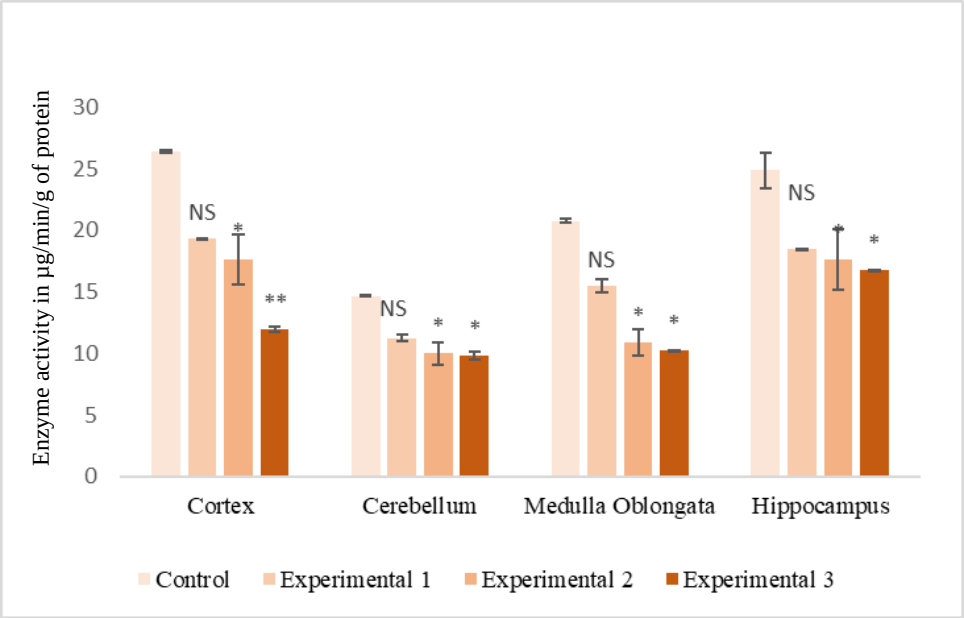


Figure 13- Effects of acute exposure to CdO NPs on the ALP content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant)

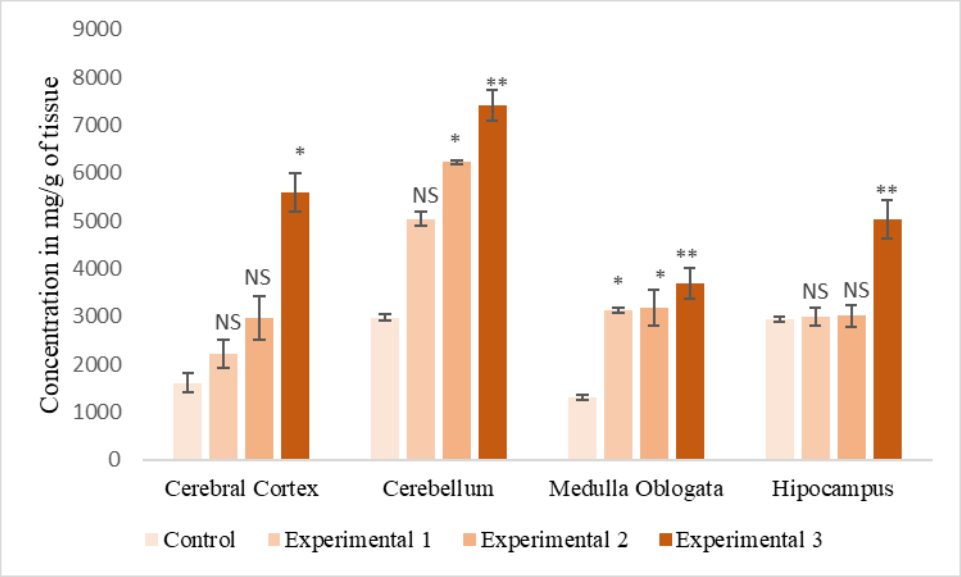


Figure 14- Effects of acute exposure to CdO NPs on the GABA content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant, **-P<0.01, highly significant)

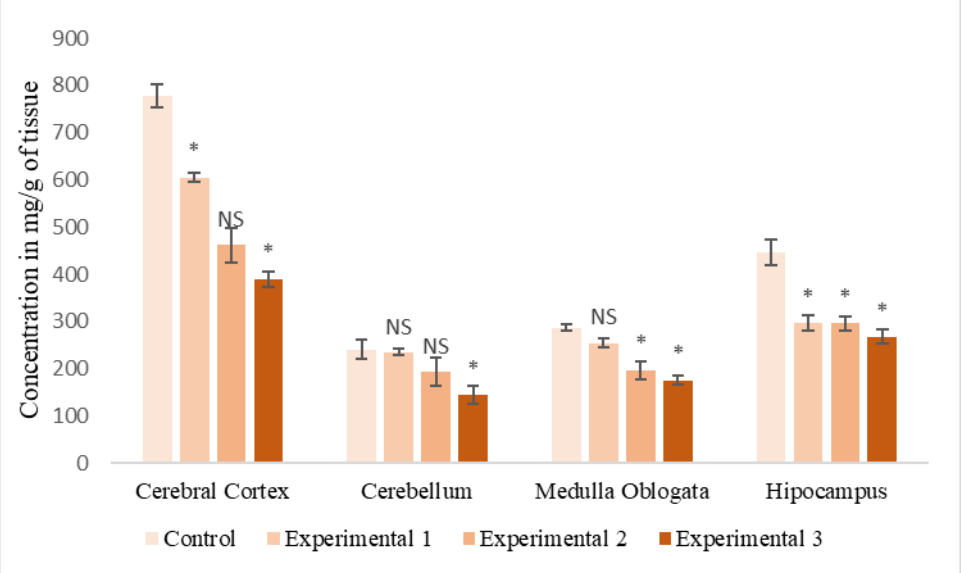
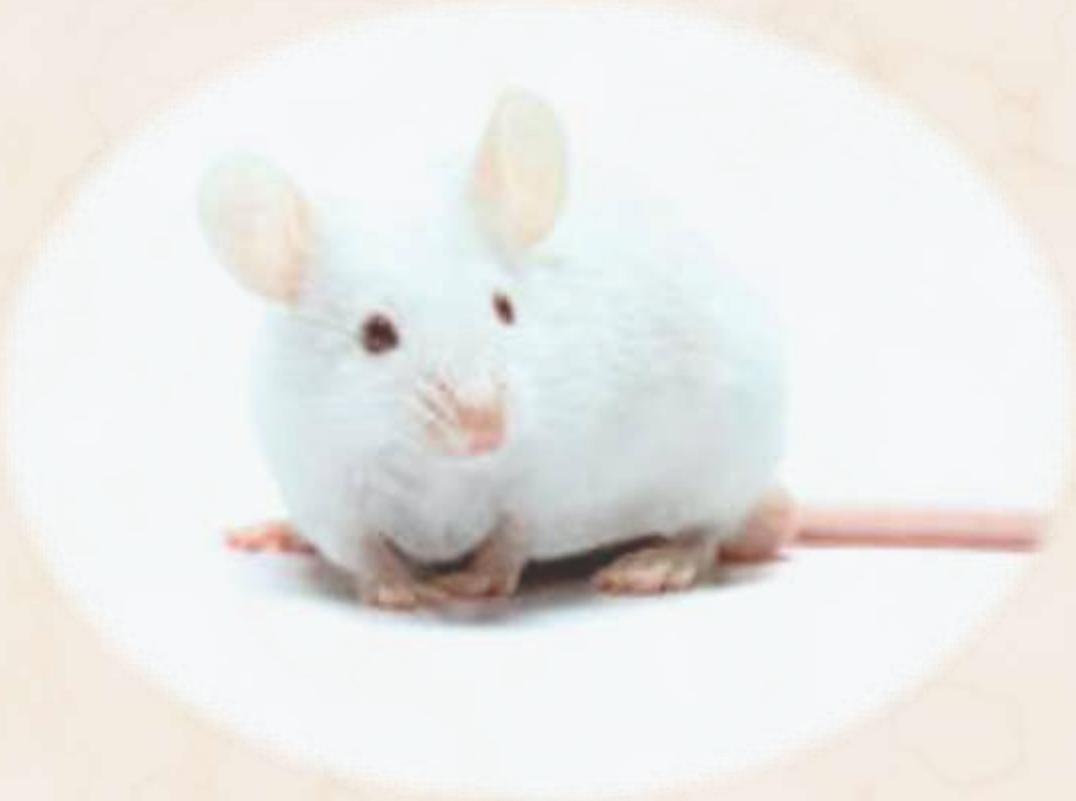


Figure 15- Effects of acute exposure to CdO NPs on the Glutamate content in the brain of mice. (NS- Non significant, *- P≤ 0.05, significant)

CHAPTER 5-

DISCUSSION



5. DISCUSSION

The CdO NPs increase the susceptibility of disorders due to their unique property of forming agglomerates attributed to high interaction between the particles. The results of the present study investigated the toxicity of CdO NPs on different parameters associated with behaviour, anatomy, and Brain functioning. CdO NPs were gavaged through differential doses which resulted in their accumulation determining the evidence of the possible pathogenicity that can occur. Thus, the experiment provides new insight into the relation between CdO NPs and their toxicity on *Mus musculus.*

5.1. LD_{50} of CdO NPs

According to the Hazardous Substances Data Bank, the median lethal dose for Cd is 890 mg/kg (Lewis, 2004). The acute toxicity of CdS nanodots, CdS nanorods, and CdCl2 was analyzed to calculate the LD_{50} values by Liu et al., (2014). The LD50 values for CdS nanodots and nanorods were shown to be 767 and 7203 mg/kg per day, respectively, while CdCl2 had a lower LD50 value of 137 mg/kg per day. Zayed & Philippe, (2009)evaluated that under the experimental conditions, the median oral lethal dose for CdTe was considered to be greater than 2000 mg/kg.

The toxicity of CdO, by exposure through the gastrointestinal tract in the mice, was investigated in the present work. It is hypothesized that various environmental pollutants including NPs might be one of the reasons for causing neurological disorders, but not many experimental pieces of evidence are available to support it (Win-Shwe & Fujimaki, 2011). The LD_{50} of CdO is reported to be 90mg/kg body weight of mice. The LD_{50} value obtained in this work revealed that CdO is moderately toxic according to Hodge and Sterner

scale (1956) but the scale given by Gosselin, Smith, and Hodge (1956) it belongs to class 4 which is very toxic and in Category 3 (Dangerous) according to Globally Harmonized system of classification and labeling of Chemicals (GHS)(Ahmed, 2015; Egorova & Ananikov, 2017; Miyagawa, 2010).

Compared to other Cd forms the LD_{50} of CdO NPs is much less that says lesser amount is enough to cause deleterious changes in an organism this toxicity might be because of its size as the properties of any compound changes with the size of nanoparticles and also the route of exposure is important here as every route will pass through different sequence of organs as a result its deposition and effect will also vary (Naser *et al.,* 2020).

5.2. Retention of CdO NPs in the brain of mice

After acute exposure of mice to CdO it was found that 0.5- 1.0% of the Cd was deposited in the brain. The results indicate that the reason that a few NPs get deposited in the brain is that the NPs can cross the blood-brain barrier (BBB) or the BBB is compromised (Fan *et al*., 2013). After the entry of NPs into the body a very small quantity of NPs is capable of traveling towards the brain and reaching the brain circulation and from that very few particles can cross the BBB and make entry into the brain. Further, as all the brain pieces were free of any blood traces, the entry of NPs into the neural tissue was either by compromising the BBB or through the leaky passages of the blood vessels or the endothelium leading to its proximity to neural tissue and eventual entry into it (Shaikh *et al.,* 2015; Vishwakarma *et al*., 2010).

5.3. Behavioural changes in mice

The mice exposed to the CdO NPs showed behavioural changes like drowsiness. This can be correlated with the changes found in the levels of neurotransmitters, i.e. the level of GABA was found to be increased while levels of Glutamate were decreased after exposing the mice to establish that activation of GABA- A receptor favours sleep (Gottesmann, 2002). Similar kind of results was found in the study done by Stefanie et al (2014) where they exposed rats to manganese for the subacute period and observed that rats showed hypoactivity (O'Neal et al., 2014). There were no other behavioural changes observed in the mice which indicated that these NPs were not able to affect any other behaviour which could be visibly observed or demarcated (Inoue *et al.*, 2014).

5.4. Anatomical changes in the brain of mice

Histological examinations serve as an identifier of the cytoarchitecture of any tissue or organ and also help in determining its normal anatomy and also the changes that have occurred in the infected ones or animals exposed to any toxicant or any other foreign substances. In the present study, different cytoarchitectural changes were observed in the mice brain when exposed to CdO NPs. The observations from the light microscope histopathology of the brain regions demonstrated that this NPs led to various pathological changes like inflammation, necrosis, and vacuolation in the discrete brain regions viz., cerebellum, cerebral cortex, medulla oblongata, and Hippocampus of mice indicating that CdO NPs cause neurotoxicity and can alter the membrane bound enzymes leading to oxidative stress further altering the anatomy, as suggested by Reddy et al., (2007).

The cerebral cortex, which is the biggest site of neuronal integration in the central nervous system, is crucial for the development of consciousness, awareness, memory, thought, perception, and language. The pyramidal cells are present in CC and they are the main excitation units of it. Any injury to these pyramidal cells would affect excitatory units of CC. The degree of damage to the pyramidal cells in the CC would decide the extent of loss of excitation (Elston, 2003; Garcia-Arenas *et al*., 1999). Other cells, i.e. glial cells surround the neurons and hold them in place, also, supply nutrients and oxygen and insulate neurons from each other which avoid short circuiting of the excitatory signals and impulses. They also destroy pathogens and phagocytize dead neurons. Further these also assist neurons to form synaptic connections with each other (Gourine *et al.*, 2010). Therefore, any degeneration or any other changes in these glial cells would affect the above-mentioned functions.

Among other brain activities, the cerebellum is crucial for memory, learning, motor skill acquisition, and motor control. Rodents' cerebellum development continues after birth, with granule neurons maturing as a result. Granular cells are responsible for not only receiving the inputs from the mossy fibers but also are responsible for encoding and interpreting the mossy fiber inputs. This process is actually termed as combinational coding which potentially allows C to make finer distinction between the input patterns for finer and correct interpretations of the inputs (Wolosker *et al.*, 2008). Any loss in the number of granular cells or even their degeneration as well as impairment would obstruct the reception of inputs, encoding of these inputs and interpretation of such inputs. In such events the functions of the C would be affected accordingly (Mescher, 2013). CdO NPs also led to degeneration or shrinkage of purkinje

cells which could interfere with the signals from the cerebellum to higher centers as these purkinje cells are the only efferent neurons of the cerebellum (Gartner, 2007).

The caudal region of the hindbrain is known as the medulla oblongata. It includes brain centres that control visceral functions and the preservation of bodily homeostasis, significant ascending and descending fibre networks, and a number of motor and interneuron populations (Hu *et al.*, 2010). In MO, the injury to the nerve rootlets either to the anterior or posterior row would affect the transmission of nerve impulses from brain to hypoglossal nerves and vice-versa and also would affect the transmission to and from impulses of the accessory nerves, glossopharyngeal nerves and vagus nerves. Such impairment of transmissions would affect the functioning of organs associated with these nerves. The deposition of NPs was also prominent in the histological sections showing the entry of NPs into the brain after crossing the BBB and the entry of NPs in the brain was also confirmed by AAS. (Shaikh *et al.*, 2015).

Hippocampus plays a key role in memory formation and consolidation, so any pathological change to its histo-morphological integrity will have an impact on the brain's normal physiological processes. Findings from histology and histochemistry confirm that CdO NPs causes oxidative stress in the brain. This conclusion was drawn from the observation of cellular clusters with pyknotic nuclei that features in some areas of the hippocampus and the distortion in the glial cells (Omotoso *et al.*, 2019). The pyramidal cells process sensory and motor cues to form a cognitive map encoding spatial, contextual, and emotional information, which they transmit throughout the brain and any changes to these

cells can lead to impairment in its functions (Graves *et al*., 2013). All these pathological changes in the brain could disturb the enzyme activities, neurotransmitters and homeostasis of this NPs directly or indirectly affecting the brain function and causing neurodegenerative diseases.

5.5. Effects of CdO NPs on brain metabolites and Enzymes

The effect of CdO NPs on the biochemical parameters such as metabolites, enzymes, neurotransmitters and antioxidants associated with the brain functioning were analyzed. The studies of acute exposure to NPs are essential to evaluate the effects of a very short-term exposure to NPs or any toxic substances before doing chronic exposure as there are more chances of individual getting a single exposure or short-term exposure accidentally or due to occupation to a lower or a higher dose of toxicants (Pompella *et al*., 2003).

The metabolites and enzymes are very essential for the normal metabolism and proper body functioning. The mice exposed to CdO NPs showed significant dose dependant increase in total carbohydrate and free sugar contents in brain regions. A known fact, that the brain necessitates two times more energy than the other organs; hence the glucose demand by brain could increase due to the stress created by CdO NPs deposition. This would step up carbohydrate metabolism to meet glucose demand by the affected regions of the brain hence increasing the levels of total carbohydrates and other sugar levels to meet the energy demand of the brain (Shaikh *et al*., 2015).

The protein and albumin contents of all the brain regions have declined under the influence of CdO NPs. The histological analyses have shown necrotic damage to the C, CC, MO and HP upon the administration of CdO NPs. The

very presence of pyknotic nuclei clearly suggests the onset of necrosis. During necrosis there is always a breakdown of cytoplasmic organelle, vacuolation of cytoplasm and under such circumstances the cell proteins which are integral part of the cell membranes, cell organelles and cytoplasm break down. The breakdown of proteins viz-a-viz necrosis of brain tissues would result in decrease in protein levels of these tissues. Also, the use of protein as a fuel would decrease the protein levels of the brain tissues. Therefore, the drop in protein level would be a culmination of all the processes/factors mentioned above under the state of acute exposure to CdO NPs. The decreasing tendency of albumin concentration, especially in the higher dose mice groups, can be due to organism's attempt to adapt to changing conditions. The most important region of ions binding by albumin is N-terminal compound's fragment, including a sequence of four amino acids: asparagine-alanine-histidine-lysine. The N-terminal fragment is very sensitive to damages, especially when additionally it is exposed to the influence of free radicals, produced during the oxidative stress, acidosis or decreased oxygen tension. Decrease of albumin level can be due to the influence of free radicals produced during the oxidative stress induced by CdO NPs (Kopańska *et al.*, 2015).

Brain is the most susceptible part of the animal body that undergoes oxidative damage, because it contains rich concentrations of unsaturated fatty acids, that are easily peroxidizable, and has disproportional large consumption of oxygen per unit weight, along with absence of having generous antioxidant defence (Saddick *et al.,* 2017). GSH in a reduced form (Reduced glutathione) is an antioxidant that can scavange free radicals or become a substrate for other antioxidant enzymes, such as glutathione peroxidase and GSH reductase.

During the detoxification process GSH (reduced form) becomes oxidated to glutathione disulfide (GSSG) which is reconverted then to reduced GSH by the enzyme GSH reductase in cells (BarathManiKanth et al., 2010). The ratio of GSH from reduced to oxidized form in the cells is oftenly used to measure the oxidative stress in the cells. The exposure of CdO, NPs led to decrease in the reduced GSH content, thereby possibly leading to the formation of complexes between radical species and cellular proteins or other biomolecules. Whereas the CdO, NPs caused elevation of TBARS which might be causing excessive lipid peroxidation indicating oxidative stress, which can be attributed to the depletion of the antioxidant system which is also proved by decrease in the antioxidant enzyme activities (Shaikh *et al*., 2015).

Antioxidant enzymes like CAT is used as markers for oxidative stress, as it is important in the maintenance of homeostasis for normal cell functioning (El Kholy *et al*., 2021). It metabolizes a toxic by product of metabolic reactions viz., hydrogen peroxide to free oxygen and water. The increase of catalase activity means involvement of reactive oxygen species production in CdO NPs toxicity. The increasing catalase activity because of CdO NPs exposure, suggested its participation in scavenging excess of H_2O_2 (Hussain *et al*., 1999). Whereas CdO NPs also led to decrease in ALP activity which may be due to a decline in the rate of protein synthesis in the brain and electrolytic imbalance caused by tissue after dehydration. ALP is localized in pre and post synaptic membranes of brain and injury to the brain can reduce the ALP activity in general due to damage to the synaptic membranes. Arun et al. (2015) reported acute decrease in ALP of rat brain after brain injury. Further, any damage to the neuronal architecture can

not only promote the rise in ACP but also decline in ALP activity as observed by Ogundele et al. (2010) and Arun et al., (2015).

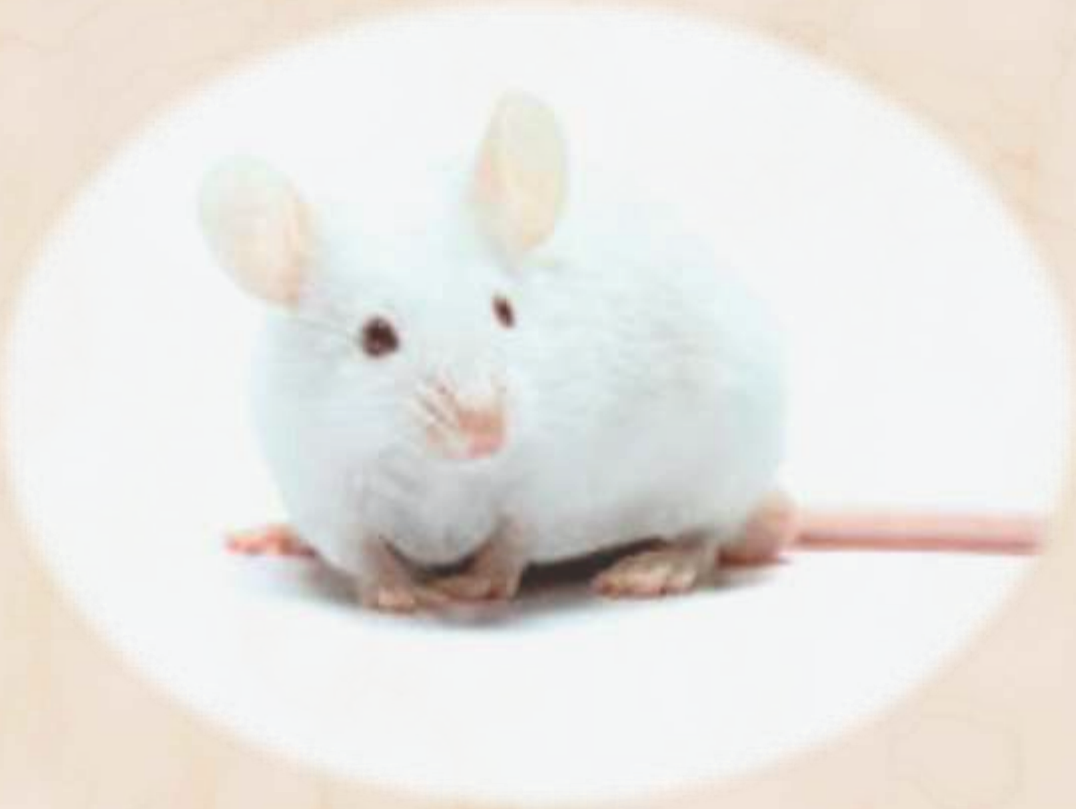
5.6. Effects of CdO NPs on brain Neurotransmitters

Release of neurotransmitters in proper amount is essential for brain signaling within and to the animal body at large. Glutamate is an excitatory neurotransmitter responsible for memory, speech, learning and cognition. The levels of glutamate were decreased when the mice were exposed to CdO NPs. Similar results were found in the study done by Akira et al (2001) during perfusion with cadmium in a dose-dependent manner, suggesting that the release of glutamate into the synaptic clefts is suppressed by inhibitory action of cadmium against the voltage-dependent calcium channels hence decreasing its levels. It is believed that Glutamate deficiency in the brain can cause symptoms like insomnia, concentration problems and mental exhaustion. GABA the so-called inhibitory neurotransmitter is responsible for calming the brain and relaxing the person in its thoughts, processes and speech. The levels of this

inhibitory neurotransmitter was significantly increased with CdO NPs exposure. This might be due to the effective inhibition of CdO NPs against the voltage-dependent calcium channels (Minami *et al.*, 2001).

CHAPTER 6-

SUMMARY



6. SUMMARY

Nanotoxicology is an emerging field of nanoscience dealing with exploring the hazards of NPs. The increased use of NPs in many industrial products poses a potential risk to the environment as well as to the handlers of such products. The main goal of nanotoxicity research is to design rules for the synthesis of safe NPs by providing information on their consequences. Some attempts have been made to synthesize stable NPs and to understand their toxicity in general to organisms and human cells. However, relatively less work has been done in understanding the effective toxicity of such stable NPs to the human brain.

CdO NPs are one of the most applicative NPs contributing to different economic fields from medicine, electronics, automobile, and so on. Due to its increasing demand, its exposure to humans is on a rapid momentum, making its investigations the need of the hour. The follow-up work was to understand the toxicity of CdO NPs on Mus musculus.

The mice were gavaged with CdO NPs using distilled water as a vehicle after determining the LD_{50} value. This helped in deciding the doses for acute exposure of mice. The mice were divided into 4 groups with 1 control group and 3 Exp. Groups. They were subjected to oral gavaging of different concentrations of CdO NPs (4.5mg/kg, 9mg/kg, and 18mg/kg body weight) for 14 days and their behaviour was observed.

After exposure of mice to NPs the brain was aseptically isolated and washed and C, CC, MO, and HP were separated. The brain was used for the assay of the deposition of NPs using AAS and brain regions were used for studying biochemical and histological changes. Under acute exposure to CdO NPs the

Carbohydrates, Free sugars, Proteins, Albumin, Reduced Glutathione, TBARS, and enzymes such as Catalase and ALP were assayed.

Behavioural changes like drowsiness were observed which was constant for 14 days. Also, different anatomical changes relaying the damage caused by CdO NPs in brain regions were seen. The neuronal enzyme activity of Catalase elevated while ALP activity decreased, as well as non-enzymatic antioxidants like Reduced Glutathione increased while TBARS decreased. Different biomolecule concentrations like Carbohydrates and Free sugars levels increased while Protein and Albumin contents showed a drop. The neurotransmitters i.e. GABA increased while Glutamate decreased under the effect of CdO NPs.

The LD_{50} value obtained in this work for the acute period revealed that CdO NPs are moderately toxic with the scale given by Gosselin, Smith, and Hodge (1984). The deposition of CdO NPs in the brain regions suggests compromising BBB and leaky capillaries. Therefore, the root cause of inflammation and neuronal injury affecting the metabolite levels, metabolic enzymes, neurotransmitters, and antioxidants, is the deposition of NPs in the brain tissue.

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CONCLUSION

CdO NPs have adverse effects on brain anatomy, physiology, and behaviour of *Mus musculus.* The oral exposure of these particles via different processes during the manufacturing, handling, and disposal of CdO NPs products can induce toxic effects on humans. They cross the blood-brain barrier and get deposited in the brain hence, promoting neurotoxicity to the brain after acute exposure.

FUTURE PROSPECTS

- The action of CdO NPs on learning and memory.
- Detoxification studies concerning CdO NPs.

CHAPTER 7-

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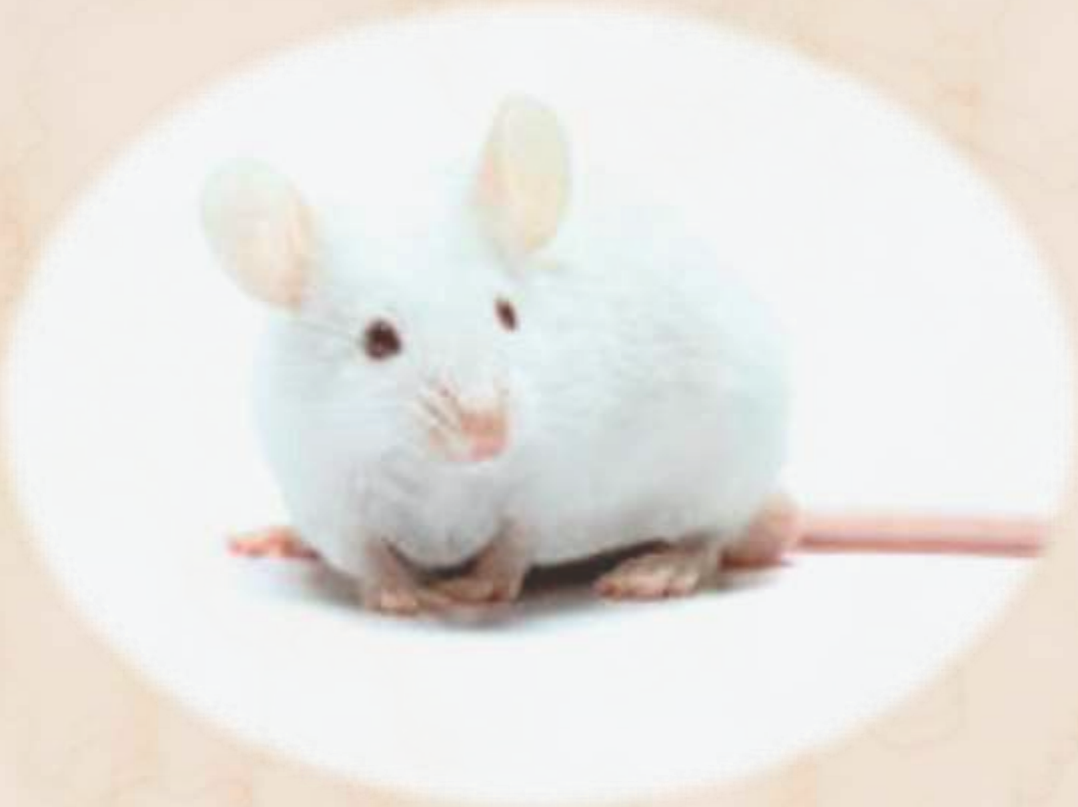

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Environmental conditions constitute one of the four major determinants of human health, and the air is the medium causing the most direct exposure to harmful substances. Airborne particulate matter can be classified as sedimenting dust which is>10 µm in size, and suspended or fine dust which is 100 nm-10 µm in size and is often called PM10 (Particulate Matter). “Nano-objects” are defined as substances that have one or more external dimensions in the nanoscale (1–100 nm)(Horie & Fujita, 2011). Whereas, a nanoparticle (NP) is defined as a small entity that acts as an entire unit concerning its properties. Currently, research in this area is of intense scientific interest due to its wide potential applications. Many NPs are synthesized or engineered in today’s world. These NPs are not new to the environment but already exist since the beginning of the Earth. They are all around us now and have been around us throughout our life history.

The entry of NPs into the environment may be anthropogenic or natural. Anthropogenic sources for the entry of NPs into the environment are primarily ore exploitation and secondarily from industrial activities, transport activities, and energy production whereas, natural sources for nanoparticles are forest fires, volcanoes, gas-to-particle conversion, photochemical reactions, soil erosion, plants and animals e.g. shed skin and hair. The chemical composition, type, particle size, coating, charge, and concentration of nanoparticles may all influence their ultimate effect on humans and animals (Dumkova et al., 2016). Due to their novel physicochemical properties, they are widely utilized in diverse areas of industry, including textiles, cosmetics, sunscreens, dietary supplements, medical devices, prescription drugs, and electronics. Since the demand for nanoparticles is increasing, they are being introduced into the environment causing humans to be exposed to NPs at an increasing rate. It is due to these reasons that nanotoxicology is being developed as a novel field to study the potential risk of NPs and their mechanisms of action (Demir et al., 2020).

1.1. Applications of Nanoparticles

The most emerging applications of nanotechnology are: Nanomedicine-which includes medical applications of nanomaterials and biological devices, nanoelectronics biosensors, and possible future uses of molecular nanotechnology such as biological machines. Nanobiotechnology means the connection of nanotechnology with biology. It involves developing nanotools for the relevant biological problems e.g. peptide nanosheets. Green nanotechnology refers to producing NPs without affecting or harming the environment or human health and producing nano-products to take care of environmental problems. Industries associated with food products and consumer goods like cosmetics, sports products, textiles, surfaces, and coatings could do wonders. Nanoparticles can also be useful for gene and drug delivery in cancer therapy because of their distinct properties (Horie & Fujita, 2011).

1.2. Metal oxide nanoparticles

Metal oxide nanoparticles are produced industrially in large volumes and are an important material in the fields of ecology, and medicine. These NPs have shown fascinating physical and chemical properties i.e., good sensitivity, catalytic and selective activity, unusual adsorptive behaviour, and superparamagnetic state (D’Amora et al., 2022). In addition to their industrial application, such as catalysts, these are also widely used in the production of sunscreens, cosmetics, Food additives, pharmaceutical products, fabrication of microelectronic circuits, sensors, piezoelectric devices, fuel cells, and coatings for the passivation of surfaces against corrosion(Horie & Fujita, 2011).

Metal oxide nanoparticles present some toxic defects as they enter into the cells and interact with DNA, proteins, and organelles wherein they induce the formation of reactive oxidative species (ROS) and interfere with the antioxidant mechanisms. The increase in the production and accumulation of ROS in

cells and tissues leads to oxidative stress and consequently to lipid peroxidation, DNA damage, inflammation, and cell death (D'Amora et al., 2022).

1.3. Background and sources of Cadmium

Cadmium (Cd) is a non-essential transition metal (Cd; atomic number 48, atomic weight 112.41) belonging to group XII of the periodic table of chemical elements possessing health risks for both humans and animals. It forms different compounds when burned in air like, cadmium oxide while, Hydrochloric, sulfuric, and nitric acids dissolve cadmium and form cadmium chloride, cadmium sulphate, and cadmium nitrate, respectively (Genchi et al., 2020).

Recently, Cadmium compounds have also been found to possess qualities as the starting material for manufacturing quantum dots, which are being extensively used in both medical diagnostic imaging and targeted therapeutics (Blum et al., 2015) As a result of the increased production and application of Cadmium NPs, the likelihood of its exposure to humans has increased considerably. The main environmental outdoor sources of Cd-containing NPs for the general population could be traffic roads, industry, power plants, and other combustion sources while the indoor sources include wall painting, consumer products, and tobacco smoke (Tulinska et al., 2020). Cd is absorbed in high concentrations from water, food, and air contamination. Depending on the level of soil contamination, food that is derived from plants generally contains a higher concentration of Cd than meat, egg, milk, and dairy products. Cd that has been used for electroplating and is also found in industrial paints is hazardous when sprayed. The atmospheric deposition of such airborne cadmium, mining, and use of certain sources of fertilizers that contain cadmium and sewage sludge on farms may lead to soil contamination and increased absorption by crops and vegetables which are then consumed by humans (Genchi et al., 2020).

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5. DISCUSSION

The CdO NPs increase the susceptibility of disorders due to their unique property of forming agglomerates attributed to high interaction between the particles. The results of the present study investigated the toxicity of CdO NPs on different parameters associated with behavior, anatomy, and Brain functioning. CdO NPs were gavaged through differential doses which resulted in their accumulation determining the evidence of the possible pathogenicity that can occur. Thus, the experiment provides new insight into the relation between CdO NPs and their toxicity on Mus musculus.

5.1. LD50 of CdO NPs

According to the Hazardous Substances Data Bank, the median lethal dose for Cd is 890 mg/kg (Lewis, 2004). The acute toxicity of CdS nanodots, CdS nanorods, and CdCl2 was analyzed to calculate the LD50 values by Lu Liu et al. (2014)(Liu et al., 2014). Joseph et al (2009) evaluated that under the experimental conditions, the median oral lethal dose for CdTe was considered to be greater than 2000 mg/kg (Zayed & Philippe, 2009).

The toxicity of CdO, by exposure through the gastrointestinal tract in the mice, was investigated in the present work. It is hypothesized that various environmental pollutants including NPs might be one of the reasons for causing neurological disorders, but not many experimental pieces of evidence are available to support it (Win-Shwe & Fujimaki, 2011). The LD50 of CdO is reported to be 90mg/kg body weight of mice. The LD50 value obtained in this work revealed that CdO is moderately toxic according to Hodge and Sterner scale (1956) but the scale given by Gosselin, Smith, and Hodge (1956) it belongs to class 4 which is very toxic and in Category 3 (Dangerous) according to Globally Harmonized system of classification and labeling of Chemicals (GHS)(Ahmed, 2015; Egorova & Ananikov, 2017; Miyagawa, 2010).

Compared to other Cd forms the LD50 of CdO NPs is much less that says lesser amount is enough to cause deleterious changes in an organism this toxicity might be because of its size as the properties of any compound changes with the size of nanoparticles and also the route of exposure is important here as every route will pass through different sequence of organs as a result its deposition and effect will also vary (Naser et al., 2020).

5.2. Deposition of CdO NPs in the brain of mice

After acute exposure of mice to CdO it was found that about 0.7% of the NPs were deposited in the brain. The results indicate that the reason that a few NPs get deposited in the brain is that the NPs can cross the blood-brain barrier (BBB) or the BBB is compromised. After the entry of NPs into the body a very small quantity of NPs is capable of traveling towards the brain and reaching the brain circulation and from that very few particles can cross the BBB and make entry into the brain. Further, as all the brain pieces were free of any blood traces, the entry of NPs into the neural tissue was either by compromising the BBB or through the leaky passages of the blood vessels or the endothelium leading to its proximity to neural tissue and eventual entry into it (Shaikh et al., 2015; Vishwakarma et al., 2010).

5.3. Behavioural changes in mice

The mice exposed to the CdO NPs showed behavioral changes like drowsiness. This can be correlated with the changes found in the levels of neurotransmitters, i.e. the level of GABA was found to be increased while levels of Glutamate were decreased after exposing the mice to establish that activation of GABA- A receptor favors sleep (Gottesmann, 2002). Similar kind of results was found in the study done by Stefanie et al (2014) where they exposed rats to manganese for the subacute period and observed that rats showed hypoactivity (O'Neal et al., 2014). There were no other behavioural changes observed in the mice which indicated that these NPs were not able to affect any other behaviour which could be visibly

observed or demarcated.

5.4. Anatomical changes in the brain of mice

Histological examinations serve as an identifier of the cytoarchitecture of any tissue or organ and also help in determining its normal anatomy and also the changes that have occurred in the infected ones or animals exposed to any toxicant or any other foreign substances. In the present study, different cytoarchitectural changes were observed in the mice brain when exposed to CdO NPs. The observations from the light microscope histopathology of the brain regions demonstrated that this NPs led to various pathological changes like inflammation, necrosis, and vacuolation in the discrete brain regions viz., cerebellum, cerebral cortex, medulla oblongata, and Hippocampus of mice indicating that CdO NPs cause neurotoxicity and can alter the membrane bound enzymes leading to oxidative stress further altering the anatomy, as suggested by Reddy et al. (2007) (Reddy et al., 2007).

The cerebral cortex is the largest site of neural integration in the central nervous system and it plays a key role in attention, perception, awareness, thought, memory, language, and consciousness. The pyramidal cells are present in CC and they are the main excitation units of it. Any injury to these pyramidal cells would affect excitatory units of CC. The degree of damage to the pyramidal cells in the CC would decide the extent of loss of excitation (Elston, 2003; Garcia-Arenas et al., 1999). Other cells, ie. glial cells surround the neurons and hold them in place, also, supply nutrients and oxygen and insulate neurons from each other which avoid short circuiting of the excitatory signals and impulses. They also destroy pathogens and phagocytize dead neurons. Further these also assist neurons to form synaptic connections with each other(Gourine et al., 2010). Therefore, any degeneration or any other changes in these glial cells would affect the above-mentioned functions.

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Among other brain activities, the cerebellum is crucial for memory, learning, motor skill acquisition, and motor control. Rodents' cerebellum development continues after birth, with granule neurons maturing as a result. Granular cells are responsible for not only receiving the inputs from the mossy fibers but also are responsible for encoding and interpreting the mossy fiber inputs. This process is actually termed as combinational coding which potentially allows C to make finer distinction between the input patterns for finer and correct interpretations of the inputs. Any loss in the number of granular cells or even their degeneration as well as impairment would obstruct the reception of inputs, encoding of these inputs and interpretation of such inputs. In such events the functions of the C would be affected accordingly (Mescher, 2013). CdO NPs also led to degeneration or shrinkage of purkinje cells which could interfere with the signals from the cerebellum to higher centers as these purkinje cells are the only efferent neurons of the cerebellum (Gartner, 2007).

The caudal region of the hindbrain is known as the medulla oblongata. It includes brain centres that control visceral functions and the preservation of bodily homeostasis, significant ascending and descending fibre networks, and a number of motor and interneuron populations. In MO, the injury to the nerve rootlets either to the anterior or posterior row would affect the transmission of nerve impulses from brain to hypoglossal nerves and vice-versa and also would affect the transmission of to and from impulses of the accessory nerves, glossopharyngeal nerves and vagus nerves. Such impairment of transmissions would affect the functioning of organs associated with these nerves. The deposition of NPs was also prominent in the histological sections showing the entry of NPs into the brain after crossing the BBB and the entry of NPs in the brain was also confirmed by AAS. (Shaikh et al., 2015). Hippocampus plays a key role in memory formation and consolidation, so any pathological change to its histo-morphological integrity will have an impact on the brain's normal physiological processes. Findings from histology and histochemistry confirm that CdO NPs causes oxidative stress in the brain. This conclusion was drawn from the observation of cellular clusters with pyknotic nuclei that features in some areas of the hippocampus and the distortion in the glial cells (Omotoso et al., 2019). The pyramidal cells process sensory and motor cues to form a cognitive map encoding spatial, contextual, and emotional information, which they transmit throughout the brain and any changes to these cells can lead to impairment in its functions (Graves et al., 2013). All these pathological changes in the brain could disturb the enzyme activities, neurotransmitters and homeostasis of this NPs directly or indirectly affecting the brain function and causing neurodegenerative diseases.

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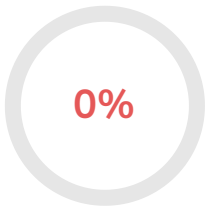

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Total Characters:	**6248**
Plagiarized Sentences:	**0**
Unique Sentences:	**44 (100%)**

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5.5. Effects of CdO NPs on brain metabolites and Enzymes

The effect of CdO NPs on the biochemical parameters such as metabolites, enzymes, neurotransmitters and antioxidants associated with the brain functioning were analyzed. The studies of acute exposure to NPs are essential to evaluate the effects of a very short-term exposure to NPs or any toxic substances before doing chronic exposure as there are more chances of individual getting a single exposure or short-term exposure accidentally or due to occupation to a lower or a higher dose of toxicants.

The metabolites and enzymes are very essential for the normal metabolism and proper body functioning. The mice exposed to CdO NPs showed significant dose dependant increase in total carbohydrate and free sugar contents in brain regions. A known fact, that the brain necessitates two times more energy than the other organs; hence the glucose demand by brain could increase due to the stress created by CdO NPs deposition. This would step up carbohydrate metabolism to meet glucose demand by the affected regions of the brain hence increasing the levels of total carbohydrates and other sugar levels to meet the energy demand of the brain (Shaikh et al., 2015).

The protein and albumin contents of all the brain regions have declined under the influence of CdO NPs. The histological analyses have shown necrotic damage to the C, CC, MO and HP upon the administration of CdO NPs. The very presence of pyknotic nuclei clearly suggests the onset of necrosis. During necrosis there is always a breakdown of cytoplasmic organelle, vacuolation of cytoplasm and under such circumstances the cell proteins which are integral part of the cell membranes, cell organelles and cytoplasm break down. The breakdown of proteins viz-a-viz necrosis of brain tissues would result in decrease in protein levels of these tissues. Also, the use of protein as a fuel would decrease the protein levels of the brain tissues. Therefore, the drop in protein level would be a culmination of all the processes/factors mentioned above under the state of acute exposure to CdO NPs . The decreasing tendency of albumin concentration, especially in the higher dose mice groups, can be due to organism’s attempt to adapt to changing conditions. The most important region of ions binding by albumin is N-terminal compound’s fragment, including a sequence of four amino acids: asparagine-alanine-histidine-lysine. The N-terminal fragment is very sensitive to damages, especially when additionally it is exposed to the influence of free radicals, produced during the oxidative stress, acidosis or decreased oxygen tension. Decrease of albumin level can be due to the influence of free radicals produced during the oxidative stress induced by CdO NPs (Kopańska et al., 2015).

Brain is the most susceptible part of the animal body that undergoes oxidative damage, because it contains rich concentrations of unsaturated fatty acids, that are easily peroxidizable, and has disproportional large consumption of oxygen per unit weight, along with absence of having generous antioxidant defense (Saddick et al., 2017). GSH in a reduced form (Reduced glutathione) is an antioxidant that can scavange free radicals or become a substrate for other antioxidant enzymes, such as glutathione peroxidase and GSH reductase. During the detoxification process GSH (reduced form) becomes oxidated to glutathione disulfide (GSSG) which is reconverted then to reduced GSH by the enzyme GSH reductase in cells (BarathManiKanth et al., 2010). The ratio of GSH from reduced to oxidized form in the cells is oftenly used to measure the oxidative stress in the cells. The exposure of CdO, NPs led to decrease in the reduced GSH content, thereby possibly leading to the formation of complexes between radical species and cellular proteins or other biomolecules. Whereas the CdO, NPs caused elevation of TBARS which might be causing excessive lipid peroxidation indicating oxidative stress, which can be attributed to the depletion of the antioxidant system which is also proved by decrease in the antioxidant enzyme activities (Shaikh et al., 2015).

Antioxidant enzymes like CAT is used as markers for oxidative stress, as it is important in the maintenance of homeostasis for normal cell functioning (El Kholy et al., 2021). It metabolizes a toxic by product of metabolic reactions viz., hydrogen peroxide to free oxygen and water. The increase of catalase activity means involvement of reactive oxygen species production in CdO NPs toxicity. The increasing catalase activity because of CdO NPs exposure, suggested its participation in scavenging excess of H2O2(Hussain et al., 1999). Whereas CdO NPs also led to decrease in ALP activity which may be due to a decline in the rate of protein synthesis in the brain and electrolytic imbalance caused by tissue after dehydration. ALP is localized in pre and post synaptic membranes of brain and injury to the brain can reduce the ALP activity in general due to damage to the synaptic membranes. Arun et al. (2015) reported acute decrease in ALP of rat brain after brain injury. Further, any damage to the neuronal architecture can not only promote the rise in ACP but also decline in ALP activity as observed by Ogundele et al. (2010) and Arun et al., (2015).

5.6. Effects of CdO NPs on brain Neurotransmitters

Release of neurotransmitters in proper amount is essential for brain signaling within and to the animal body at large. Glutamate is an excitatory neurotransmitter responsible for memory, speech, learning and cognition. The levels of glutamate were decreased when the mice were exposed to CdO NPs. Similar results were found in the study done by Akira et al (2001) during perfusion with cadmium in a dose-dependent manner, suggesting that the release of glutamate into the synaptic clefts is suppressed by inhibitory action of cadmium against the voltage-dependent calcium channels hence decreasing its levels. It is believed that Glutamate deficiency in the brain can cause symptoms like insomnia, concentration problems and mental exhaustion. GABA the so-called inhibitory neurotransmitter is responsible for calming the brain and relaxing the person in its thoughts, processes and speech.

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1.4. Application of cadmium oxide

One important metal oxide nanoparticle (NP) widely used in industries is cadmium oxide (CdO). They have long been used in the manufacture of batteries, dyes, fire retardants, solar sensors, biosensors, photodiodes, phototransistors, photovoltaic cells, transparent electrodes, liquid crystal displays, infra-red-detectors, nanostructured films, anti-reflection coats, antibacterial age, and as a major ingredient for electroplating baths and pigments (Blum et al., 2015; Genchi et al., 2020; Tulinska et al., 2020)

1.5. Cadmium resorption into the body (Gastrointestinal tract, Respiratory system, and Dermal)

The absorption of Cd into the body is mainly via the respiratory tract and to a smaller extent via the gastrointestinal tract, while absorption through the skin is found to be very rare. Once Cd enters the body, it is transported to the bloodstream via erythrocytes and albumin and is then later accumulated in the kidneys liver, and gut (Satarug, 2018; Tinkov et al., 2018). Unlike metals like copper (Cu) or zinc (Zn) which are essential micronutrients, Cd has no known biological function in higher organisms and can damage numerous biochemical pathways, even at low concentrations. It is an extremely toxic element of ongoing concern due to its worldwide anthropogenic mobilization, its diverse toxic effects, extremely long biological half-life (approximately 20–30 years in humans) (Dumkova et al., 2016) low rate of excretion from the body via kidneys, urine, saliva, milk (during lactation)(Tinkov et al., 2018), and storage predominantly in soft tissues such as liver and kidneys (Dumkova et al., 2016).

Amounts of Cd consumed by humans are absorbed by the digestive system at a rate of about 5%. Gastrointestinal absorption of Cd in the diet is determined by the content of essential elements (zinc, magnesium, selenium, calcium, and iron), vitamins, polyphenols, antioxidants, and other active biomolecules. Enhanced intake of these elements may prevent the absorption and toxic effects of Cd while deficiency of the same may increase gastrointestinal absorption and accumulation of Cd in the body (Genchi et al., 2020). Furthermore, an increase in a diet containing high fibre can lead to an increase in dietary cadmium intake (Berglund et al., 1998). The most important metabolic parameter for the uptake of Cd is lack of iron as it has been seen that people with low iron supplies showed a 6% higher uptake of iron as compared to those with a balanced iron stock (Flanagan et al., 1978). This is the main reason for the higher uptake of Cd in anaemic individuals (Godt et al., 2006).

About 2-50% of Cd in the airborne forms is absorbed from the respiratory tract and this depends primarily on particle size (Papp et al., 2012). The major source of Cd intoxication via inhalation is considered to be cigarette smoke. The human lung reabsorbs about 40-60% of Cd in tobacco smoke (Godt et al., 2006), and smokers are found to have 4-5 times higher levels of Cd in their bloodstream as compared to non-smokers (Berglund et al., 1998). Workers that are exposed to Cadmium-containing fumes develop acute respiratory distress syndromes (ARDS) (Papp et al., 2012).

The human skin is exposed to soil and water during daily activities where chemicals from both water and soil, including metals can get into the skin and have the potential to be absorbed into the body (Wester et al., 1992). But there has been little research done on the dermal absorption of cadmium (Godt et al., 2006). Two mechanisms facilitate Cd absorption via skin i.e. in epidermal keratins, either free Cd ion binds to cysteine sulfhydryl radicals or Cd promotes and combines with metallothionein. (Godt et al., 2006).

1.6. Central Nervous System (Brain)

In all animals, the brain serves as the centre of the nervous system. It is the most complex part of the animal’s body. It has centralized control over the other organs of the body that allow muscle activity, secretion of hormones as well as coordinate responses to changes in the environment. Neurotoxicity

occurs when neurotoxins (natural or artificial toxic substances) alter the normal activity or functioning of the nervous system causing injury to nervous tissue which can eventually damage, disrupt or kill the neurons that help in transmitting neuronal signals. Neurotoxicity can occur due to exposure to different substances used during radiation treatment, chemotherapy, certain drug abuse, organ transplants, drug therapies, exposure to heavy metals, certain foods and pesticides, some naturally occurring chemicals, cosmetics, industrial and cleaning solvents, and food additives. The symptoms/effects of these exposures may appear immediately or may be delayed(Kakkar & Kaur, 2011; Wu et al., 2013).

1.7. Mus musculus as an animal model

Fig 1. Classification of Mus musculus

The mice share many features with humans at the anatomical, cellular, biochemical, and molecular level. Anxiety, hunger, circadian rhythm, aggression, memory, sexual behaviour, and other emotional reactions are additional tasks that the brain shares with other organs. Because of all the above reasons many studies use mice models to approximate human behavioural responses under physiological and pathological conditions. In addition, mice are relatively cheap, easily accommodated, and easy to handle, they breed in captivity and the generation time is relatively short; they reach adulthood in approx. in 3 months, and typically they live for 2 years (Meer, 2005).

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2. LITERATURE REVIEW

A non-essential metal called cadmium has no recognised biological use.. It burns in the air and produces various compounds, such as cadmium oxide, whereas hydrochloric, sulfuric, and nitric acids dissolve cadmium and produce, respectively, cadmium chloride, cadmium sulphate, and cadmium nitrate.Genchi et al., 2020). Ronald, et al (1985) estimated that cadmium has adverse effects on fishes, wildlife and invertebrates.

2.1. Effect of CdNPs on aquatic organisms

In a study conducted by Verma et al., (2020), the effect of CdSNPs was studied in the gills of a freshwater fish Channa punctatus by exposing the fish to CdSNPs for 15 and 30 days. At the same doses and exposure times, CdSNPs were found to cause a higher level of lipid peroxidation as determined by thiobarbituric acid reactive chemicals than the CdS bulk particles.(Verma et al., 2020). Another study by Sangeetha et al. (2017) revealed that CdNP-exposed liver tissues in C. catla displayed parenchymal cells arc, hepatocyte degeneration, enlarged intrahepatic spaces, unique vacuoles, and pyknosis and cytoplasmolysis,and necrosis among other findings. While the epithelial cells lining the renal tubules degenerated, the glomerulus shrank, Bowman's gap widened, and there was localised necrosis with bleeding in the kidney tissues.(Sangeetha et al., 2017).

2.2. Effect of Cd NPs toxicity to higher organisms

The tissue distribution and toxic effects of cadmium telluride quantum dots (CdTe-QDs) in male BALB/c mice were examined in a study by Nguyen et al., (2019) via single-dose intravenous injections. These CdTe-QDs were detected mostly in the liver, followed by the spleen and kidney. Histological analysis of the tissues showed hepatic hemorrhage and necrotic areas in the spleen when exposed to high doses. While a significant increase in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin levels, as well as a reduction in albumin, was observed (Nguyen et al., 2019).

The main routes of CdO NPs absorption are the respiratory and gastrointestinal (GI) tract and little CdO penetrates through the skin. Usually, the greatest industrial hazard for humans and animals is from pulmonary absorption of CdO. About 10 to 40% of inhaled Cd is retained with the exact amount influenced mainly by mucociliary and alveolar clearance in the lungs.

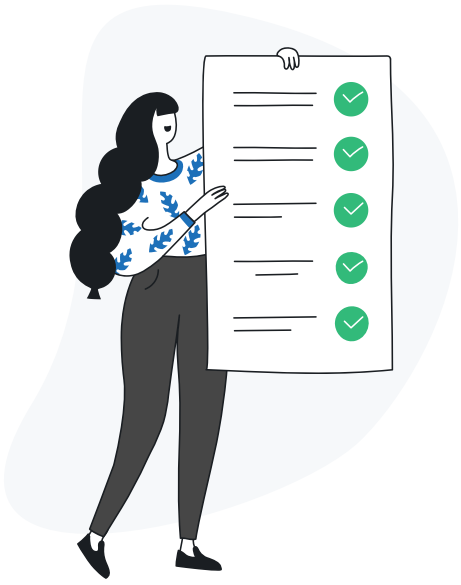
2.3. Toxicity of CdO NPs via intratracheal route

Intratracheal instillation of CdO in the rat showed induction of lung metallothionein (MT) and a slight increase in reduced GSH concentration as well as the activity of glucose-Q phosphate dehydrogenase (GCIPDH). Consequently, the activity of SOD decreased but that of GPx and GR remained unchanged .According to these findings, antioxidant enzymes performed a much less role in the detoxification of implanted CdO than did metallothionine (Hirano et al., 1990). Cadmium exposure has also been shown to cause behavioural and neurological disorders (Institó Ris et al., 2002). A study conducted by Papp et al., (2012) in which Cd-containing fumes were exposed to Wister rats by subacute intratracheal instillation. Their study showed the spectrum of spontaneous cortical electrical activity shifted to higher frequencies, the latency of sensory-evoked potentials lengthened, and the frequency following ability of the somatosensory evoked potential was impaired even without any detectable Cd deposition in the brain demonstrating the role of Cd NP in causing nervous system damage and showing the possibility of modelling human neurotoxic damage in rats (Papp et al., 2012).

2.4. Toxicity of CdO NPs via the intraperitoneal route

Only study done on outbred male rats which were repeatedly injected intraperitoneally with either a

combination or separately with lead oxide and/or cadmium oxide nanoparticles to study its effects on the contractile characteristics of isolated right ventricle trabeculae and papillary muscles showed that these NPs reduced the mechanical work produced by both types of myocardial muscles (Klinova et al., 2021).



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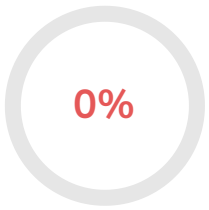

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2.5. Toxicity of CdO NPs via the inhalation route

A multitude of research has been done on the toxicity of Cadmium oxide mostly administered through the inhalation route. When metal compounds are inhaled, the penetration into the lung is determined by the particle size and chemical form. In another experimental study on rats, exposure to CdO dust for 90 mins caused a decrease in the number of macrophages. While a 15-minute exposure to CdO and aluminum microparticles caused the absolute number of alveolar macrophages to decrease at first and later increase (Bouley et al., 1977). In 2016, Lebedová et al. documented the gradual uptake and distribution of short and longer-term exposure to CdO NP in mice through inhalation and found a greater portion of NP retained in the lungs while longer exposure led to the redistribution of Cd to kidneys, spleen, liver and at low levels to the brain (Lebedová et al., 2016). A study by Takenaka et al., (2004), indicated that inhalation of ultrafine CdO particles resulted in an efficient deposition in rat lungs, and the adverse effects of ultrafine CdO and fine CdO appear to be comparable.

A study by Dumkova et al., (2016), reported that inhaled CdO NP in the lungs caused significant alterations in parenchyma tissue including hyperaemia, enlarged pulmonary septa, congested capillaries, alveolar emphysema and small areas of atelectasis. The inhaled NP was found to cause periportal inflammation and local hepatic necrosis in the liver while only minor changes such as diffusely thickened filtration membrane with intramembranous electron-dense deposits were observed in the kidney (Dumkova et al., 2016). Furthermore, Tulinska et al., (2020) reported that kidneys from CdO NPs treated mice showed an increase in pro-fibrotic factors TGF-β2 (transforming growth factor-β2), α-SMA (α-smooth muscle actin), and collagen I, but not in TGF-β1 or CTGF (connective tissue growth factor). All these factors are involved in the formation of renal tubular interstitial fibrosis which can lead to chronic renal failure(Tulinska et al., 2020).

Cd also has the potential to affect reproduction and development in many different ways (Thompson et al.,2008). Blum et al., (2012), demonstrated that inhalation of CdO during pregnancy decreased the incidence of pregnancy, altered placental weight, and showed a significantly slower body weight gain in neonates exposed to CdO NP (Blum et al., 2012). Another study by Blum et al., (2015), also revealed that inhaled CdO NP increased Kim 1 mRNA expression in the kidneys of directly exposed pregnant rats and their new born offspring (Blum et al., 2015). In a study by Baraikski, (1984), female rats were exposed to CdO aerosols (0.02 and 0.16 mg Cd/m3) which showed a reduction of exploratory motor activity in 3-month-old pups from the 0.16 mg Cd/m3 group and male offspring from 0.02 mg Cd/m3 group. The offspring of the female mice exposed to low concentrations of CdO also showed central nervous system dysfunction (Baraikski, 1984). Both compounds after 72h exposure have been shown to cause multifocal, interstitial pneumonitis but the CdO lesion was found to be more severe (Grose et al., 1987).

A study done by Hadley et al., (1979) exposed rats to an aerosol of cadmium oxide for a period of one year. The rats have been found to exhibit a significant increase in blood pressure, blood glucose levels, urinary protein, and seminiferous tubule degeneration. Lung tumour was observed among 34 of the rats exposed to CdO (Hadley et al., 1979). In a study by Boisset et al., (1978), Young male rats were exposed to CdO-containing particles and sacrificed at different times in a three-month exposure period. For this period, the growth of the lungs was damaged showing some degree of permanent damage to pulmonary lobes whereas the liver was found to be affected to a lesser extent followed by no effects seen on the growth of kidneys. A total of 12% of the inhaled cadmium was found to be deposited in the lungs and the clearance of pulmonary cadmium was slow, exponential, and monophasic. A slight

accumulation of Cd was seen in the liver and kidney as well (Boisset et al., 1978).

2.6. Toxicity of CdO NPs via the oral route

In a study, the toxic effect of metal nanoparticles such as Ag, Cu, ZnO, CdO, and Sn NPs was investigated on the reproductive system in adult male rats. Results have shown a significant elevation of luteinizing hormone by most nanoparticles except AgNPs. While all showed to cause a rise in the follicular stimulating hormone but increase in testosterone was caused only by Cu and Sn NPs (Naser et al., 2020). In another study, done on Wister rats, which were fed with three concentrations of CdO NPs for a period of 10 days via oral gavaging and the results indicated that the enzyme concentration (creatine phosphate kinase, Alanine aminotransferase, and Aspartate transaminase) in the blood increased with increased concentrations of NPs (Masoumeh et al., 2017).

In the present study Cadmium oxide nanoparticles (CdO NP) will be administered in different doses through the oral gavaging exposure method to study the sub-chronic effects of CdO NP on mice. Oral gavaging turns out to be one important route of exposure due to contamination of nanoparticles in food or succeeding swallowing or because of hand-to-mouth contact or accidental intake during the manufacture of CdO NP or related products.

In the present study, CdO NPs will be administered in different doses through the oral route for 14 days. The effects of CdO NPs will be evaluated in Mus musculus to understand and analyze the degree of toxicity caused by these nanoparticles.