SHORT-TERM EFFECT OF CADMIUM OXIDE NANOPARTICLES ON THE LIVER AND KIDNEY OF *Mus musculus*

Dissertation submitted

To

GOA UNIVERSITY

In partial fulfillment of the degree of

Masters of Science in Zoology

BY

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M.Sc. Zoology

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2022-2023

Short-term effect of Cadmium Oxide nanoparticles on the liver and kidney of *Mus musculus*

A Dissertation for
Course code and Course Title: ZOO 438 Dissertation
Credits: 8
Submitted in partial fulfilment of Masters Degree
M.Sc. in Zoology
by

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21P044022

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation / Internship report entitled, "**Short-term effect of Cadmium oxide nanoparticles on the liver and kidney 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 of 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 be 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 **"Short-term effect of Cadmium Oxide nanoparticles on the liver and kidney of *Mus musculus*"** is a bonafide work carried out by **Ms. Anouska Mascarenhas** under my supervision in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in the Zoology Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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DEDICATION

I would like to dedicate my dissertation work wholeheartedly to my family, who have always been a source of inspiration, and strength and have continually provided their moral, spiritual, emotional, and financial support

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ACKNOWLEDGMENT

This dissertation work became a reality with many individuals' kind support and help. I would like to extend my sincere thanks to all of them.

Foremost, I would like to offer this endeavor to our God Almighty for the wisdom he has bestowed upon me, the strength, peace of my mind, and good health in order to finish this research.

I would first like to express my gratitude to my project guide Dr. Shamshad Bi M. Shaikh for her support, advice, guidance, valuable comments, and constant encouragement. Her kind and moral support was a catalyst for this project's completion.

I am also thankful to Dr. Savita S. Kerkar, Dean, SBSB for the facilities equipped to make this project smoothly feasible.

I also express my gratitude to Dr. Nitin S. Sawant, Programme Director of Zoology discipline for his keen trust in providing the laboratory, and animal house facilities to conduct the work and his encouragement to bring out the best of my ability.

I'm deeply grateful to Dr. Shanti N. Dessai, Dr. Minal D. Shirodkar, Dr. Avelyno H. D'costa, and Ms. Gandhita V. Kundaikar, Dr. Preeti Pereira Assistant

professors of the Zoology Department, for their endless support and priceless suggestions offered during the entire tenure of my project.

I would also like to express my sincere gratitude to the laboratory staff and non-teaching staff for their patient cooperation in providing all the chemicals and equipments that were needed for my study.

I am affectionately thankful to all my friends, Neeha Borker, Melvita Alvares, Riya Gawas and Feazel Dias, Caren Noronha, and Sarita Rebello who have been my constant support, providing me the strength to deal with all the adversities that came along my way during this project.

I would also like to thank Ashwini Pathology Lab for providing me with histological slides and Ita Lab for providing me with atomic absorption spectrometry.

Most importantly, I would like to express my deepest gratitude to my parents, for patiently dealing with my tantrums, understanding me, and encouraging me with their unconditional love, time, and moral support.

Anouska Mascarenhas

24th April 2023

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ABBREVIATIONS

µm- micrometer

µmole- micromole

µl- microliter

ALP- Alkaline Phosphatase

ALT- Alanine Transaminase

ANOVA- Analysis of Variance

AST- Aspartate amino transferase

ARDS- Acute Respiratory Distress Syndromes

AgNPs- Silver Nanoparticles.

AAS- Atomic Absorption Spectrometry

Cd- Cadmium

Cu- Copper

CAT- Catalase activity

CdO NPs- Cadmium oxide Nanoparticles

CdNPs- Cadmium Nanoparticles

CdTe- Cadmium Telluride

CdS- Cadmium sulphate

CPCSEA- Committee for Control and Supervision on Experiments on Animals

DNA- Deoxyribonucleic Acid

DCT- Distal Convoluted Tubules

DNPH- 2,4-Dinitrophenylhydrazine

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

DW- Distilled Water

dl- decilitre

Exp.- Experimental

Fig.- Figure

$FeCl_3$ – Ferric chloride

FSH- Follicle Stimulating Hormone

g- Gram

GPx- Glutathione Peroxidase

GNP- Gold Nanoparticles

GI- Gastrointestinal

GSH- Reduced Glutathione

H_2O- Water

H_2O_2- Hydrogen peroxide

H_2SO_4- Sulphuric Acid

HCl- Hydrochloric Acid

ISO- International Organisation for Standardisation

IU- Unit

Kg- Kilogram

LH-Luteinizing Hormone

LD_{50} – Lethal Dose

M- Molarity

mg- Milligram

MgCl2- Magnesium chloride

MT-Metallothione

min- Minutes

ml-milliliters

MONPs- Metal Oxide Nanoparticles

N- Normality

NaOH- Sodium Hydroxide

nm- Nanometer

NP- Nanoparticles

NS- Non-significant

NM- Nanomaterials

OECD- Organization for Economic Cooperation and Development

OD- Optical density

pH- Potential of hydrogen

PCT- Proximal Convoluted Tubules

ROS- Reactive Oxygen Species

SOD- Super Oxide Dimutase

SnNPs- Tin Nanoparticles

TiO_2 NPs- Titanium di-oxide Nanoparticles.

TCA- Trichloroacetic Acid

Zn- Zinc

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 Bi M. Shaikh, Assistant Professor of Zoology Discipline, 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 increasing rapidly during either its 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 NPs effect on *Mus musculus* will provide data about its toxicity that can be further used to generate preventive, measures to reduce CdO NPs exposure to humans. There are very few studies, undertaken to analyze the toxicity of CdO NPs via oral gavaging. There are almost no reports on their toxicity to the liver and kidney functioning in animals. This thesis is contributing to the knowledge investigating their toxicity in behavior, morphology, anatomy, liver, and kidney functioning of *Mus musculus*. The thesis is divided into five main chapters. The first chapter's introduction gives an overview about Cd and CdO nanoparticles, their applications, and their routes of exposure to humans. The 2nd chapter includes a survey of literature and the aims and objectives of the work. Chapter 3 gives the material and methods used for the study. The exposure techniques along with different biochemical estimations. Chapter 4 represents the results embodying observations of animal

behavior, morphology, anatomy, liver, and kidney functioning of Mus musculus. Chapter 5 gives elaborate discussions about the reasons and effects of changes occurring in the mice as a result of CdO NPs toxicity. Conclusion with a summary, future work references, and contributions from the thesis follows chapter 5.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

"Nano-objects" are defined as substances which are one or more external dimensions in the nanoscale (1–100 nm). Among these objects, particle-shaped substances having a diameter of 100nm or less are known as 'nanoparticles'(NPs) (Horie & Fujita, 2011). 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 utilised in diverse areas of industry, including textiles, finishes, cosmetics, sunscreens, dietary supplements, medical devices, prescription drugs, and electronics. Since, the demand for nanoparticles are increasing, they are being introduced into the environment causing humans to be exposed to NPs at increasing rate. It is due to these reasons that nanotoxicology is being developed as a novel field to study the potential risk of NMs (Nanomaterials) and their mechanisms of action (Demir et al., 2020). In the recent years, one of the most potential candidates for scientific applications are the Metal oxide nanoparticles that are widely used for biomedical and industrial applications.

1.1. Metal oxide nanoparticles: properties and applications

Metal oxide nanoparticles (MONPs) are produced industrially in large volumes and are an important material also in the fields of ecology, and medicine (Fernández-Garcia & Rodriguez, 2007). These MONPs have physical and chemical properties of good selective and catalytic activity, sensitivity, unusual adsorptive behaviour, and superparamagnetic state (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, piezoelectic devices, fuel cells and coatings for the passivation of surfaces against corrosion (Fernández-Garcia & Rodriguez, 2007, 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 lead to oxidative stress and consequently to lipid peroxidation, DNA damage, inflammation, and cell death (D'Amora et al., 2022).

1.2. 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 risk for both humans and animals. This metal being soft silvery-white show similar physical and chemical properties to zinc and mercury. Cd burns in air to form CdO while Hydrochloric, sulfuric, and nitric acids dissolve cadmium and form cadmium chloride, cadmium sulfate, and cadmium nitrate, respectively (Genchi et al., 2020).

Cd ions have shown to exhibit strong affinities for biological structures that include – SH groups (cysteine and glutathione GSH) as well as disulfide – S – S-groups (cystine and reduced GS-SG glutathione) that may interfere with their function (El Kholy et al., 2021). 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 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 road, 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. High concentration of cadmium has been found to be present in crustaceans and bivalves such as molluscs, oysters, cephalopods and crabs. It is also found in offal products like liver and kidneys, in oil seeds, cocoa beans and certain wild mushrooms. Apparently, depending on the level of soil contamination, food that is derived from plants generally contain higher concentration of Cd than meat, egg, milk, and dairy products. Among these foods, it has been found that higher concentration of cadmium is found in rice, wheat, green leafy vegetables, potatoes, carrot, and celery as compared to other food from plants. Higher concentration of Cd has also been found in vegetarians and shellfish consumers as compared to omnivores. Cd that has been used for electroplating and 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).

1.3. Cadmium resorption into the body

The absorption of Cd into the body is mainly via the respiratory tract and to a smaller extent via the gastro-intestinal 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 (Satarug, 2018) liver, and gut (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) and storage predominantly in soft tissues such as liver and kidneys (Dumkova et al., 2016 , Tinkov et al., 2018).

1.3.1. Uptake of Cadmium through Gastrointestinal tract

The uptake of Cd via the human gastrointestinal tract is found to be approximately 5% of an ingested amount of Cd. 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 bio-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 the 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 6% higher uptake of iron as compared to those with a balanced iron stock (Flanagan et al., 1978). This is the main reason for higher uptake of Cd in anaemic individuals as low iron levels

stimulate the expression of DCT-1 (Divalent Cation Transporter 1), a metal transporter in the GI tract that serves as a gate for Cd resorption (Godt et al., 2006).

1.3.2. Uptake of Cadmium through Respiratory system

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 level 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). The inhaled Cd reached the bloodstream usually in the form of cadmium-cysteine complexes. After its entry into the bloodstream, it targets several organs like lungs, kidneys, testis, placenta as well as the nervous system (Papp et al., 2012).

1.3.3. Resorption of cadmium through dermis

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 dermal absorption of cadmium (Godt et al., 2006). In 1991, Wester et al. demonstrated that cadmium will partition from both water and soil into human skin and subsequently will be absorbed into the body. They demonstrated the penetration of Cd into the skin to be about 8.8 % and 12.7% from the soil and water respectively. The plasma uptake of the

cadmium into the skin from soil was found to be 0.01% and 0.07% from water (Wester et al., 1992). Cd absorption via the skin can be facilitated by two mechanisms i.e. either it binds to the sulfhydryl radicals of cysteine in epidermal keratins or it induces and complexes with metallothionein (Godt et al., 2006).

1.3.4. Handling and effects of Cadmium in the body

Once Cd enters the body it is taken up by the blood and transported bound to proteins, such as Albumin and Metallothionein. The first organ it reaches after its uptake is the liver where it induces the production of metallothionein (Godt et al., 2006). These metallothionein are small metal-binding proteins that are very rich in cysteines and its major physiological functions include the homeostasis of essential metal Zn and Cu, it protects against the cytotoxicity of Cd as well as other toxic metals, and helps to scavenge free oxygen radicals generated in oxidative stress (Genchi et al., 2020). After causing hepatocyte necrosis and apoptosis, parts of Cd from the Cd-metallothionein complexes enter the entero-hepatic cycle from the sinusoidal blood. This occurs via the secretion into biliary tract in the form of Cd-Glutathione conjugates. The Cd-cysteine complexes that are enzymatically degraded in the biliary tree re-enters the small intestine.

The main organ for long-term Cd accumulation is the kidney where its half-life is approximately 10 years. A life-long intake can therefore lead to a its accumulation in the kidney, thereby leading to tubulus cell necrosis (Godt et al., 2006). The highest concentration of Cd in the kidney is found in renal cortex. Its concentration in the kidney is found to increase with age up to about 50-60 years, after which it is found to decrease(Berglund et al., 1998). Concentration

of Cd in the blood serves as an reliable indicator for any recent exposure to the metal while urinary concentration indicates past exposure, body burden and renal accumulation (Godt et al., 2006).

Exposure to Cd in humans can lead to a variety of adverse effects such as renal and hepatic dysfunction, pulmonary edema, testicular damage, osteomalacia, damage to the adrenals (Tinkov et al., 2018) and hemopoietic system, coronary heart disease, peripheral artery disease and atherogenic changes in lipid profile (IARC, 1993). In addition, Cd is a proven human carcinogen (group I of International Agency for Research on Cancer classification) (Tinkov et al., 2018) wherein occupational or environmental cadmium exposure has been related to lung, breast, prostate, pancreas, urinary bladder, and nasopharynx cancers (Mezynska & Brzóska, 2018). A lot of ecotoxicity and physiochemical results indicate Cd as one of the harmful environmental pollutants in terms of cytotoxicity and bio accessibility. Despite the extensive studies conducted on Cd toxicity, there continues to be a gap regarding the mechanism of its action, environmental exposure and intracellular damage. With the evolution of nanotechnology, the scientific community is provided with another aspect of cadmium toxicity i.e., effects of cadmium nanoparticles on the biological systems of organims. Furthermore, since nanoparticles affect the biological systems via various mechanisms and reactions which are very much different from their bulk counterparts, so it becomes very important to understand its effect on human health. There exist different forms of Cadmium having various sizes, one of which is cadmium oxide nanoparticles (CdO NPs) which are widely used in the industries in recent years.

1.4. Application of cadmium oxide

They have long been used in the manufacture of batteries, dyes, and fire retardants, solar sensors, biosensor (Blum et al., 2015), photodiodes, phototransistors, photovoltaic cells, transparent electrodes, liquid crystal displays, infra-red-detectors, nanostructured films, anti-reflection coats, antibacterial age (Tulinska et al., 2020), and as a major ingredient for electroplating baths and pigments (Genchi et al., 2020). Cadmium oxide nanoparticles. Besides its industrial application, CdO NPs are also widely used as a starting material for the manufacture of quantum dots thereby finding its way in both medical diagnostic imaging and targeted therapeutics (Demir et al., 2020) . Since the release of these nanoparticles can be exposed to human via several routes thereby causing deleterious effects, it is very important to understand the toxicity of these nanoparticles so as to protect the environment as well as humans from its adverse effects. In order to understand the toxicity of any chemical, mice have been extensively used as an animal model due to its physiological and anatomical similarities to humans.

1.5. *Mus musculus* as a model organism

As of today, Mice and rats make up approximately 95% of all laboratory animals, with mice the most commonly used animal in biomedical research. These animals are considered to be ideal models for biomedical research and comparative medicine studies due to their similarities to humans in terms of physiology and anatomy. Rats, mice and humans have approximately 30,000 genes of which about 95% are shared by all the three species

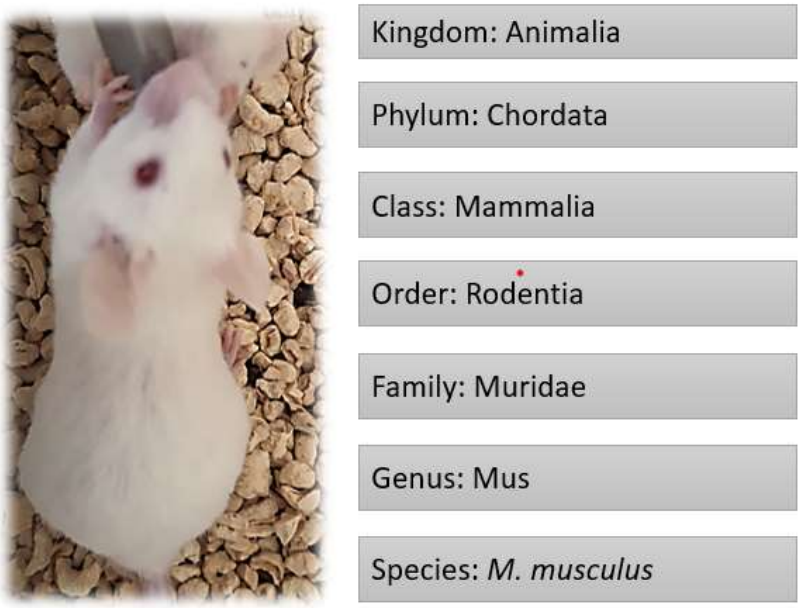

Fig 1: Classification of Mice

Utilization of these animals for research purpose has economic advantages also which can be summarized as follow:

- They are relatively small therefore require little space or resources to maintain.
- Have short gestation time but relatively large number of offspring
- Exhibit rapid development to adulthood.
- Have relatively short life spans

In the current study, mice was used as an experimental model to understand the toxicity of CdO NPs because of its similarities with human beings (Bryda, 2013).

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

Besides its industrial application, CdO nanoparticles have gained rising interest in its use in biomedical and pharmaceutical research including drug delivery, attack and elimination of cancer cells, identification and location of tumors, and magnetic resonance imaging. Therefore, due to their increasing demand, multitude of research has been conducted to access the toxicity of these nanoparticles via various routes.

2.1. Study of CdNPs in different animal models

In a study conducted by Verma et al., (2020), the effect of CdSNPs (Cadmium sulphide nanoparticles) was studied in the gills of a fresh water fish *Channa punctatus* by exposing the fish to CdSNPs for 15 and 30 days. CdSNPs was observed to induce higher degree of lipid peroxidation than CdS bulk particles at similar dosage and period of (Verma et al., 2020). Another study by Sangeetha et al., (2017) showed that CdNP exposed liver tissues in C.catla showed parenchymal cells arc, degeneration of hepatocytes, dilated intra hepatic spaces, distinct vacuoles while kidney tissues showed severe degeneration of epithelial cells lining the renal tubules, disorganization of tubules, , reduction in glomerulus, dilation of Bowman's spaceand focal necrosis with hemorrhage among the renal parenchyma (Sangeetha et al., 2017).

Balmuri et al., (2017), studied and compared two different CdO nanoparticles, i.e. calcination of Cd(OH)2 without any organic molecule (CdO-1) and calcination of Cd-citrate coordination polymer (CdO-2) using Zebrafish as a model organism. Results show reduced toxicity in zebrafish exposed to CdO-2 NPs as compared to CdO-1 NPs. This can also be supported by the

histopathological observation where CdO-1 NPs showed higher toxicity as compared to CdO-2 NPs (Balmuri et al., 2017).

In the study by Niitsuya et.al (2011), alveolar macrophages obtained from bronchoalveolar lavage of Syrian golden hamsters were concurrently exposed in vitro to Fe_3O_4, as an indicator for magnetometry, along with various concentrations of cadmium oxide (CdO). A rapid decrease of the remnant magnetic field, called relaxation, was observed in macrophages exposed to phosphate-buffered saline or CdO at 0.1 µg/ml, while relaxation was delayed in those exposed to 1, 25, or 50 µg/ml CdO. Therefore, the concentration of CdO that is affecting relaxation was estimated in-vitro between 0.1 and 1 µ g/ml (Niitsuya et al., 2003).

Kholy et al., (2020), exposed Drosophila melanogaster for 7 days to a sub lethal concentration of CdO, to study whether short episodes of CdO NPs exposures have long-lasting effects on life history traits such as fecundity. It was found that life history as well as climbing behavior and fecundity were adversely affected by exposure to CdO NPs (El Kholy et al., 2021).

2.2. Study of Cadmium NPs in rodent models

Cadmium nanoparticles are an important occupational and environmental pollutant that is translocated through the food chain to the animals and man thereby entering the body via inhalation or ingestion. Due to its ability to accumulate, cadmium causes a variety of toxic effects in both experimental animals as well as humans. Therefore, complete knowledge of its distribution and metabolism is essential to elucidate the mechanisms of these toxicological actions.

2.2.1. Toxicity of different forms of Cd NPs

Male Wister rats treated with CdSNPs showed significant changes in the structure and function of liver with increased values of serum enzymes and reactive species that altered membrane integrity and induced oxidative stress thereby activating cell death pathways in hepatocytes. (Rana et al., 2021).

A study by Nguyen et al., (2019), investigated the tissue distribution and toxic effects of cadmium telluride quantum dots (CdTe-QDs) wherein higher doses, revealed hepatic hemorrhage and necrotic areas in the spleen. 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).

2.2.2. Toxicity of CdO NPs via 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. A study by Takenaka et al., (2004), indicated that inhalation of ultrafine CdO particles resulted in 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 deep lung showed to stimulate an inflammatory response where neutrophils and monocytes enter the lungs to ingest inhaled material (Takenaka et al., 2004).In another experimental study on rats, exposure to CdO dusts 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 increase (Koshi et al., 1978,

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 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 Dumkova et al., (2016), reported that inhaled CdO NP in lungs caused significant alterations in parenchyma tissue including hyperemia, 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 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 that 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 significant 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 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 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 $CdCl_2$ and CdO on pulmonary biochemical function were compared in rats and rabbits. Both compounds after 72h exposure have shown to cause multifocal, interstitial pneumonitis but CdO lesion was found to be more severe. Both the compounds also caused a significant increase in lung weight, lung-to-body weight ratio, GSH reductase, CSH transferase, and C-6-PDH. The response in both, rabbit and rat showed a similar response pattern (Grose et al., 1987).

Hadley et al., (1979) exposed rats to aerosol of cadmium oxide and 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 tumor 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 time period, the growth of the lungs was damaged showing some degree of permanent damage of pulmonary lobes whereas 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. Slight accumulation of Cd was seen in the liver and kidney as well (Boisset et al., 1978).

2.2.3. Toxicity of CdO NPs via intratracheal route

Cadmium exposure has also 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 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 the causing nervous system damage and showing the possibility of modelling human neurotoxic damage in rats (Papp et al., 2012).

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, activity of SOD decreased but that of GPx and GR remained unchanged. These observations suggested that metallothionine played an important role in detoxification of instilled CdO, but that the antioxidant enzymes had a minimal role (Hirano et al., 1990).

2.2.4. Toxicity of CdO NPs via intraperitoneal route

Outbred male rats that were repeatedly injected intraperitoneally with either a combination or separately with lead oxide and/or cadmium oxide nanoparticles for the purpose of studying 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).

2.2.5. Toxicity of CdO NPs via oral route

Wister rats were fed with three concentrations of CdO NPs for a period of 10 days via oral gavaging. The results indicated that the enzyme concentration (creatine phosphate kinase, Alanine aminotransferase and Aspartate transaminase) in blood increased with increased concentrations of nanoparticles (Masoumeh et al., 2017). In another study, toxic effect of various metal nanoparticles including, CdO were investigated on the reproductive system in adult male rats. Results have shown a significant elevation of leuteinizing 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).

From the above literature review, even though a lot of research is done on evaluating the toxicity of CdO NPs, but majority of the studies have been dealt with inhalation toxicity employing rodent models (Balmuri et al., 2017) . While there are very few studies that emphasize its toxicity through oral administration.

2.3. Lacunae

Most of the studies have evaluated the toxicity of CdO NPs via inhalation and intratracheal route of exposure. Since the toxicity of the nanoparticle depends on the size as well as the route of exposure oral gavaging also turns out to be one important route to be studied due to contamination of nanoparticles in food

or accident intake during the manufacture of CdO NP or related products. Therefore, in the present study Cadmium oxide nanoparticles (CdO NP) will be administered in different doses through oral gavaging exposure method to study the acute effects of CdO NP on mice.

2.4. Hypothesis

The study hypotheses that CdO NPs can cause adverse toxic effects on the behaviour, morphology, anatomy and physiology of liver and kidney functioning.

2.5. Objectives of the study

- To determine the LD_{50} of CdO NPs in *Mus musculus* via oral gavaging.
- To analyze the acute effects of CdO on the morphology of *Mus musculus.*
- To estimate the retention of CdO NPs in liver and kidney of *Mus musculus*
- To evaluate the structural changes in the liver and kidney.
- To evaluate its effect on kidney and liver functioning

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Glasswares

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

3.2. Instrumentation

Weighing balances (Wensar PGB 200), water bath, pH meter (TOSHCON TMP3), Vortex (CM 101), Refrigerator, Deep freezer (-20^{o}C), Centrifuge (REMI R-24), hot air oven (MIC-165), light microscope (Olympus, bx53) Colorimetric spectrophotometer, used in the study are available in the Zoology Discipline, Goa University. Microtome for histology was utilized from Ashwini Pathological lab and atomic absorption spectroscopy was utilized from Ita lab.

3.3. Chemicals

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

3.4. Procurement of the nanoparticles

The cadmium oxide nanoparticles (CdO NPs) having a molecular weight of 128.41g/mol were obtained from Nanoshel LLC (Product code: NS6130-03-379) with the following specifications (Table no 1):

Table no 1: Specifications of Cadmium Oxide Nano Powder (CdO).

Appearance	Brown, Red-brown or Rust
Purity	99.7+%
Grain size (nm)	<100nm
Specific surface area (m^2/g)	>90
Loss of weight in drying (%)	<0.3
Loss of weight in burning (%)	<0.2
Trace of metal analysis	≤ 5000 ppm (meets requirement)
% Cadmium	85.8-89.3 Wt %

Table no. 2: Administration of different doses to experimental groups of mice

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

3.5. Evaluation of CdO Nanoparticle toxicity

3.5.1. Animal model: Mouse (*Mus musculus*)

Mice are widely used as experimental animal in studies because they share high degree of anatomical, physiological and genetical 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).

3.5.2. Procurement of animals

A total of 26 healthy male Swiss Albino Mice (*Mus musculus*), 6-8 weeks of age having average body weight of 23-28g, were obtained from 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 prior to the start of the experimentation.

3.5.3. 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, animals of uniform body weights i.e., 23-28 g were selected for the present study. The mice were housed in polypropylene cages with stainless steel lid, 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 commercial diet and tap water provided ad libitum (Tulinska et al., 2020).

3.5.4. LD_{50} measurement for acute toxicity

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

In order to obtain the LD_{50} for CdO NPs, the experiments 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 LD50. 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. The $LD_{50,}$ was determined for a period of 14 days.

3.5.5. Experimental set up

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 with 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 (Sulaiman et al., 2015) (Table no.2).

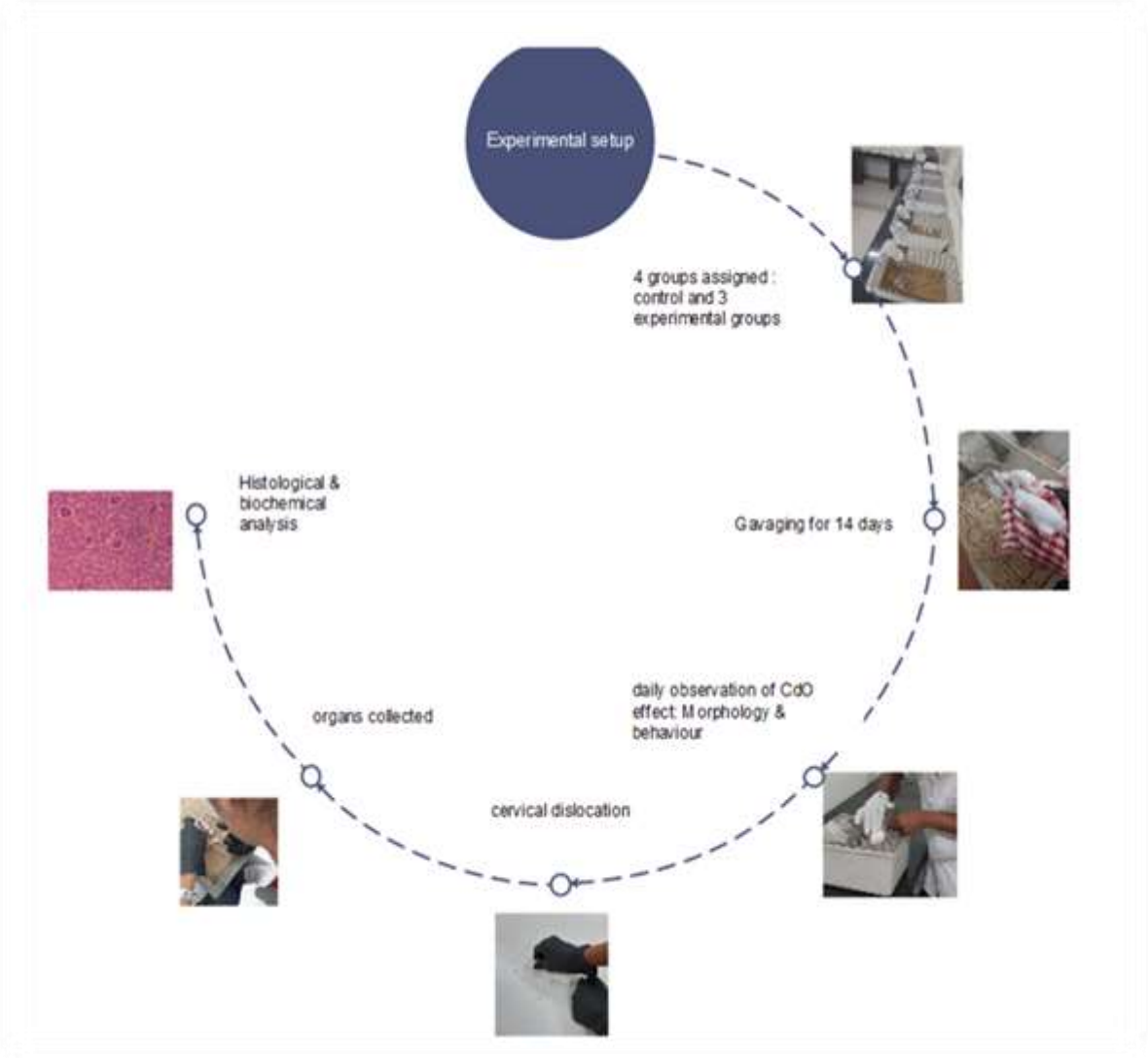

Fig 2: Experimental design

3.5.6. General examination

For a period of 14 days, the mice were routinely examined for their survival, evident behavior or motor impairments and morphological changes in hair, skin texture and colour.

3.5.7. Morphology

Animals were also observed routinely for any morphological changes such as changes in the weight of the body, skin colour, hair texture, eye colour, and tail spots during the 14 days exposure period.

3.5.8. Body weight

The change in body weight was recorded every 3 days during the exposure period to see the effect of CdO NPs administered orally.

3.6. Collection of tissues

After 14 days of exposure period, the mice were sacrificed by cervical dislocation. In this technique, pressure was applied to the neck dislocating the spinal column from the skull or brain. Blood was collected from mice by cardiac puncture with moderate suction. The blood was then transferred into tubes for the separation of serum (Roy & Dattatraya Pujari, 2010). After opening the general viscera, tissues like liver and kidney were removed and washed with mammalian saline to remove all the traces of blood, weighed and quickly stored at -20^0C for biochemical analysis.

3.7. Histology

Kidney and liver for histological examination were washed thoroughly 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 prepared and stained with haematoxylin and eosin by Ashwini Pathology Lab. The prepared slides were further analyzed at 20x under a light microscope (Olympus bx53).

3.8. Retention of CdO NPs in liver and kidney

Tissue samples were dried completely in a clean oven at 60–70 ^{0} C. Each dried tissue was digested to white ash using 30% hydrogen peroxide (GFS Chemical; Powell, OH) at 50–60 ^{0}C followed by digestion with concentrated ultra-pure trace-metal free nitric acid (0.1 mL, GFS Chemicals). The digested white ash was dissolved in 0.25 N nitric acid (1–4 mL, depending on the weight of the digested tissue). After digestion, the samples were diluted with distilled water to 50ml (Lasagna-Reeves et al., 2010). A 5 ml aliquot was made which was then used for the analysis of cadmium using atomic absorption spectrophotometry. A hollow cathode lamp for cadmium was used and the absorption was recorded at 228.8nm where metallic cadmium (Hi-media) was used as a standard (Rana et al., 2021).

3.9. Extraction and estimations of biomolecules

Liver, kidney and serum were collected aseptically and used for biochemical estimations.

3.9.1. Total Proteins and albumin

Extraction: The Kidney and liver were thawed and homogenized in PBS for protein estimation. The homogenization was done in a cold chamber set at 4°C. The homogenates were then centrifuged at 3000 rpm for 15-20 min. The resulted supernatants were stored at -4° C in clean sterile micro centrifuge tubes until analysis.

3.9.1.1. 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 color. The intensity of the blue color 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 sulfate 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-colored 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).

3.9.1.2. Albumin: *At pH 4.1, Albumin present in the sample binds specifically with bromocresol green to form green colored 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 900 m1 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 l000 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 color 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).

3.9.2. Total carbohydrates, Free sugars and urea

Extraction: 10% tissue (liver and kidney) 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 urea, total carbohydrates and free sugars.

3.9.2.1. 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 deprotenized 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 et al.,1955). Quantification of the total carbohydrate content was done with the help of a standard curve of total carbohydrate (100pg of glucose/ml).

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

3.9.2.2. Free sugars: *When sugars are heated with alkaline copper reagent it forms 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 arseno-molybdate colour reagent was added. The mixture was diluted with 7 ml 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 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 the solutions a and b were mixed, 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, stored in amber coloured bottle at 37°C for 48 hours.
- **DNPH reagent**: Dissolve 0.25 gm of 2-4 DNPH in a mixture of 42 ml cone. HC1 and 50 ml DW by warming on water bath.

0.1ml sample with 1 ml of DNPH reagent was incubated for 15 min at 37°C. 4 N NaOH (1.0 ml) was added to the mixture and was kept at RT for 5 min and the OD was read at 440 nm. The standard curve of pyruvate was used for calculating the concentration of pyruvate in the samples and expressed in mM/gm of tissue.

3.9.2.3. Urea (Friedman, 1953): *In the hot acidic medium with the presence of ferric ion, diacetyl monoxime reacts with urea and gives a pink color substance. The intensity of the color depends upon the amount of urea present, which can be measured at 520nm*.

- **Acid mixture**: 10.0mg of ferric chloride was dissolved in 100ml of orthophosphoric acid.
- **Diacetyl monoxime reagent:** 2.5g of diacetyl monoxime and 0.12g of thiosemicarbazide was dissolved in 100ml distilled water.

0.1ml of serum was diluted up to 3ml with distilled water. 2ml of the acid mixture and 0.2 ml of diacetyl, monoxime reagent were added. It was then incubated in a boiling water bath for 20 mins and then cooled at room

temperature. The intensity of color was measured at 530nm. The amount of urea present in various samples was calculated using standard curves of urea 10µg/ml.

3.9.3. Creatinine (Brod and Siruta, 1948): *Creatinine reacts with picric acid in an alkaline medium to form a reddish yellow complex, the intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 520nm.*

- **Reagent Alkaline picric acid:** it is prepared fresh by mixing four parts of picric acid and one part of sodium hydroxide. This working reagent is stable for one day.

0.1ml of Serum or 0.1ml urine was diluted to 3.0 ml with distilled water. Then 0.5 ml 2N sulphuric acid was added followed by 0.5ml sodium tungstate. The content was centrifuged in a centrifuge tube for 3000rpm for 10mins. The supernatant was collected and 0.1 ml alkaline picric reagent was added. The contents were mixed thoroughly and were kept at room temperature for 20 minutes. The intensity of color was measured at 520nm. Quantification of serum creatinine was done with the help of a standard curve creatinine.

3.9.4. Reduced glutathione (GSH)

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

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

Extraction: 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'- dithiobis, 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. Reduced glutathione content of the samples was quantified with the help of a standard curve of reduced glutathione (0.2mnole/m1 in 5% TCA).

3.9.5. Catalase (Sinha, 1972)

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

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.

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

1.5 ml 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.

3.9.6. Alanine aminotransferase (ALT)

a-ketoglutaric acid reacts with alanine undergoing a transamination reaction where alanine donates its amino group to a-ketoglutaric acid. Resulting in alanine becoming pyruvate and a-ketoglutaric acid to glutamic acid. Pyruvate reduces 2, 4- dinitrophenyl hydrazine to dinitrophenyl hydrazone which in an alkaline medium, produces a colored complex. The intensity of the color is measured at 505nm.

Alanine+ Alpha-ketoglutaric acid ---------> Pyruvate+ glutamic acid

- **ALT substrate**- 0.532 g alanine and 6.0 mg a-ketoglutaric acid were added to 0.1 ml of 1N NaOH prepared in phosphate buffer (0.01M, pH 7.5). Final quantity was adjusted to 20 ml with phosphate buffer (pH 7.5).

0.5 ml of ALT substrate prepared in phosphate buffer (0.01 M, pH 7.0) was incubated at 37°C for 5 minutes. To this 0.1 ml of serum or 5% liver tissue homogenate (in 0.01M phosphate buffer, pH 7.0) was added to assay the enzyme activity. A suitable enzyme blank was also prepared by taking 0.5m1 of substrate and 0.1 ml of distilled water. They were incubated further for another 15 minutes. The reaction was stopped by the addition of 0.5 ml dinitrophenyl hydrazine reagent. They were mixed thoroughly and kept at room temperature (25°C ± 5°C) for 20 minutes. 5 ml of 0.4 N NaOH was added to develop the

colour, intensity of which was read against the enzyme blank at 540 nm (Reitman & Frankel, 1957). The product formed (pyruvate) during this reaction time was quantified with the help of a standard curve of pyruvate (220 μg/ml). The enzyme activity was expressed as μg of pyruvate formed / min reaction / mg of enzyme protein. Protein content in the enzyme was estimated as described earlier.

3.9.7. Aspartate amino transferase (AST)

a-ketoglutaric acid reacts with aspartate and undergoes transamination reaction. By donating its amino group to a-ketoglutaric acid aspartate becomes oxaloacetic acid and a-ketoglutaric acid becomes glutamic acid. Oxaloacetic acid reduces 2, 4-dinitrophenyl hydrazine to dinitrophenyl hydrazone. In alkaline medium, hydrazone produces a coloured complex. The intensity of the colour can be measure at 540nm.

Aspartate transaminase Aspartate + Alpha-ketoglutaric acid Oxaloactate+glutamic acid

- **AST substrate**- 0.35 g aspartate and 6.0 mg a-ketoglutaric acid were added to 0.1 ml of 1 N NaOH prepared in phosphate buffer (0.01 M, pH 7.5). Final quantity was adjusted to 20 ml with phosphate buffer (pH 7.5).
- **DNPH reagent**-10 mg of dinitrophenyl hydrazine was added to 4.25 ml conc. HC1 and the final quantity was adjusted to 20 ml with distilled water.

Using AST substrate by following the same method mentioned above, activity of aspartate amino transferase was assayed (Reitman & Frankel, 1957). Activity

of the enzyme was quantified with the help of a standard curve of sodium oxaloacetate (220 μg/ml).

3.9.8. Alkaline phosphatase (ALP)

Paranitrophenyl phosphate is colourless. The enzyme splits off the phosphate group from it to form p-nitrophenol, which in the acid medium is also colourless. Under alkaline conditions this is converted to p-nitrophenoxide ions, which exhibit yellow colour. The intensity of the yellow colour is directly proportional to the enzyme present in the specimen and can be measured at 405mn.

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

- **ALP substrate** - 680 mg of p-nitrophenyl phosphate was added to 8.0 mg of MgC12 solution which was prepared by adding 30mg of MgC12 to 10 ml glycine buffer.

To 2.7 ml of glycine buffer, 0.2 ml of substrate (freshly prepared) was added and incubated at 37°C for 5 minutes. To this 0.1 ml of serum or 5% liver tissue homogenate (prepared in 0.2 M glycine buffer, pH 7.0) was added to assay the enzyme activity. Simultaneously an enzyme blank was prepared by mixing 2.7 ml buffer, 0.2 ml substrate and 0.1m1 of distilled water. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 5 ml of 0.25 N NaOH. The intensity of the products of this reaction (p-nitro phenol) was measured at 405 nm (King and Armstrong, 1934). The amount of the p-nitrophenol released by the action of alkaline phosphatase was quantified by prepared p-nitro phenol standard curve (250 μg/m1). Protein content in the

enzyme was estimated as described earlier. The enzyme activity was expressed as (IU/mg protein). 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.

3.10. Statistical analysis

Statistical analyses were done using GraphPad Prism 9.5 software. The results were expressed as mean ± SEM (standard mean error). Student t-test, One-way and two-way ANOVA analysis of variance was carried out to compare the differences of means among multi-group data calculating P and F values. A statistical software system was used to perform a post hoc multiple comparison test (Dunnett's test) after ANOVA. $P < 0.05$ was considered to be significant, $P \leq 0.01$ as highly significant and $P < 0.001$ as very highly significant.

CHAPTER 4

RESULTS

4. RESULTS

4.1. Determination of LD_{50}

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

4.2. Effects of CdO NPs on the morphology of *Mus musculus*

The mice were continuously observed for any morphological changes throughout the 14-day study period for all 4 groups viz., control, Exp 1, Exp 2 and Exp 3.

Weight of the body: The initial average weight of the mice in all 4 groups was around 23-28g. The weight decreased significantly with doses ($P \leq 0.01$, $F=5.485$) and the decline was non-significant with respect to days. The interaction between doses and days was also observed, wherein the weight was decreasing with respect to doses as well as days, but this decline was non-significant.

Loss of hair: At the beginning of the exposure, all the mice showed perfect hair consistency. After a week of exposure to CdO NPs, the mice in the experimental groups displayed loss of hair near the head region which was more evidently observed in the Exp. Group 3 as compared to other experimental groups (Fig 4).

Skin colour: The animals had a pink skin colour at the beginning of the study period which remained constant throughout the study period in all 4

groups.

Table No. 3: Body weights of mice after 14 days oral exposure to CdO NPs.

Groups	**Body weight**		
	Day 0	Day 7	Day 14
Control	26.96 ± 1.57	32.2 ± 2.49	33.58 ± 1.29
Exp. Group 1	27.9 ± 1.55	27.5 ± 1.42	30.4 ± 1.57
Exp. Group 2	26.46 ± 0.98	25.96 ± 1.35	23.72 ± 1.66
Exp. Group 3	27.88 ± 1.59	24.72 ± 1.89	22.66 ± 1.83

Values represent mean ± S.E.M of five animals

Fig 3: Hair loss in the head region

Table No 4: Acute effects of CdO NPs on the morphology of *Mus Musculus*

Morphological parameters	**Control**	**Exp. Group 1**	**Exp. Group 2**	**Exp. Group 3**
Weight of the body	Increase	Decrease	Decrease	Decrease
Skin color	White	White	White	white
Hair texture	Smooth	Smooth	Smooth	Smooth
Eye color	Red	Red	Red	Red
Tail spots	No spots	No spots	No spots	No spots
Loss of hair	No loss	Hair loss observed	Hair loss observed	Hair loss observed

Table No. 5: Retention of CdO NPs in liver and kidney.

Cadmium accumulation	**Treatment group**	
	Control	**Exp. Group 3**
Total cadmium intake at the end of 14 days of the exposure period (mg/kg)	0	252
Cadmium retention (whole organ (mg/kg))		
Liver	0.1 ± 0.008	11.36 ± 0.48
Kidney	0.1 ± 0.01	2.73 ± 0.15
Percentage of cadmium uptake		
Liver	-	4.2 - 4.8 %
Kidney	-	0.96 – 1.16 %

All values are expressed as mean ± SEM of three animals, ($P < 0.0001$)

Eye colour: All the mice exhibited red eye colour at the beginning of the study period which also remained constant in all 4 groups.

Hair texture: Before the exposure, all the mice were coated with smooth white fur which also did not show changes throughout the study period in all 4 groups.

Tail spots or any other markings. There was no presence of any spots or marks on the tail or any other region of the body in the mice in any of the groups throughout the 14 days of the exposure period (Table No. 4).

4.3. Retention of CdO NPs in kidney and liver

The total intake of CdO NPs for the highest dose was 252 mg/kg of the body weight. The significant retention of CdO NPs in the whole liver and kidney was found to be 11.36 ± 0.48 mg/kg and 2.73 ± 0.15 mg/kg respectively. The percentage of CdO NPs in the given tissue was found to be 4.4 – 4.8 % for liver and 0.96 – 1.16 % for kidneys (Table No. 5).

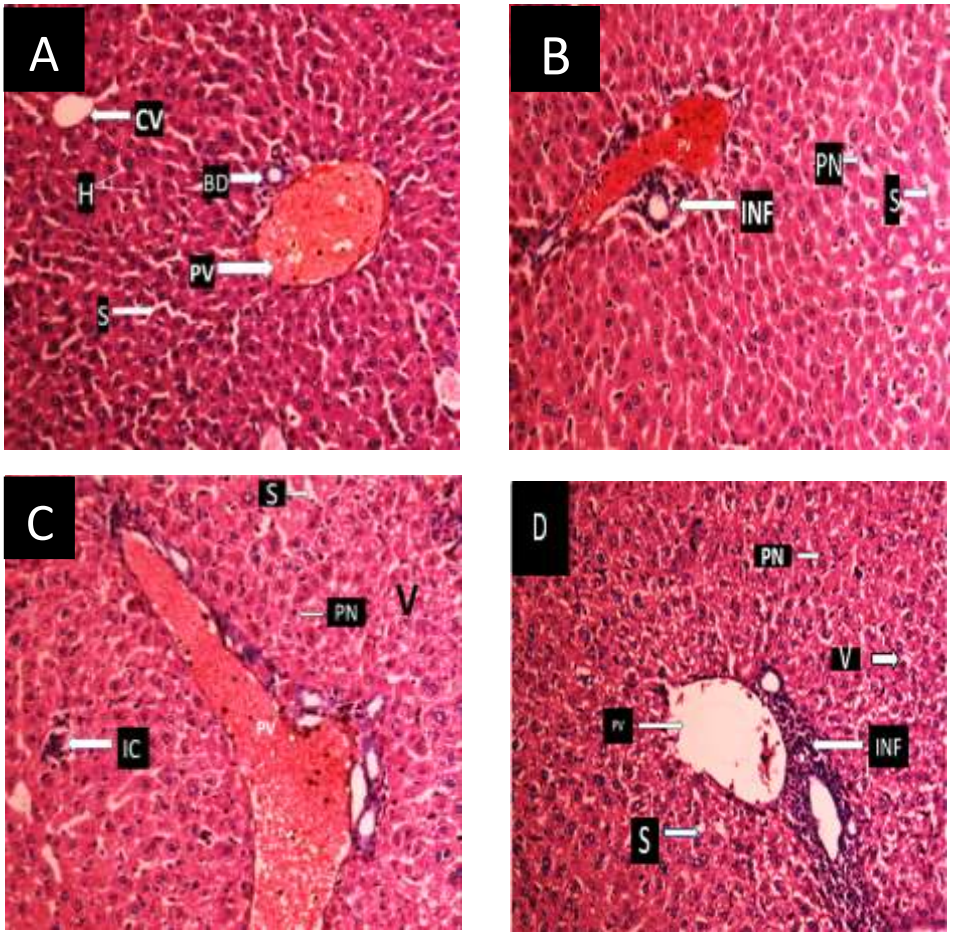

Fig 4: CdO NPs effect on histopathology of liver

A-Control mouse liver showing normal hepatic architecture with normal central vein (CV), Portal vein (PV), Bile duct (BD), hepatocytes (H) and sinusoids (S) B-Exp. Group 1 mice showing liver section with pycnotic nucleus (PN), dilated sinusoids (S), inflammatory cells (INF) and congested hepatic portal vein (PV). C-Exp. Group 2 mice showing liver sections with, pycnotic nucleus (PN), dilated sinusoids (S), aggregation of inflammatory cells (IC), enlarged and congested portal vein (PV) and hepatocytes with cytoplasmic vacuolation (V). D-Exp. Group 3 mice showing hepatocytes showing cytoplasmic vacuolation (V), pyknotic nucleus (PN), inflammatory cells (INF) and enlarged and disrupted portal vein (PV).

4.4. Histopathological examination of major organs of *Mus musculus*.

4.4.1.Liver

The light micrographs of the control group showed normal hepatic architecture with normal central vein, intact sinusoids, normal portal vein with bile duct near its vicinity (Fig 4A). The mice treated with 4.5mg/kg of body weight showed a slight disruption in the hepatic architecture along with pycnotic nucleus, dilated sinusoids, and accumulation of inflammatory cells in the vicinity of the portal vein (Fig 4B). Mice treated with 9mg/kg showed comparatively more damage to the hepatocyte structure along with cytoplasmic vacuolation, pyknotic nucleus, and elongated and congested portal vein surrounded by inflammatory cells (Fig 4C). Mice treated with 18mg/kg body weight showed a highly disrupted hepatic architecture as compared to the control with excessive cytoplasmic vacuolation, activated Kupffer cells, aggregation of inflammatory cells, and disrupted epithelial lining of the large congested portal veins. hepatic portal vein (Fig 4D).

4.4.2.Kidney

The light micrographs of the control group showed normal Glomerular with intact bowman's capsule, distal and proximal convoluted tubules (Fig 5A). The mice treated with 4.5mg/kg of body weight showed cytoplasmic vacuolization in the epithelium of renal tubules and enlarged congested blood vessels (Fig 5B). Mice treated with 9mg/kg of body weight showed comparatively much more aggregation of inflammatory cells, congested

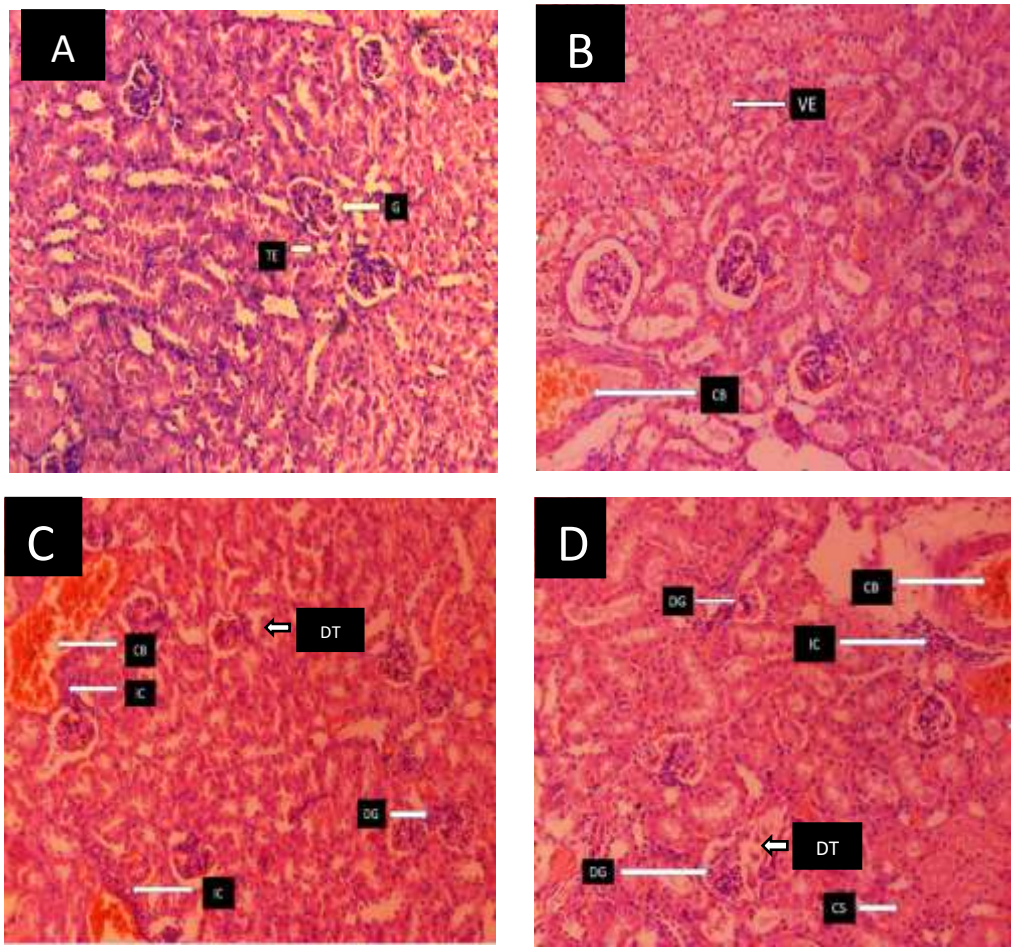

Fig No 5: CdO NPs effect on histopathology of kidney

A-Control mouse kidney showing normal glomerulus (G) with abundant capsular space and intact tubular epithelium (TE). B-Exp. Group 1 mice Kidney section showing cytoplasmic vacuolization (VE) and enlarged congested blood vessels (CB). C-Exp. Group 2 mice showing enlarged congested blood vessels (CB) with an accumulation of inflammatory cells (IC) and distorted glomerulus (DG) and tubular epithelium (DT). D-Exp. Group 3 mice showing distorted glomerulus (DG), distorted tubular epithelium (DT), congested enlarged blood vessels with aggregation of inflammatory cells (IC) and cloudy swelling (CS)

blood vessels, and distorted glomerulus (Fig 5C). Mice treated with 18mg/kg body weight showed highly disrupted glomerulus with excessive accumulation of inflammatory cells throughout the tissue, enlarged congested blood vessels, and cloudy swelling of the epithelial lining of the renal cell (Fig 5D).

4.5. Effects of CdO NPs on the Liver and its Functioning

The changes in the concentration of the different biomolecules and enzymes of mice exposed to three different concentrations of CdO NPs are revealed from Fig 6 to Fig 14.

The mice gavaged with CdO NPs showed a significant dose-dependent decrease in the protein concentration ($F= 11.24$, $P \leq 0.01$) in Exp. Group 2 and Exp. Group 3 whereas Exp. Group 1 showed a non-significant decrease as compared to the control (Fig 6) Similarly, a significant decline was observed in the concentration of Albumin in Exp. Group 2 and Exp. Group 3 mice ($F=9.964$, $P \leq 0.01$) while Exp. Group 1 showed a non-significant decline as compared to control (Fig 7).

The concentrations of total carbohydrates showed a significant dose-dependent increase ($F=15.39$, $P \leq 0.001$) in Exp. Group 2 and Exp. Group 3 while in Exp. Group 1 the increase was non-significant increase as compared to the control (Fig 8). Further, a significant and dose-dependent increase in the level of free sugar content was observed in all the experimental groups ($F=9.752$, $P \leq 0.01$) as compared to the control (Fig 9)

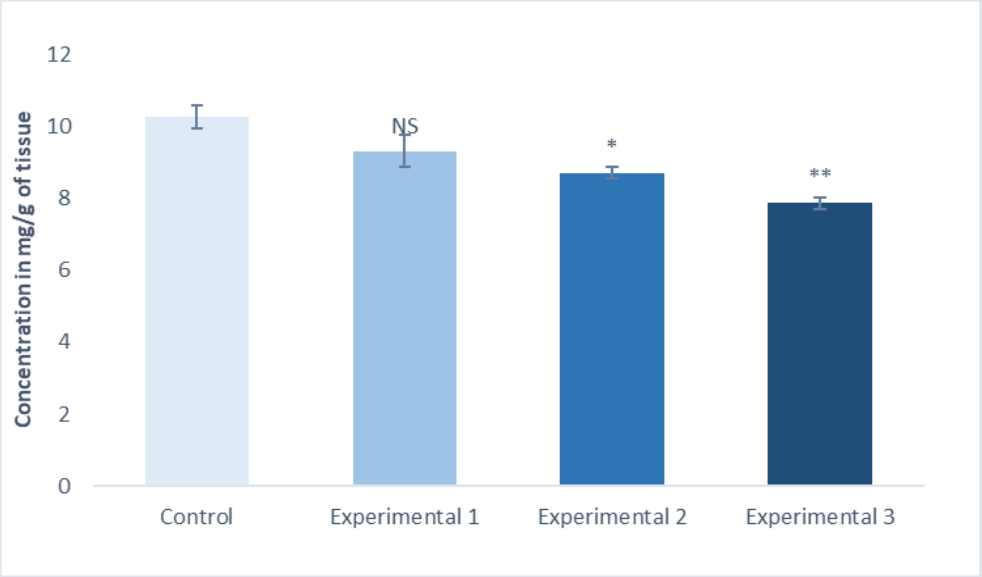

Fig 6: Effect of CdO NPs on Total Proteins content in the liver of mice. NS (non- significant). * ($P \leq 0.05$, significant); ** ($P \leq 0.01$, highly significant)

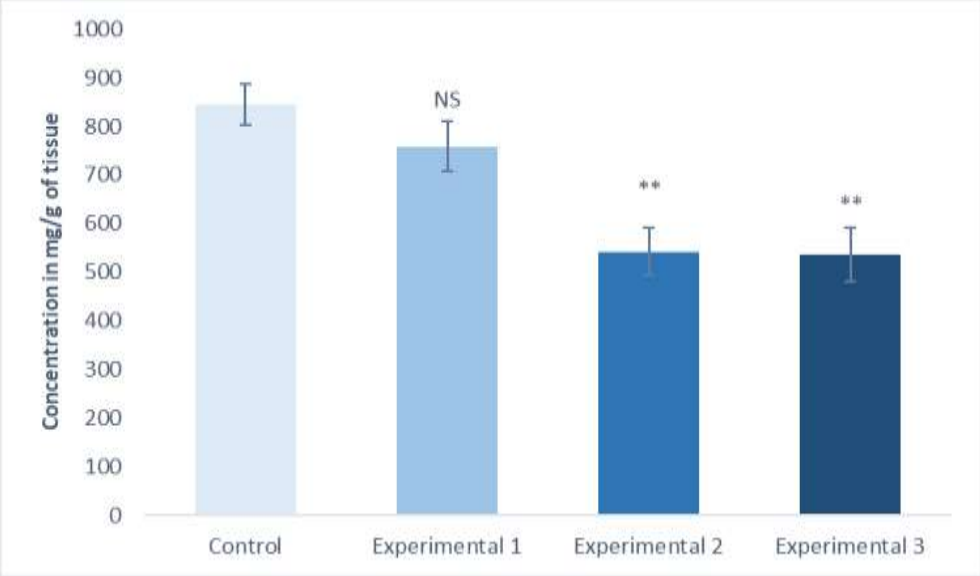

Fig 7: Effect of CdO NPs on Albumin content in the liver of mice. NS (non-significant); **($P \leq 0.01$, highly significant)

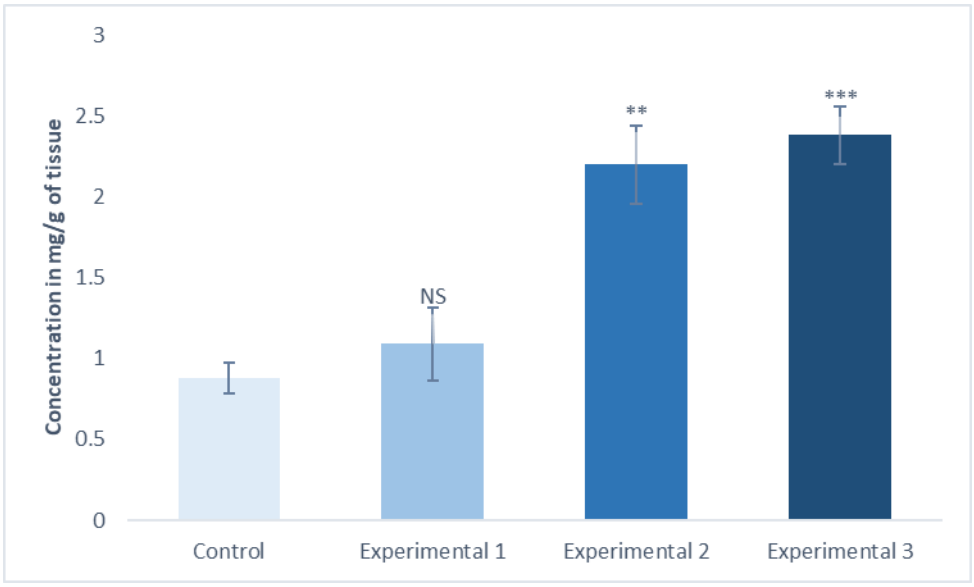

Fig 8: Effect of CdO NPs on Total carbohydrate content in the liver of mice. NS (non-significant); **(P ≤ 0.01, highly significant);***(P≤0.001, very highly significant)

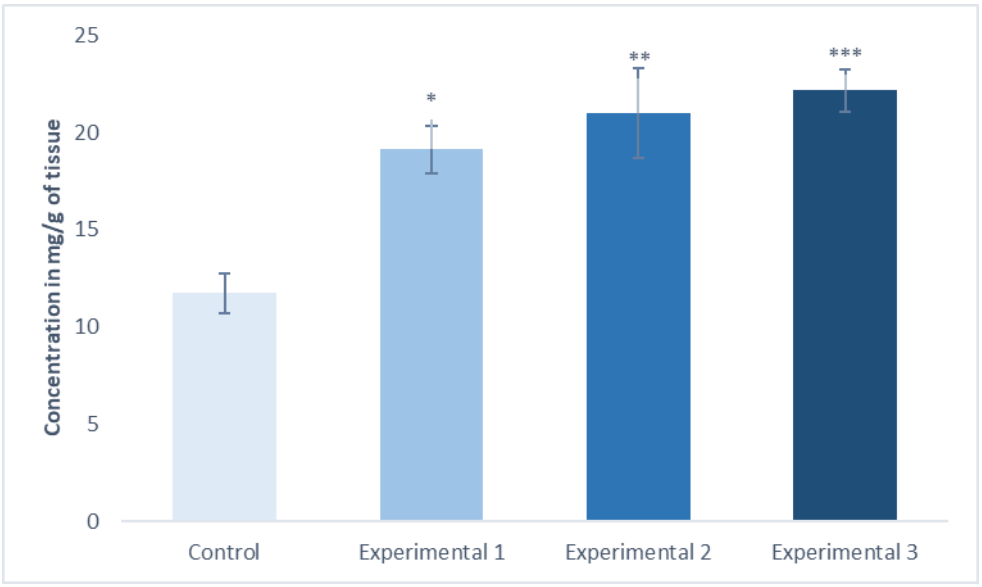

Fig 9: Effect of CdO NPs on Free sugar content in the liver of mice. * (P ≤ 0.05, significant); **(P ≤ 0.01, highly significant); *** (P ≤ 0.001, very highly significant)

Upon exposure to CdO NPs, a significant increase in reduced glutathione ($F=9.497$, $P \leq 0.01$) was observed in Exp. Groups 2 and Exp. Group 3 while Exp. Group 1 showed a minute non-significant increase as compared to the control (Fig 10). The activity of Catalase in Exp. Group 2 and Exp. Group 3 showed a significant increase ($F=15.19$, $P \leq 0.01$) while the increase in Exp. Group 1 was non-significant as compared to the control (Fig 11).

The levels of ALT showed a significant and dose-dependent increase in the Exp. Group 2 and Exp. Group 3 ($F=8.051$, $P \leq 0.01$) while the increase in Exp. Group 3 was non-significant as compared to the control (Fig 12). Upon exposure of mice to CdO NPs, AST concentrations in the blood serum have shown to have a significant increase ($F=10.32$, $P \leq 0.01$) only in Exp. Group 3 while the increase in Exp. Group 2 and Exp. Group 1 was non-significant as compared to the control (Fig 13). The activity of ALP increased significantly in all the experimental groups ($F=20.82$, $P \leq 0.001$) as compared to the control (Fig 14).

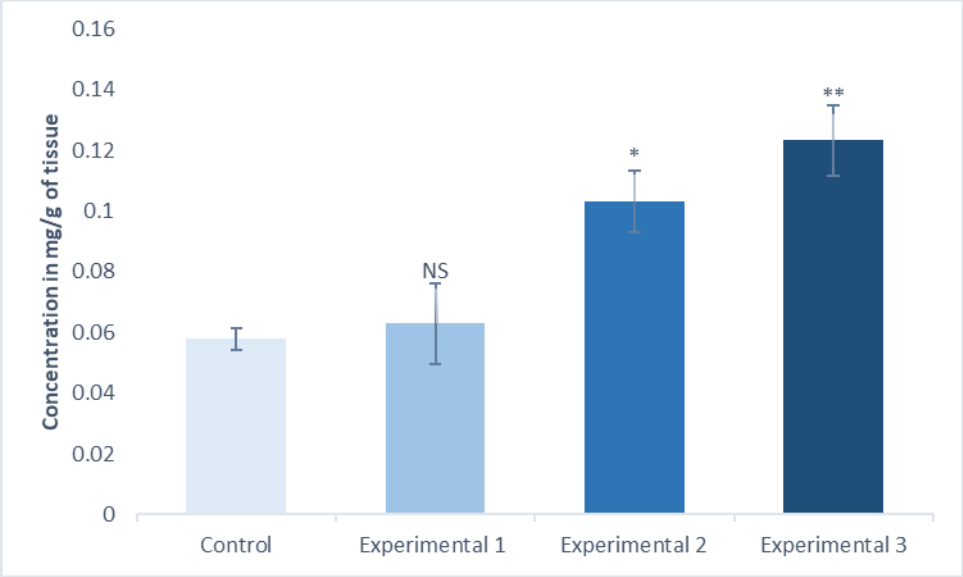

Fig 10: Effect of CdO NPs on Reduced Glutathione content in the liver of mice. NS (non-significant); *($P \leq 0.05$, significant); ** ($P \leq 0.01$, highly significant)

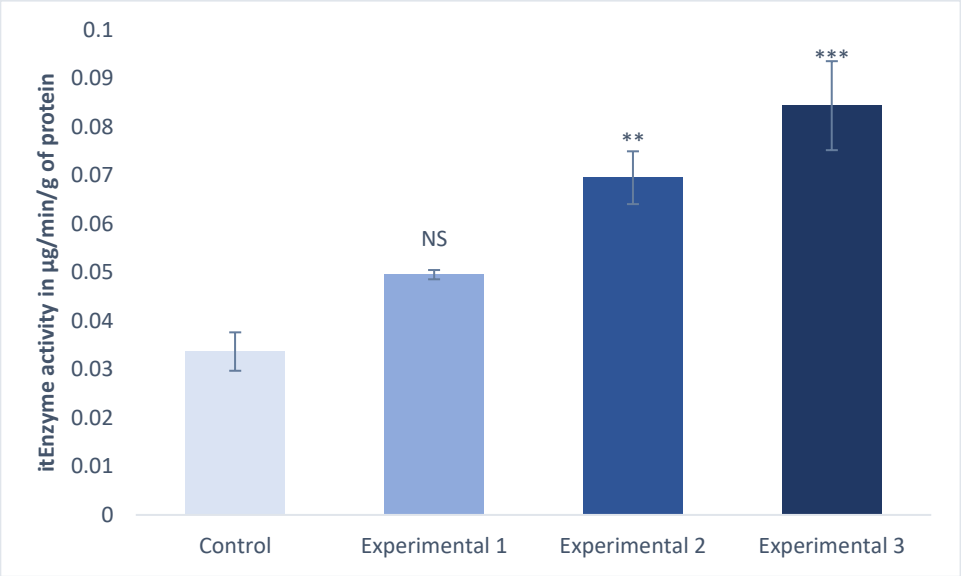

Fig 11: Effect of CdO NPs on Catalase activity in the liver of mice. NS (non-significant); ** ($P \leq 0.01$, highly significant); ($P \leq 0.001$, very highly significant)

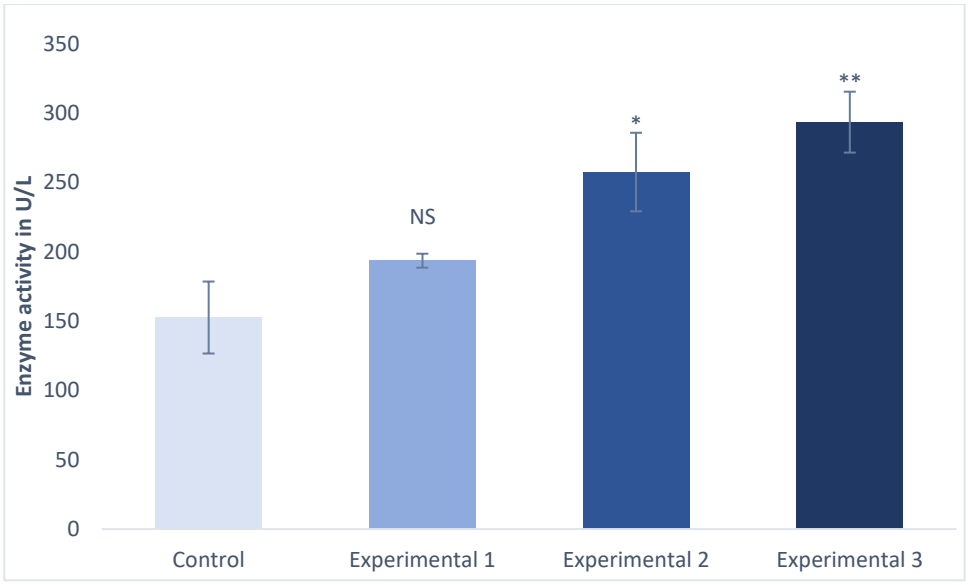

Fig 12: Effect of CdO NPs on ALT in blood serum of mice. NS (non-significant); * ($P \leq 0.05$, significant) ** ($P \leq 0.01$, highly significant)

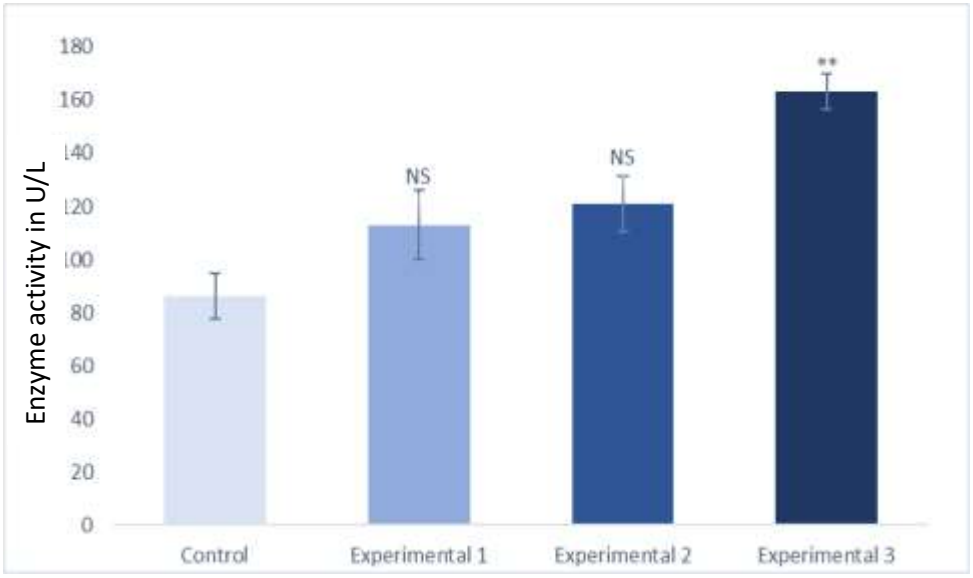

Fig 13: Effect of CdO NPs on AST in blood serum of mice. NS (Non-significant); ** ($P \leq 0.01$, highly significant)

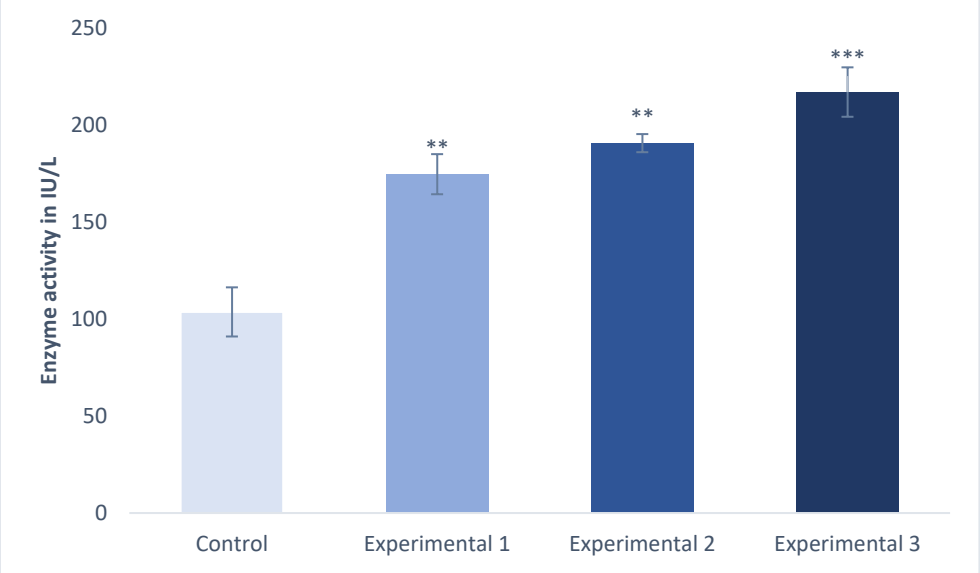

Fig 14: Effect of CdO NPs on ALP in blood serum of mice. ** ($P \leq 0.01$, highly significant); *** ($P \leq 0.001$, very highly significant)

4.6. Effects of CdO NPs on the kidney and its functioning

The changes in the concentration of the different biomolecules and enzymes in mice exposed to CdO NPs are revealed from Fig 15 to Fig 22.

The concentration of protein showed a significant decrease ($F=3.953$, $P\leq 0.05$) only in Exp. Group 3 while Exp. Group 1 and 2 showed minute variations where their decrease was non-significant as compared to the control (Fig 15). Furthermore, the concentrations of Albumin showed a significant and dose-dependent decrease in Exp. Group 2 and Exp. Group 3 ($F= 5.44$, $P= \leq 0.05$) (Fig 16).

The concentration of carbohydrates has shown a significant increase ($F=5.511$, $P \leq 0.05$) only in Exp. Group 1 while the increase in Exp. Group 2 and Exp. Group 3 was non-significant as compared to the control (Fig 17). Similarly, free sugars also showed a significant dose-dependent increase in Exp. Group 2 and Exp. Group 3 was exposed to CdO NPs ($F=7.723$, $P \leq 0.01$) while the increase in Exp. Group 1 was non-significant as compared to the control (Fig 18).

Upon exposure to the nanoparticles, all the experimental groups showed elevated levels of reduced glutathione as compared to the control with the highest elevation observed in Exp. Group 3 (8.33, $P \leq 0.01$) (Fig 19). Catalase activity was shown to increase significantly in Exp. Group 2 and Exp. Group 3 ($F=11.61$, $P \leq 0.01$) while Exp.Group 1 showed a minute but non-significant increase as compared to the control (Fig 20).

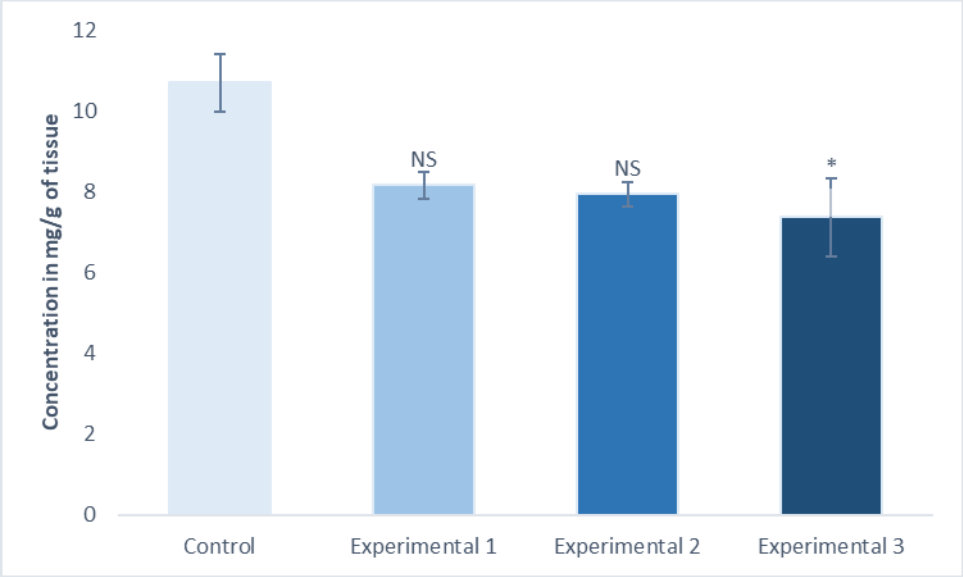

Fig 15: Effect of CdO NPs on Total Proteins content in kidney of mice. NS (non- significant); * ($P \leq 0.05$, significant)

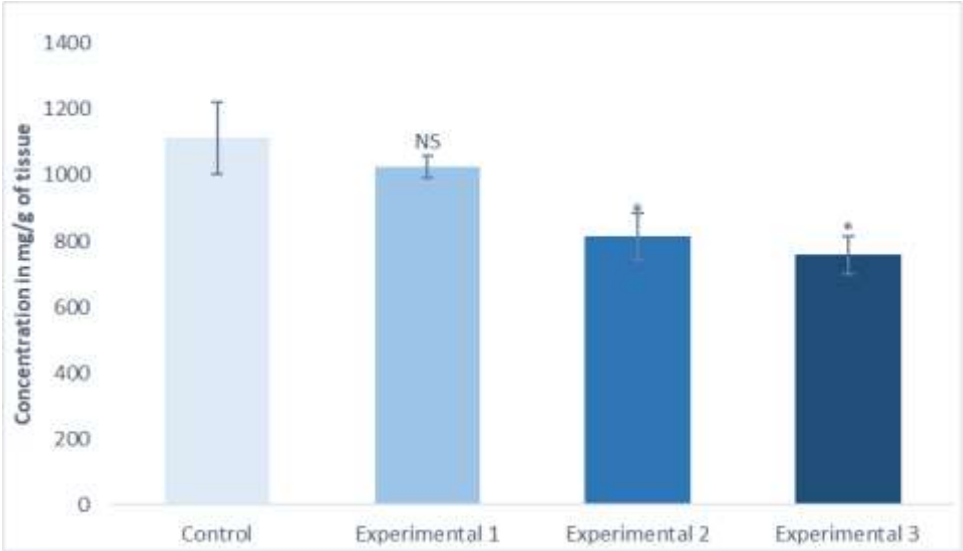

Fig 16: Effect of CdO NPs on Albumin content in kidney of mice. NS (non-significant); * ($P \leq 0.05$, significant)

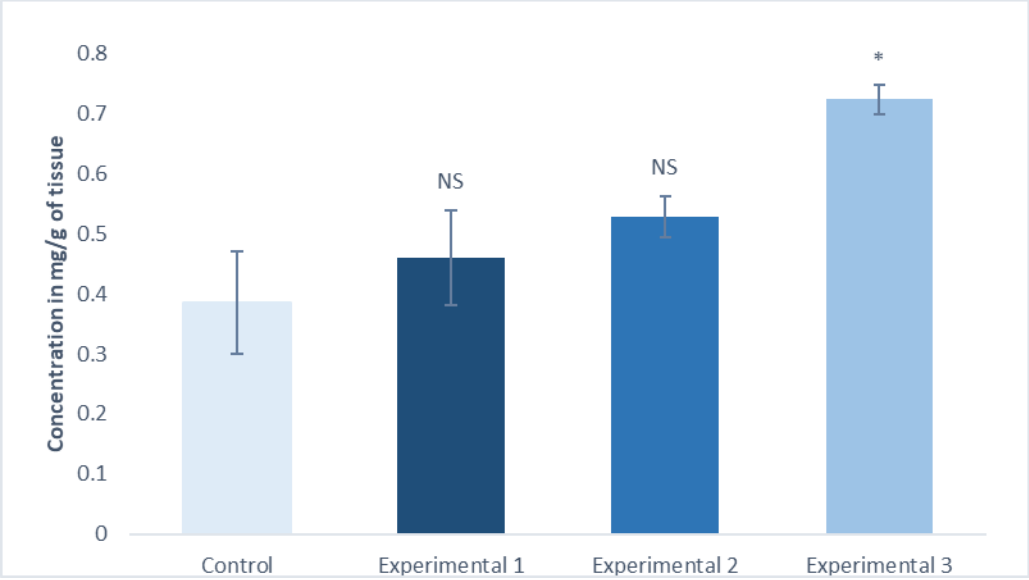

Fig 17: Effect of CdO NPs on carbohydrate content in the kidney of mice. NS (Non- significant); * ($P \leq 0.05$, significant)

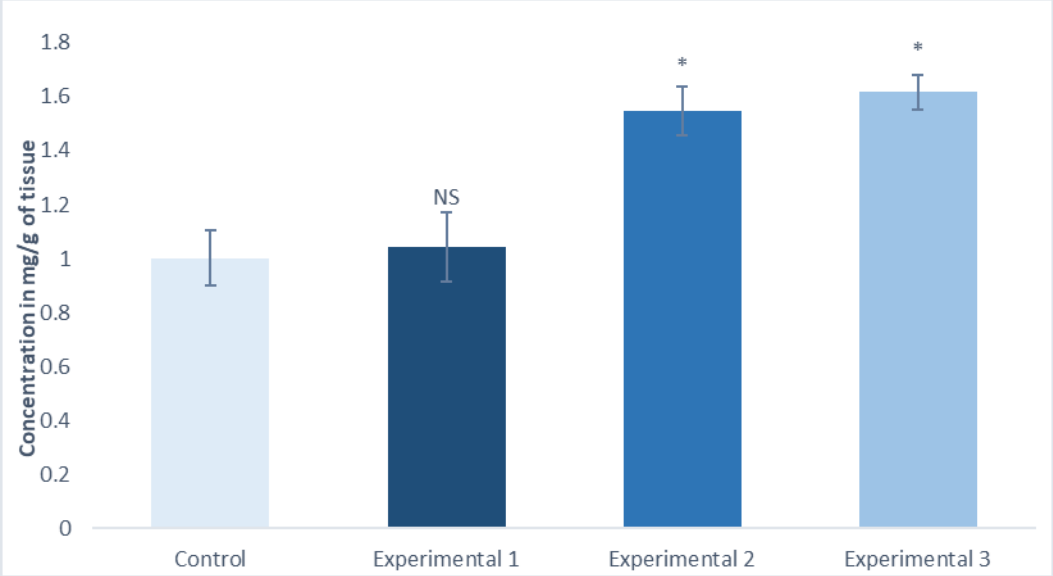

Fig 18: Effect of CdO NPs on Free sugar content in kidney of mice. NS (non-significant); * ($P \leq 0.05$, significant)

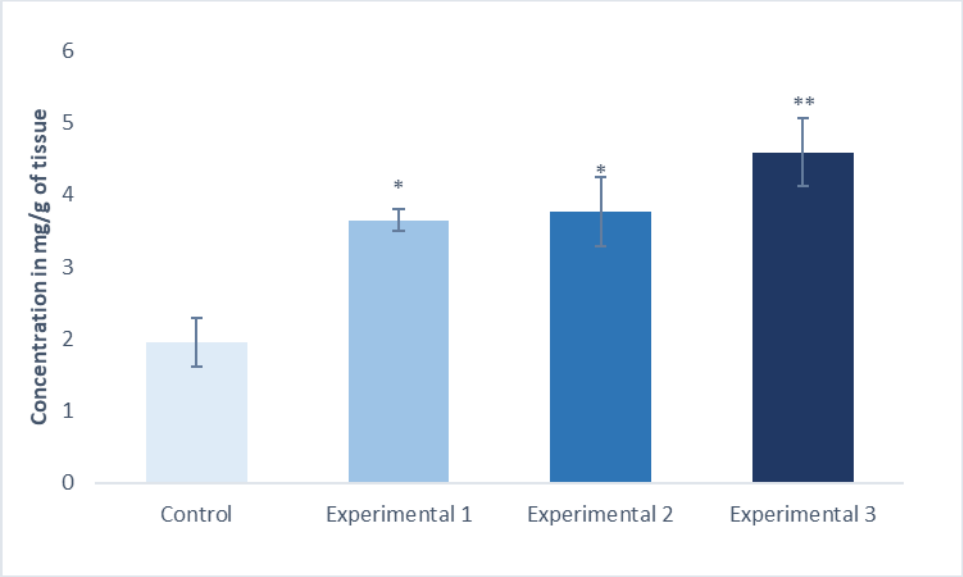

Fig 19: Effect of CdO NPs on reduced glutathione content in kidney of mice. * (P ≤ 0.05 , significant); **(P≤ 0.01, highly significant).

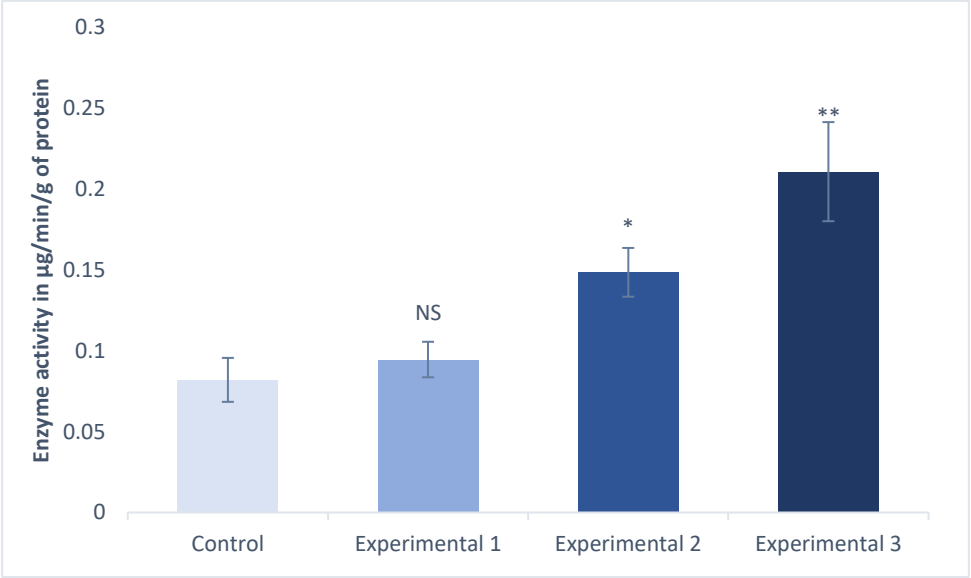

Fig 20: Effect of CdO NPs on catalase activity in kidney of mice. NS (Non-significant); *(P≤ 0.05, significant); **(P≤ 0.01, highly significant)

Urea has shown a gradual significant dose-dependent increase in the blood serum levels of exposed mice ($F= 9.507$, $P \leq 0.01$) with the highest significant increase observed in Exp. Group 3 followed by Exp. Group 2 while Exp. Group 3 showed a non-significant increase as compared to the control (Fig 21). Upon exposure, the creatinine levels have shown a significant increase in the blood serum ($F=12.35$, $P \leq 0.01$) of Exp. Group 2 and Exp. Group 3 while the increase in Exp. Group 1 was non-significant as compared to the control (Fig 22).

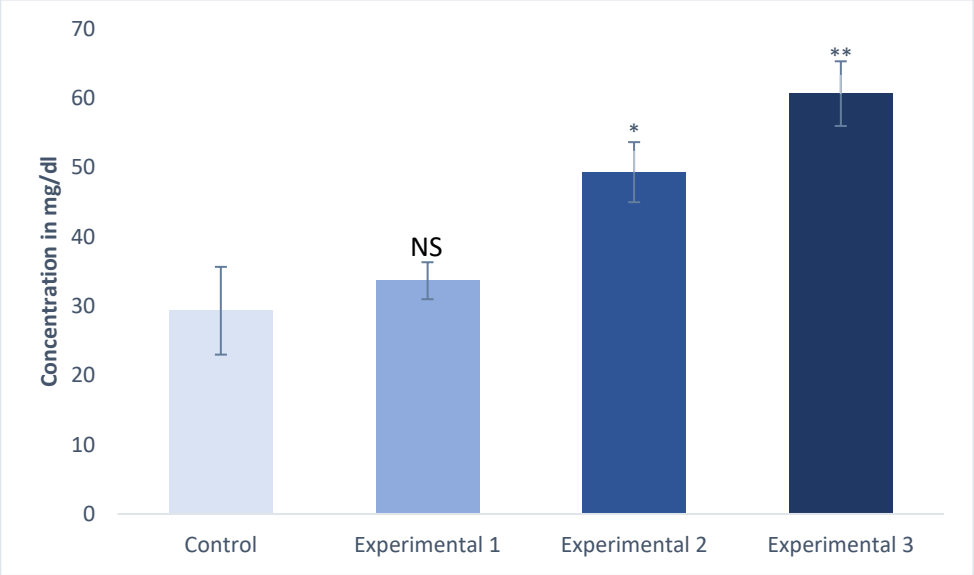

Fig 21: Effect of CdO NPs on Urea content in blood serum of mice. NS (Non-significant); * (P≤ 0.05, significant) **(P≤ 0.01, highly significant).

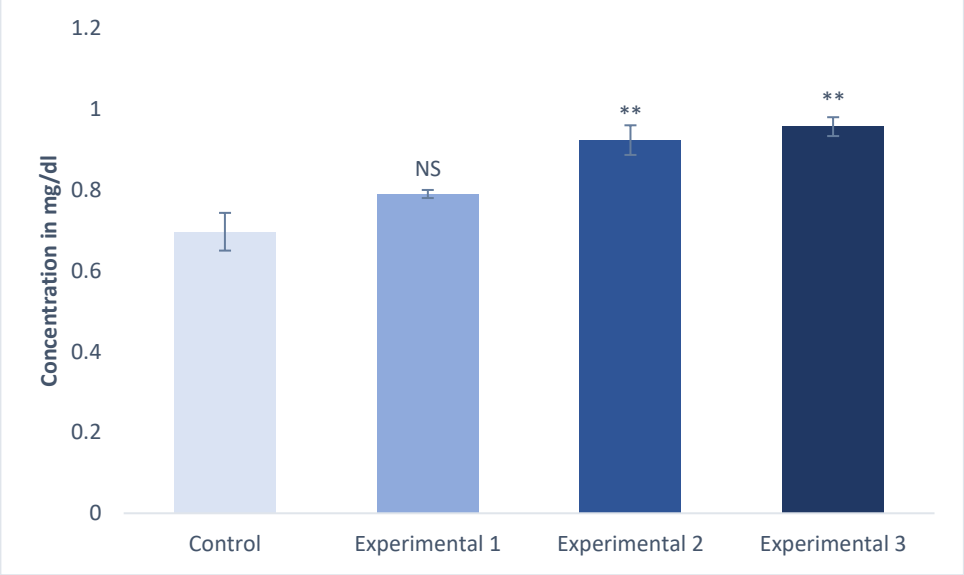

Fig 22: Effect of CdO NPs on creatinine levels in blood serum of mice. NS (Non-significant); **(P≤ 0.01, highly significant).

CHAPTER 5

DISCUSSION

5. DISCUSSION

There has been an increasing demand for nanoparticles for biomedical purposes but our knowledge and understanding of the effect of these nanoparticles on living cells and the biochemical indices still remain insufficient. Hence there is a need to encourage studies aiming to determine biochemical evaluations of nanoparticles (Sulaiman et al., 2015).

Cadmium compounds have long been used in the manufacture of batteries, dyes, and fire retardants, among other things with its most recent use is that it has been found to possess qualities that make them attractive in a wide range of industrial and medical uses. While the potential for occupational exposure to Cd-based NP remains a possibility, little is known about their toxicity or whether adverse effects are similar to those produced by other Cd forms. Hence, this study investigated the toxicity of CdO NPs on different parameters associated with morphology, behavior, anatomy, and liver and kidney functioning.

5.1. Determination of LD_{50}

This is the first paper reporting the LD_{50} of CdO nanoparticles. By following the OECD guidelines, the LD_{50} of CdO NPs was found to be 90 mg/kg which according to Hodge and sterner scale is considered to be class 3 (moderately toxic) but according to Gosselin, Smith and Hodge it belongs to class 4 (very toxic). According to "Globally Harmonized System of Classification and Labelling of Chemicals", it was found that the LD_{50} of CdO NPs belongs to 'Category 3' for acute toxicity (M. Ahmed, 2015, United Nations. Economic Commission for Europe. Secretariat., 2011).

The median lethal dose for Cd as mentioned by "Hazardous Substances Data Bank" is 890 mg/kg of body weight. The LD_{50} for other cadmium nanoparticles such as $CdCl_2$ was found to be lower than 137mg/kg, CdTe were found to be higher than 2000 mg/kg, CdS nanodots and CdS nanorodes were found to be 767 and 7203 mg/kg respectively (Zayed & Philippe, 2009, Liu et al., 2014). Therefore, compared to the other Cd forms, the LD_{50} of CdO NPs was found to be much less attributing to the fact that lesser amounts of this nanoparticle are enough to cause deleterious changes in an organism. This toxicity could be due to its size and route of exposure as the property of any compound changes with respect to its size as well as the route of exposure since the NPs will pass through a different sequence of organs depending on their route (Zayed & Philippe, 2009).

5.2. Effect of CdO NPs on Morphology of *Mus musculus*

CdO NPs has shown no effect on skin colour, hair texture, eye colour, and tail spots. Only loss of hair was observed in the head region which was more evident in the group that received the highest dose. Thinning hair and hair loss are common signs of poisoning attributing to the ability of these nanoparticles to damage the hair follicles resulting in excessive hair shedding and impaired hair growth (Hounkpatin et al., 2013).

The weight of the mice showed a significant decrease as compared to the control which was more evidently seen in Exp. Group 2 and Exp. Group 3. The decrease in the weight may form part of the symptoms of toxic events consequent upon CdO NPs exposure. Studies have linked changes in body or organ weight to toxicity of administered chemical or toxicant (Sulaiman et al., 2015).

5.3. Retention of CdO NPs in liver and kidney

The nanoparticles when ingested into the body can be distributed to different regions because of their small size. Many tissues have fenestrations present between the endothelial cells, thus making it possible for nanoparticles to pass into the surrounding. The size of the fenestrae varies, for instance liver have fenestrae up to 150nm while kidneys have a fenestra of 20-30nm. Under disease conditions, the endothelium becomes leakier, thereby allowing larger particles to exit from the circulation and accumulate in the organs (Skotland et al., 2010) Only about 4.4- 4.8 % and 0.96 – 1.16 % of the total amount gavaged has been found to retain in the liver and kidney respectively. The rest of the nanoparticles have possibly been absorbed by the other body tissues or have been excreted out. The CdO NPs were mainly found to be retained in the liver as compared to the kidney after 14-day sub-acute exposure (Sharma et al., 2012). Abdel-Moneem Ali et al., (2021), attributed the trapping of NPs by the hepatic tissue to remove them from circulation. Additionally, the glomeruli in the kidney have slit pores of 3.5nm in diameter hence allowing NPs accumulate inside the kidney.

5.4. Histopathological examination of major organs of *Mus musculus*

Histopathological examinations help to identify and characterize any test-article-related changes that occur in tissues and organs thereby helping to decide their pathologic importance to the overall health of the animal and also predict their relevance to humans (Sills et al., 2019).

In accordance with histopathological changes, the liver is known to be a primary target organ of the toxic effects due to its function in drug biotransformation, high metabolic potential, and ability to clear xenobiotics from the blood therefore, it can be used as a toxicity index for various toxic materials (M. S. Ahmed et al., 2020). In this study, the liver of the control group (A) showed normal, intact hepatic architecture without any pathological changes. The histopathological evaluation of the groups treated with CdO NPs showed disruption in the hepatic architecture, pycnotic nucleus, dilated sinusoids, aggregation of inflammatory cells, enlarged and congested Portal vein and hepatocytes showing cytoplasmic vacuolation. These findings are in line with Hegazy et al., (2018), Ghonimi et al., (2022) and Kassab et al., (2020) who worked on the toxicity of GNPs, CuO NPs and ZnO NPs respectively thus justifying our present results as liver damage could be a consequence of CdO NPs toxicity.

The disruption in the hepatic architecture along with dilations of the sinusoids could indicate the necrotic effect of CdO NPs on liver tissue (A. Hegazy et al., 2018). In our study, liver cells showed cytoplasmic vacuolation which is an indicator of acute liver injury. This could be attributed to the effect of CdO NPs to damage the cell membrane thereby causing Na^+ and water influx resulting in cellular swelling, lysosomal damage and cytoplasmic degeneration. Moreover, CdO NPs also induced nuclear changes such as pyknotic nuclei which is an indicator of cell death and liver injury due to protein metabolism disturbances. Another observation was the increase in the number of Kupffer cells in the liver exposed to CdO NPs. This could attribute to the ability of these nanoparticles to activate the phagocytic activity of the Kupffer cells to remove these

nanoparticles. This may be a defense mechanism and may also contribute to hepatic oxidative stress. Inflammatory cellular infiltration was also observed possibly due to the ability of these nanoparticles to interact with hepatic interstitial tissue thereby interfering with the antioxidant defense mechanism and initiating an immune response (Kassab et al., 2021). Furthermore, the portal area revealed dilation and congestion of central and portal vein which could be caused due to portal hypertension (A. Hegazy et al., 2018).

Another major organ 'Kidney' is responsible for basic function of excretion and metabolism and is a potential target of CdO NPs since it is one of the vital organs in addition to its innate function as a blood filter. The control group showed normal architecture with intact glomeruli, renal tubule and interstitial tissues of both cortex and medulla. The mice treated with CdO NPs showed enlarged and congested blood vessels, cytoplasmic vacuolization, accumulation of inflammatory cells around the distorted glomerulus and cloudy swelling of the epithelial cells of renal tubules. These findings are in agreement by Al. Doiass et al., (2019), Elbanna et al., (2017), and Elwan et al., (2018), who worked on the toxic effects of TiO_2 NPs, food dyes and GNP on kidney respectively. The experimental groups more evidently the Exp. Group 2 and Exp Group 3 showed enlarged and congested blood vessels which could be resulted from the vasodilator effect of these nanoparticles and might indicate impact on the cell membrane permeability of renal blood vessel endothelia (Al-Doaiss et al., 2019, Elbanna et al., 2017). The cytoplasmic vacuolation maybe caused due the disturbance of renal cell membrane function leading to massive influx of Na^+ ions (Elwan et al., 2018). Infiltration of inflammatory cells in the CdO NPs treated groups may be due the ability of these nanoparticles to interfere with the

antioxidant defence mechanism hence inducing oxidative stress in the renal tissue thereby leading to the induction of inflammatory response. The cloudy swelling of the renal tubules was more evident in Exp. Group 3 which indicated that acute renal injury could induce disturbances of membranes function leading to leakage and accumulation of water due to CdO NPs toxicity and cytoplasmic degeneration and macromolecular crowding (Al-Doaiss et al., 2019).

5.5. Effect of CdO NPs on liver and kidney functioning

The liver serves as an important core source for albumin synthesis and major source of proteins (Johnston, 1999). The total proteins and albumin levels in the liver have shown a gradual decrease in the experimental groups as compared to the control. This could possibly be due to the reduced ability of the liver to synthesize proteins because of the possible CdO NPs induced damage to the liver cells (Sulaiman et al., 2015, Ghonim et al., 2017). These results can be linked to the ability of the nanoparticle to activate endopeptidases hence leading to protein destruction (Abdel-Moneem Ali et al., 2021). Other studies have also reported a decline in serum total proteins and albumin suggesting the possibility that serum albumin can attach to SiNPs upon the entrance of these NPs inside the blood, however these claims require more investigations in order to be confirmed (Hassankhani et al., 2015).

Similarly, a decrease in total proteins was also observed in the kidney, this could be attributed to increased protein loss resulted from oxidative damage mediated renal insufficiency (Abdel-Daim et al., 2019). The decrease of albumin levels in the kidney could be due to albumin leakage due to glomerular basement damage of membrane combined with increase in the pressure of trans glomerular

filtration (Suhailah & Soheir, 2017). These findings are in line with other studies that also reported decreased total protein and albumin levels due to renal dysfunction (Abdel-Daim et al., 2019, Suhailah & Soheir, 2017, Abdel-Moneem Ali et al., 2021).

The total carbohydrates and free sugars have been found to increase significantly in the liver and kidney of mice gavaged with CdO NPs. This increase could be due to the stress caused by these NPs onto the organs thereby resulting in an elevation of carbohydrate metabolism to meet the energy demands in the affected organs. Ahmed et.al.,(2020), reported that ALP plays an integral role in glycogen metabolism in the liver by stimulating glucose synthesis to overcome energy requirements during stress conditions (M. S. Ahmed et al., 2020).

The NPs have a great potential to interact with the biological system and causing undesirable effects, one of which are the disturbances in the natural balance between oxidative stress and antioxidant defense indices, which in turn can lead to various pathological effects. The oxidative stress has been identified as a likely mechanism of nanoparticle toxicity (Anwar Kassem Abdelhalim et al., 2015). Reduced Glutathione (GSH) serves as one of the most important antioxidants converting peroxides into oxidized glutathione thus contributing in significantly eliminating peroxides in the enzymatic reactions. In the present study, exposure to the CdO NPs has caused a significant increase in GSH, which could be due to increase in lipid peroxidation in liver and as a preventive measure in kidney (Anwar Kassem Abdelhalim et al., 2015). This antioxidant biomarker was possibly initiated to antagonize free radicles i.e., over production

of H_2O_2 in mice exposed to nanoparticles (Wang et al., 2009). Similar findings have been reported by Wang et al., (2009), who reported increased levels of GSH-Px, GSH, GSSG and SOD in TiO_2 exposed groups thereby indicating oxidative stress.

Catalase (CAT) is an important enzyme that uses hydrogen peroxide, a nonradical ROS, as its substrate. This enzyme plays a role in neutralization through decomposition of hydrogen peroxide, thereby maintaining an optimum level of the molecule in the cell which is also essential for cellular signaling processes (Nandi et al., 2019). Current study revealed a dose dependent increase in the activity of CAT in both liver and kidney as a defense mechanism against excess H_2O_2 levels as CAT needs high levels of H_2O_2 to commence its activity (Reddy et al., 2017, Anwar Kassem Abdelhalim et al., 2015). Increase CAT activity is supported by other findings where Reddy et al., (2017) reported a significant increase in CAT, GR, GPx, GST and decrease in SOD activity in rat treated with iron oxide nanoparticles. Similarly, Prabhakar et al., (2012) also reported increase in the activities of CAT and GST with depleted SOD activity in rat tissues exposed to aluminum oxide NPs.

Transaminases and phosphatases are critical enzymes in biological processes and are considered specific biochemical indicators of liver damage (M. S. Ahmed et al., 2020). Alkaline phosphatase plays an integral role in glycogen metabolism in the liver by stimulating glucose synthesis to overcome energy required during stress conditions (M. S. Ahmed et al., 2020). While enzymes such as ALT and AST are the metabolic enzymes in liver, these enzymes are dysfunctional in serum and plasma. The level of these enzymes in the cytoplasm

of liver cells is number of times more than extracellular fluid but when hepatic cells are damaged the amount of these enzymes raise in the blood stream and this elevation is an indication of the liver damage (Vasantharaja et al., 2015). Thereby, elevation of all these enzymes may reflect some necroinflammatory disease of the liver. In our present study, mice exposed to different doses of CdO NPs, showed elevated levels of ALT, AST and ALP as compared to the control thereby indicating liver damage hence causing these enzymes in the hepatocyte to be released into the bloodstream, thus this positively correlated with the observed histopathological changes in the liver tissue. Many studies are in line with these results showing increase in ALT and AST activity due to liver damage caused by ZnO (Sharma et al., 2012), Abdel-Daim et al., 2019), TiO_2 NPs (Vasantharaja et al., 2015), SiNPs (Azouz & Korany, 2021), ZrO_2 NPs (Arefian et al., 2015), AgNPs (Parang & Moghadamnia, 2018) and Cymoxanil (M. S. Ahmed et al., 2020).

Meanwhile, kidney helps in the elimination of unsafe substances from the blood and thus NPs that are absorbed in the circulatory system can be filtered by renal system (Vasantharaja et al., 2015). However, kidney dysfunction was found in the mice treated with CdO NPs due to elevated creatinine and urea levels in the serum. Urea and Creatinine is the major constituent of the kidney accounting for up to 80- 90% of non-protein nitrogenous waste excretion. They play a major role in evaluating renal function thus are used as markers to evaluate kidney functioning. Current study showed increased levels of urea and creatinine levels possibly reflecting reduction of glomerular filtration rate (Azouz & Korany, 2021). Histologically, this was evidenced by the observed glomerular alterations in mice administered with CdO NPs. The increase in urea and creatinine levels

are consistent with evidence provided by Sadek et al., (2017), Najafzadeh et al., (2013), Azouz & Korany, (2021), and Abdel-Moneem Ali et al., (2021).

SUMMARY

6. SUMMARY

Nanotechnology is become the new hotspot of research in the world due to its increasing applicative domains. However, we cannot only pay attention to its benefit to the society and economy, because its wide use has been bringing potential environmental and health effects that should be noticed. CdO NPs is 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 divided into 4 groups with 1 control group and 3 Exp. Groups. In order to estimate the dose to be given, the LD_{50} was carried out following the OECD guidelines. Once the doses were determined, the mice were subjected to oral gavaging of different concentrations of CdO NPs (4.5 mg/kg body weight, 9 mg/kg body weight, and 18 mg/kg body weight) for a period of 14 days.

The effects of these CdO NPs were evaluated on the morphology, anatomy, and liver and kidney functioning of mice. Oral gavaging of CdO NPs had significant effects on *Mus musculus*. The retention of CdO NPs was found to be higher in liver as compared to kidney.

Different anatomical changes were also of focus relaying the damage caused by CdO NPs in both liver and kidney via aggregation of inflammatory cells in the tissue and distortion of the intact hepatic and renal architecture was observed. The liver and kidney functioning were affected by the dysfunctioning of

different enzymatic (catalase) as well as non-enzymatic antioxidants (Reduced glutathione) therefore indicating that these nanoparticles caused oxidative stress in the tissues.

The elevation of liver function enzymes in the serum (ALT, AST and ALP) indicated hepatic damage. Additionally, other metabolites such as total proteins, Albumin, Total carbohydrates and free sugars were also observed to be altered significantly due to these nanoparticles. Renal function tests showed high levels of creatinine and urea in the serum, hence indicating impaired renal function. Thus, the current study report as a whole reveals the adversities caused by CdO NPs when exposed to oral gavaging

CONCLUSION

In this study, the potential toxicological effects of orally gavaged CdO NPs were investigated on major organs (kidney and liver) using three different concentrations. The slightly pathological changes of the liver and kidney was induced by the lowest dose while severe pathological injury to the organs was observed after the exposure to middle and high-dose of CdO NPs which are consistent with the changes of serum biochemical parameters. The altered antioxidant status in the study can be possibly due to the accumulation of CdO NPs in the tissues that trigger ROS generation since CdO with small aggregated size have been found to aggregate in both liver and kidney. Thus, the present study CdO has shown to have adverse effects on the physiology and anatomy of Mus musculus and therefore, the oral exposure of these nanoparticles can cause detrimental effects on humans.

FUTURE PROSPECTS

- Further studies can be conducted to check if the toxicity of CdO NPs differs with respect to genders.
- The clearance rate of CdO NPs from different organs can also be investigated.

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