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AN *IN-VIVO* COMPARATIVE STUDY OF CERIUM TOXICITY IN BULK FORM AND IN NANOPARTICULATE FORM

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Key words:

Nanotechnology, Cerium, Toxicity, Hematological and Biochemical parameters.

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ABSTRACT: Scope: In recent years, governments and industries worldwide have invested billions of dollars in nanotechnology research and development, and proponents predict that it will offer numerous benefits to society. **Objectives:** To evaluate the toxicity of Cerium in nanomaterials and in bulk form in rats by repeated daily oral treatment for 28 days by monitoring the effects on animal and also to assess the hematological and various biochemical parameters in serum and targeted biochemical parameters. **Results and discussion:** The 28 days repeated dose oral toxicity showed CeO_2-NM and CeO_2-Bulk did not affect the body weight gain and feed intake of the treated rats. Further, CeO_2-NM significantly inhibited some of the hematological parameters like platelets count, granulocytes, % hematocrit and biochemical parameters in both male and female rats in dose dependent manner. However, CeO_2-Bulk could not induce any significant alterations in these parameters and the changes were near to control in both male and female treated rats. **Conclusion:** These results suggested that as particle size decreases, showed increased toxicity, even though the same material is relatively inert in bulk form. These studies highlight the need for further experimentation to fully elucidate the mechanisms responsible for the behavior of nanomaterials specifically to develop a comprehensive evaluation, of the human health implications, from exposure to nanoscale materials.

INTRODUCTION: Nanotechnologies are defined as activities that include "the manipulation, precision placement, measurement, modeling, or manufacture of sub-100 nanometer scale matterni, Nano materials are typically defined as materials that have at least 1 dimension < 100 nano meters. They have properties that make them useful for many applications such as high conductivity, strength, durability, and reactivity.

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'Moreover, a variety of different kinds of nanotechnologies and nanomaterials often called "bionanotechnologies," are being developed for biomedical purposes.) Bionanotechnologies are modeled after biological substances and structures, or combine nanomaterials with biological substances. They include materials such as biochips, drug release systems, nanofibres and hybrid nano-biodevices, molecular electronics and biomimetics (synthetic genes, proteins, and viruses). Nanoparticles have been manufactured from various materials, with unique architectures to serve as a possible drug vehicle to treat a particular disease. Generally, nanoparticles have been made of polymers, ceramics, metals, and biological materials. Nanoparticles might adopt spherical,

branched, or shell structures, depending on the particular therapy to be employed. Nevertheless, pharmaceutical nanocarriers must be manufactured from biocompatible materials, and their quality, safety and efficacy have to be demonstrated by appropriate pre-clinical and clinical studies.

Applications of Nanotechnology: Discovery of biomarkers: Nanotechnology is being applied to biomarker based proteomics and genomics technologies. Nanoparticles (NP) can be used for qualitative or quantitative *in vivo* or *ex vivo* diagnosis by concentrating, amplifying and protecting a biomarker from degradation in order to provide more sensitive analysis. For instance, *in vitro* streptadivin coated fluorescent polystyrene nanoparticles have been used to detect the epidermal growth factor receptor (EGFR) in human epidermal carcinoma cells by flow cytometry. A large amount of free serum prostate serum antigen (PSA) could be detected in patients suffering from prostate cancer or even women suffering from breast cancer with a great improvement in tumor screening and diagnosis [25, 29, 30].

Molecular diagnosis:
Nanoparticles are being currently tested for molecular imaging in order to achieve a more precise diagnosis with high quality images. In fact, contrast agents have been loaded on to nanoparticles for tumor and atherosclerosis diagnosis. The physicochemical characteristics of the nanoparticles allow the redirection and the concentration of the marker at the site of interest. Different nanoparticles have been used for molecular imaging and magnetic resonance images (MRI), ultrasound, fluorescence, nuclear and computed tomography imaging. It has been shown that ultra small superparamagnetic iron oxide particles enhanced the MRI signal of the thrombus in an experimental animal model developed in rabbits.

Drug delivery:

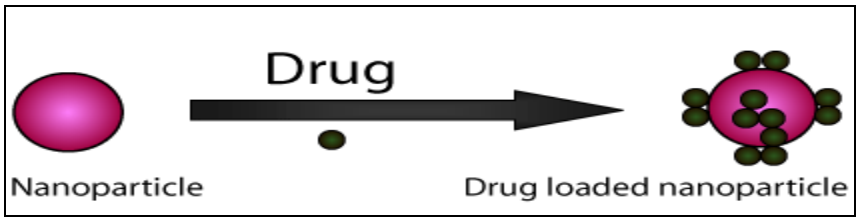

FIG. 1: SHOWING TARGETED DRUG DELIVERY

Site-specific targeted drug delivery is important in the therapeutic modulation of effective drug dose and disease control; targeted encapsulated drug delivery using NP is more effective for improved bioavailability, minimal side effects, decreased toxicity to other organs, and is less costly [9, 14].

Anticancer therapy:
Conventional anticancer treatments are non specific to target killing of tumor cells, may induce severe systemic toxicity, and produce drug resistant phenotypic growth. An exciting potential use of nanotechnology in cancer treatments is the exploration of tumor specific thermal scalpels to heat and burn tumors.

Gene therapy:
Attempts to cure genetic diseases by transfer of somatic cells transfected with normal genes gained popularity in the last two decades. In gene therapy a normal gene is inserted in place of an abnormal disease causing gene using a carrier molecule. Conventional uses of viral vectors are associated with adverse immunogenic, inflammatory reactions, and diseases in the host in this regard found(STP based gene therapy to be effective in systemic gene treatment of lung cancer using a novel tumor suppressor gene, FUS1 thitosan, a polymer long used in gene therapy, was reported to have increased transfection efficiency and decreased cytotoxicity.

From these studies, it is apparent that nanotechnology will profoundly affect human health through advances in medicine, science and industry. The potential human benefits of nanotechnology are innumerable and include many aspects of human life with wide variety of products.

Development of Nanotechnology:

Nanotechnology is a natural end-result of scientific development and our ability to understand and manipulate matter at smaller and smaller levels. Just as computers have gone from bulky, room-filling monstrosities to handheld computers, such reductions in size will continue until we reach fundamental physical limits. The National Nanotechnology Initiative in the United States defines nanomaterials generally as follows: Nanomaterials is a term that includes all nano sized materials, including engineered nanoparticles, incidental nanoparticles and other nano-objects, like those that exist in nature. Meanwhile, the American Chemistry Council--Nanotechnology Panel has proposed a separate definition for Engineered Nanomaterials.

"The ACC Nanotechnology Panel believes that definitions used to describe Engineered Nanomaterials are important because they will be used to guide the public when information requests are made by regulators and NGO's. It is desirable that the definitions be as simple as possible yet not so broad that the collection of meaningless information is encouraged." For this reason, the ACC has proposed the following definition for Engineered Nanomaterials: An Engineered Nanomaterial is any intentionally produced material that has a size in 1, 2, or 3-dimensions of typically between 1-100 nanometers. It is noted that neither 1 nm nor 100 nm is a "bright line" and data available for materials outside of this range may be valuable. Buckyballs are also included even though they have a size <1 nm. Various compounds were used in form of nanoparticles [42, 47].

Cerium: Cerium is a member of the lanthanide series of metals and is the most abundant of the rare earth elements in the earth's crust (avg concentration of 50 ppm), Elemental Cerium is an irony –gray, ductile, malleable metal. Cerium metal is very reactive and is a strong oxidizing agent that is stabilized when associated with an Oxygen Ligand. When present in compounds, cerium exists in both trivalent state (Ce^{+3}, Cerous) and the tetravalent state (Ce^{+4}, Ceric). Cerium is found in nature along with other Lanthanide elements in the minerals alanithe, bastanite, monazite, cerite, however, only bastanite and monazite are important sources commercially. Cerium(IV) oxide is formed by the calcination of cerium oxalate or cerium hydroxide.

Biomedical applications [17, 31]:

As Neuroprotective: Cerium oxide nanoparticles are able to rescue cells from oxidative stress-induced cell death in a manner that appears to be dependent upon the structure of the particle but independent of its size within the 6–1000 nm range. There are three alternative explanations for the observation that the cerium oxide particles protect from oxidative stress. It may act as direct antioxidants, they may block ROS production in HT22 cells by inhibiting a step in the programmed cell death pathway, or they may directly cause a low level of ROS production that rapidly induces a ROS defense system before the glutamate-induced cell death program is complete.

As Anti Inflammatory agent:

ROS are a major constituent of inflammation that can affect normal cellular function and have pathogenic consequences. ROS can directly damage cell membranes by oxidatively modifying fatty acid components of the phospholipid bilayer. They can also damage DNA and proteins, compromise cellular repair mechanisms, cause premature aging, and trigger apoptotic processes. Quenching ROS production can decrease inflammation and subsequent tissue damage. In our current studies, we observed the quenching of ROS and the iNOS inflammatory mediator in immune cells by the unique scavenging ability of nanoceria. The paramount characteristic of cerium oxide is its ability to scavenge free radicals, which is accomplished through its ability to reversibly switch between the 3þ and 4þ oxidation states. The reversibility of the oxidation states at relatively lower oxidation potentials also renders regenerative properties to nanoceria.

These regenerative antioxidant properties are due, in part, to the valence structure of the cerium atom combined with inherent defects in the crystal lattice and oxygen defect structure. [14, 16] Ceria nanoparticles undergo easy, fast, reversible reduction to substoichiometric phases and can readily take up and release oxygen, alternating between CeO_2 and $_{CeO2}$–x. [7, 27] Analysis of cerium

oxide nanoparticles has shown that maximizing the 3p oxidation state in nanoceria is imperative to its radical scavenging ability. Generation and proliferation of free radicals inside the body is caused by expression of pro-inflammatory enzymes such as iNOS. The iNOS protein can be activated by macrophages and other ROS to produce NO.

As Antioxidant:

In the last years, increasing biological interest is emerging for nanotechnology that can improve pharmacological treatments, by using nanomaterials. In particular, cerium oxide nanoparticles, considered one of the most interesting nanomaterials for their catalytic properties, show a promise for application in therapy. Due to the presence of oxygen vacancies on its surface and auto regenerative cycle of its two oxidation states, Ce3+ and Ce4+, nanoceria can be used as an antioxidant agent. Because many disorders are associated with oxidative stress and inflammation, cerium oxide nanoparticles may be a tool for the treatment of these pathologies.

As Anticancer agent:

The diverse abilities of cerium oxide nanoparticles (CONPs) have encouraged researchers to pursue CONPs as a therapeutic agent to treat a number of diseases, including cancer [24]. *In vitro* and *in vivo* studies have shown CONPs to be toxic to cancer cells, inhibit invasion, and sensitize cancer cells to radiation therapy [3-5, 22].

Review of literature:

Effects of nanoparticles in humans:

Humans have been exposed to nanoparticles throughout their evolutionary period; however, this exposure has been increased to a greater extent in the past century because of the industrial revolution [11, 16, 41]. Nanoparticles constitute a part of Particulate Matter (PM). Epidemiological studies have shown that urban pollution with airborne PM deriving from combustion sources such as motor vehicle and industrial emissions contribute to respiratory and cardiovascular morbidity and mortality [20].

The growing use of nanotechnology in high-tech industries is likely to intentionally generated engineered nanoparticles [23]. Nanotechnology is also being applied in medical sciences. However, the same properties (small size, chemical composition, structure, large surface area and shape), which makes them so attractive in medicine may contribute to the toxicological profile of nanoparticles in biological systems. One of the most important portals of entry and organ target for nanoparticles is the respiratory system. It is well known that lungs are easily exposed to atmospheric pollutants such as particulate matter and many other products of thermo degradation. In this regard, combustion-derived nanoparticles have been largely studied as a possible etiologic factor for several adverse health effects, including exacerbations of airways disease as well as deaths and hospitalization from cardiovascular disease along with cytotocxic effects [15].

One of the main mechanisms of lung injury caused by combustion-derived nanoparticles is via oxidative stress leading to activation of different transcription factors with up regulation of pro-inflammatory protein synthesis [8, 10, 37]. In fact, activation of mitogen-activated protein kinase and nuclear factor-kappa B signal pathways by combustion-derived nanoparticles can culminate in transcription of a number of pro-inflammatory genes such as IL- 8, IL-6 and TNF a. It has been found that nanoparticles can induce increased lung toxicity compared to larger particles with same chemical composition at equivalent mass concentration. In addition, it has been also shown that nanoparticles of different diameters can induce inflammatory reactions in lungs of experimental animals. In fact Single Walled Carbon Nano Tubes (SWCNT) has been found to be more toxic compare to other nanoparticles in inducing doses-dependent epithelioid granuloma and intestinal inflammation in lungs [38]. Therefore, these results indicate that nanoparticles can lead to inflammatory and granulomatous responses in lungs and this could have important applications for human risk assessment [18, 36].

Berry et al. (1977) showed for the first time that nanoparticles can be rapidly observed in rat platelets after intratracheal instillation of particles of colloidal gold (30 nm). Nemmar et al. (2002) also found that inhaled (99 m) Tc-labelled carbon particles (<100 nm) pass to the blood circulation 1

minute after exposure. However once nanoparticles are translocated into the blood stream they could induce adverse biological effects. It has been previously found that mixed carbon nanoparticles and nanotubes, both Multi Walled Carbon Nano Tubes (MWCNT) and SWCNT, are able to induce platelet aggregation *in vitro* and, in addition accelerate the rate of vascular thrombosis in rat carotid artery.

The translocation of nanoparticles to CNS may not only take place as a result of systemic distribution. The other mechanism involves the uptake of nanoparticles by sensory nerve endings embedded in airway epithelia, followed by axonal translocation to ganglionic and CNS structures. In addition, nanoparticles can be taken up by the nerve endings of the olfactory bulb and translocated to the CNS [12]. It has been found that C60 fullerenes can induce oxidative stress in the brain of largemouth bass via the olfactory bulb. Recent studies have indicated that this translocation pathway is operational for inhaled nanoparticles. It has been shown that the exposure of rats to 13C ultrafine particles (35nm) for 6 hour resulted in a significant increase of 13C in the olfactory bulb on day 1 and this increase was even greater on day 7 day exposure. This result contrasts with 15-day inhalation of larger-sized MnO_2 particles in rats (1.3 and 18um median diameter) where no significant increase in olfactory Mn was found the latter observation could have been expected given that the individual axons of the fila olfactoria (forming the olfactory nerve) are only 100-200 nm in diameter.

However, there are substantial differences between humans and rodents and therefore, these results should be interpreted with caution. In humans, the olfactory mucosa comprises only 5% of the total nasal mucosal surface, where as in rats this amounts to 50%. Interestingly, human associated with increased rate of Parkinson's disease [43]. Recently, it has been found that exposure of PC-12 neuroendocrine cell line to nanosized Mn induced an increased in reactive oxygen species and dopamine depletion. However, further studies are required to evaluate whether Mn nanoparticles can induce dopamine depletion *in vivo*. In it was recognized by Kumagai(1926). That particles could translocate from the lumen of intestinal tract via aggregations of intestinal lymphatic tissue (Peyer's patches) PP, containing M-cells (specialized phagocytic enterocytes). Particulate up take happens not only via the M-cells in the PP and the isolated follicles of the gut-associated lymphoid tissue, but also via the normal intestinal enterocytes. There have been a number of excellent reviews on the subject of intestinal uptake of particles. Uptake of inert particles has been shown to occur trans-cellularly through normal enterocytes and PP via M —cells, and to a lesser extent across para- cellular pathways the kinetics of particle translocation in the intestine depends on diffusion and accessibility through mucus, initial contact with enterocyte or M- cell, cellular trafficking, and post translational events.

Szentkuti observed that cationic nanometer- sized latex particles became entrapped in the negatively charged mucus, whereas repulsive carboxylated fluorescent latex nanoparticles were able to diffuse across this layer. The smaller the particle diameter the faster they could permutate the mucus to reach the colonic enterocytes; 14 nm diameter permeated within 2 min, 415 nm particles were unable to translocate this barrier. Particulates, once in the sub-mucosal tissue, are able to enter both lymphatic and capillaries. Particles entering the lymphatic are probably important in the induction of secretory immune responses while those which enter the capillaries become systemic and can reach different organs.

In one study, the body distribution after translocation of polystyrene particles was examined [13]. Polystyrene spheres(ranging from 50 nm to 3 micron) were fed by gavage to female Sprague Dawley rats daily for 10 days at a dose of 1.25 mg/kg. As much as 34% and 26% of the 50 and 100 nm particles were absorbed respectively. Those larger than 300 nm were absent from blood. No particles were detected in heart or lung tissue. It has been suggested that the disruption of the epithelial barrier function by apoptosis of enterocytes is a possible trigger mechanism for mucosal inflammation. The patho-physiological role of M cells is unclear; e.g., it has been found that in Crohn's disease M cells is unclear; e.g., it has been found that in Crohn's disease M cells are lost from

the epithelium. From the literature cited above it is clear that engineered nanoparticles can be taken up via intestinal tract. In general the intestinal uptake of particles is better understood and studied in more detail than pulmonary and skin uptake. For those nanoparticles designed to stabilize food or to deliver drug via intestinal uptake other, more demanding, rules exist and should be followed before marketing these compounds. Damage to the genomic DNA, both from endogenous and exogenous sources, may have major patho-physiological implications for cells, tissues and organisms. Currently, several NP have been shown to be capable of eliciting damage the nuclear DNA[1]. On a single cell level, such particle induced damage may principally have three major consequences, usually depending on the type and extent of DNA damage, i.e.:

1. Induction and fixation of mutations,
2. Induction of DNA cell cycle arrest, and,
3. Activation of signal transduction pathways which promote apoptosis.

Role of various parameters:

Blood [49] is a complex tissue fluid of red colour derived from embryonic mesoderm. It is slightly alkaline p^H ranges from 7.3 to 7.5. The blood consists of two principal components namely the fluid Plasma and the Blood cells or corpuscles which are found suspended but unattached to one another in the plasma. The blood cells that circulate in the bloodstream are generally divided into three types: Red Blood Cells (Erythrocytes), White Blood Cells (Leukocytes), and Platelets (Thrombocytes). Red blood cells (RBC's) are the most prevalent biconcave disc shaped corpuscles and are nucleated except in mammals with an average diameter of 6-9 µm. They are also called as Red blood Corpuscles, Erythrocytes, Haematids or Erythroid cells.

These cells are synthesized reticuloendothelial cells in red bone marrow of bones. Inside each corpuscle there is an elastic substance called stroma containing a red coloured pigment called Hb hence appears red colour. The normal range of RBC is $4.5\text{-}5.5\times10^6/\mu L$. The biological function of RBC is transport of O_2 from lungs to tissues and CO_2 from tissues to lungs. It also maintains pH of the blood as the Hb acts as buffer system and it also maintain the viscosity of blood. White Blood Cells (WBC) are rounded or irregular, nucleated, colourless due to the absence of pigment Hb. Its size is 12 to 20 µm wide. They are also called as Leukocytes or white blood corpuscles. These cells are synthesized in red bone marrow, lymph node, spleen and thymus. The normal range of WBC is $4.1\text{-}10.9\times10^3/\mu L$. They are called as the cells of the immune system involved in defending the body against both infectious disease and foreign materials. Platelets or Thrombocytes are small oval to spherically shaped, colourless, clear cell fragments (i.e. cells that do not have a nucleus containing DNA) of 2–3 µm in diameter, derived from fragmentation of precursor megakaryocytes. They are a natural source of growth factors. They circulate in the blood of mammals and are involved in haemostasis, leading to the formation of blood clots. They serve as both structural and molecular function in blood clotting.

Haemoglobin (Hb) is the red blood pigment in RBC and it is a conjugated protein consisting of two parts namely the Globulin (apoprotein) and the Haeme (non protein moiety-prosthetic group). It is a tetrameric allosteric protein with two alpha (α) and two beta (β) polypeptide chains held by non covalent interactions. Each subunit contains haeme with iron in ferrous state (Fe^{+2}). The important biological function of Hb is delivery of oxygen (O_2) from lungs to tissues and transport of carbon dioxide (CO_2) and protons (H^+) from tissues to lungs for excretion, hence it is referred to as a respiratory pigment. Hb estimation is one of the most common screening tests for the diagnosis of anemia. If the concentration of Hb is below normal range it is called as anemia and if higher called as polycythemia.

The haematocrit (HCT) or packed cell volume (PCV) or erythrocyte volume fraction (EVF) is the percentage of blood volume that is occupied by red blood cells. A low haematocrit with a low Mean Corpuscular Volume (MCV) with a high Red blood cell Distribution Width (RDW) suggests a chronic iron-deficient erythropoiesis, but a normal RDW suggests a blood loss that is more acute, such as a

hemorrhage. Higher haematocrit value is a danger sign of an increased risk of dengue shock syndrome. If a patient is dehydrated, the hematocrit may be elevated.

Clotting time (CT): is the time required for a sample of blood to coagulate under standard conditions. Clotting is the formation of a jelly like substance over the valves of the vessels which result in the stoppage of blood flow. Clotting is one of the natural defense mechanism to prevent blood loss from the body. A clot is usually formed within 5 minutes after injury. The fibrinogens are essential for the clotting of the blood. Clotting time is affected by calcium ion and many diseases. The normal value of clotting time is 5-8 minutes. Increase in the value causes heat stroke, hemophilia, and leishmaniasis where as decreased values causes eclampsia.

Biochemical parameters: Calcium (Ca^{+2}) is an important component of a healthy diet and a mineral necessary for life [51]. It is an essential element for living organisms, particularly in cell physiology, where movement of the calcium ion (Ca^{2+}) into and out of the cytoplasm functions as a signal for many cellular processes. It is also a major material used in mineralization of bones. [48] The National Osteoporosis Foundation says, "Calcium plays an important role in building stronger, denser bones early in life and keeping bones strong and healthy later in life." Its normal range is 8.5-10.5 mg/dL. Elevated calcium values are associated with hyperparathyroidism, multiple myeloma, and neopalasias of bone and rapid demineralization of bone. Lowered Calcium values are associated with hypoparathyroidism, tetany and pancreatitis. Either elevated calcium or lowered calcium values cause severe nephritis and uremia.

Creatinine (CRE) is a chemical waste product formed during muscle metabolism from high energy storage compound creatine phosphate. It is usually produced at a constant rate by the body (depending on muscle mass). Its normal range is 0.5-0.9 mg/dL in females and 0.6-1.1 mg/dL in males. CRE is a reliable indicator of renal function. CRE is transported through the bloodstream to the kidneys where the kidneys filter (glomerular filtration and proximal tubular secretion) out most of the creatinine and dispose of it in the urine. If the filtering of the kidney is deficient, creatinine levels in the blood rises. Increased serum CRE is an indicator of kidney failure or muscular dystrophy [46]. Low serum Creatinine increased the risk of type 2 diabetes. Chloride ion (Cl^-) is an essential electrolyte located in all body fluids responsible for maintaining acid/base balance, transmitting nerve impulses and regulating fluid in and out of cells. The amount of chloride in the blood is controlled by the kidneys. Its normal range is 98-107 mEq/L.

Determination of the chloride concentration in serum has an important diagnostic value in reviewing the maintenance of osmotic pressure, water distribution and pH of the body. Increase in the concentration of chloride causes hyperchloremia (dehydration, acute renal failure, metabolic acidosis associated with prolonged diarrhoea and respiratory alkalosis) where as decrease in the concentration causes hypochloremia [40].

Glucose also known as D-glucose, dextrose, or grape sugar is a simple sugar (monosaccharide) and an important carbohydrate in biology. Cells use it as the primary source of energy and a metabolic intermediate. It is one of the main products of photosynthesis and starts cellular respiration. It is a primary source of energy for the brain, and hence its availability influences psychological processes. It is a common medical analyte measured in blood samples. Higher than usual glucose levels may be a sign of prediabetes or diabetes mellitus. When glucose is low, psychological processes requiring mental effort (e.g., self-control, effortful decision-making) are impaired (American Diabetes Association, 2010,).

It is used to detect both hyperglycemia and hypoglycemia, to help diagnose diabetes. Other diseases and conditions that can result in elevated glucose levels include acromegaly, acute stress (response to trauma, heart attack, and stroke for instance), chronic renal failure and cushing syndrome. Low blood glucose levels (hypoglycemia) are also seen with adrenal insufficiency, extensive liver disease, hypopitutarism and hypothyroidism.

Alkaline Phosphatase (ALP/ALKP) is a hydrolaseenzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. Its normal range is 60-170 U/L. The ALP is used to detect liver disease or bone disorders. In conditions affecting the liver, damaged liver cells release increased amounts of ALP into the blood. It is also used to detect blocked bile ducts because ALP is especially high in the edges of cells that join to form bile ducts. If one or more of them are obstructed, for example by a tumor, then blood levels of ALP will often be high. Signs and symptoms of liver are weakness, fatigue, loss of appetite, nausea, abdominal swelling and/or pain. Signs and symptoms of a bone disorder are bone and/or joint pain, increased frequency of fractures, deformed bones.

Proteins are the most abundant compounds in our body. They are the building blocks of all cells and body tissues. They are the basic components of enzymes, many hormones, antibodies and clotting agents. They act as transport substances for hormones, vitamins, minerals, lipids and other materials. In addition, they also help in the balance of the osmotic pressure of the blood and tissue. It also plays a major role in maintaining the delicate acid-alkaline balance of the blood. Serum proteins serve as a reserve source of energy for the tissues and muscle. The concentration of protein in the serum is elevated due to liver dysfunction, collagen vascular disease, hypersensitivity states, haemolysis, respiratory distress and leukemia. The concentration of protein in the serum is decreased due to malnutrition and malabsorption (insufficient intake and/or digestion of proteins), liver disease (insufficient production of proteins). The major measured serum proteins are divided into two groups, albumin and globulins. A typical blood panel is provided with four different measurements - the total protein, albumin, globulins, and the albumin globulin ratio.

Albumin is the most abundant globular un-glycosylated blood plasma protein and is synthesized by the liver using dietary protein[59]. Its normal range is 3.5-5.0 g/dL. It is essential for maintenance of the osmotic pressure which is needed for proper distribution of body fluids between intravascular compartments and body tissues. Its presence in the plasma creates an osmotic force that maintains fluid volume within the vascular space. It functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a major role in stabilizing extracellular fluid volume. Low albumin concentration (hypoalbuminemia) may be caused by liver disease, nephrotic syndrome, burns, protein-losing enteropathy, malabsorption, malnutrition, severe hemorrhage, and malignancy. High albumin concentration (hyperalbuminemia) is almost always caused by dehydration. Hyperalbuminemia and elevated transaminases are associated with high protein diet [44].

Globulin is another type of a heterogeneous group of blood plasma protein where some of the globulins are produced in the liver, while others are made by the immune system. There are four major types of globulins, each with specific properties and actions. They are the protein antibodies that provide immunity to various diseases. It also functions as carrier of cholesterol, phospholipids, Vitamin-A, steroid and thyroid harmones. It binds with plasma free Hb and prevents its excretion and it also participates in blood coagulation. Globulin levels are elevated in chronic infections (parasites, some cases of viral and bacterial infection), liver disease (biliary cirrhosis, obstructive jaundice), rheumatoid arthritis and kidney dysfunction. Globulin level are lowered in nephrosis (A condition in which the kidney does not filter the protein from the blood and it leaks into the urine), acute hemolytic anemia and hypogammaglobulinemia or agammaglobulinemia [39].

Albumin/Globulin (A/G) ratio is elevated in hypothyroidism, high protein/high carbohydrate diet with poor nitrogen retention, hypogammaglobulinemia (low globulin) and glucocorticoid excess (can be from taking medications with cortisone effect, the adrenal gland overproducing cortisol, or a tumor that produces extra cortisol like compounds, low globulin). The A/G ratio is decreased in liver dysfunction. Lipid profile or lipid panel, is the collective term given to the estimation of Total Cholesterol, High-Density Lipoprotein Cholesterol (HDL-C), Low-Density

Lipoprotein Cholesterol (LDL-C), and triglycerides. Lipid profile is a group of tests that are often ordered together to determine risk of Coronary Heart Disease (CHD) and they have been shown to be good indicators of whether someone is likely to have a heart attack or stroke caused by blockage of blood vessels or hardening of the arteries (atherosclerosis) Cholesterol is a waxy steroid of fat which is manufactured in the liver or intestines. It is found exclusively in animals hence also called as animal sterol. It is an essential structural component of cell membranes and it is required to establish proper membrane permeability and fluidity.

In addition it is an important component for the manufacture of bile acids, steroid hormones, and Vitamin D and it is an essential ingredient in the structure of lipoprotein [95]. Its normal range is less than 200 mg/dL. Although cholesterol is important and necessary for mammals, high levels of cholesterol in the blood can damage arteries and are potentially linked to heart diseases, hyperlipoproteinemia, hypothyroidism, diabetes mellitus and various liver diseases. Low serum cholesterol (hypo cholesterol) is found in pernicious anemia, malnutrition, acute infections and hyperthyroidism. High Density Lipoproteins (HDL) is often called as good cholesterol because it contains more protein and less cholesterol. HDL is synthesized primarily in the liver and intestine, and it travels in the bloodstream and transports cholesterol from peripheral tissues to liver. Its normal range is 30-70 mg/dL. HDL-Cholesterol levels are a strong inverse predictor of cardiovascular events.

Hence, Low concentrations of HDL-Cholesterol are associated with higher risk of CHD. Thus HDL-Cholesterol in combination with total cholesterol is a good index of the risk of CHD [6]. Low Density Lipoprotein (LDLs) is often called as bad cholesterol as it mainly consists of cholesterol. It transports cholesterol from liver to other tissues. Diets high in saturated fats and cholesterol decrease the uptake of LDL particles by the liver.

Triglycerides or Neutral fats are the esters of glycerol and fatty acids. They are the most abundant lipids comprising 85-90% of body lipids. They serve as energy source and are later stored in adipose tissue. Its normal range is less than 150 mg/dL. Serum Triglycerides values are found elevated in secondary hyperlipoproteinemia, atherosclerosis (hardening of the arteries), glycogen storage diseases, greatly elevated diabetic mellitus, chronic hepatitis and nephrotic syndrome. Cerium is member of the lanthanide series of metals and is the most abundant of the rare earth elements in the earth's crust (avg concentration of 50 ppm). Elemental Cerium is an irony –gray, ductile, malleable metal. Cerium metal is very reactive and is a strong oxidizing agent that is stabilized when associated with an Oxygen Ligand. When present in compounds, cerium exists in both trivalent state (Ce^{+3}, Cerous) and the tetravalent state (Ce^{+4}, Ceric).

Pharmacokinetic parameters:

Absorption:

In adult rats, cerium compounds are poorly absorbed following oral exposure. Following inhalation exposure, cerium, a poorly – soluble particle, behaves like other air borne particles, depositing within the respiratory tract based on aerodynamic character. Cerium has been detected in lung tissues and in alveolar macrophages of subjects believed to have been exposed occupationally. Once absorbed into the body, cerium accumulate primarily in the bone, liver, heart and lungs.

Distribution: Oral exposure:

Although cerium appears to be poorly absorbed from the GI tract, the bone and liver were the organs with highest cerium levels in the rats following oral gavage of cerium chloride. The concentration of cerium in the kidney, liver, lung and spleen of male ICR mice was significantly elevated relative to controls following 12 weeks of oral exposure to 200 ppm cerium chloride [2].

Inhalation exposure:

As poorly soluble particles, Cerium oxide particles behave like other airborne particles, depositing within the respiratory tract based on aerodynamic character. Once deposited in lung, insoluble cerium compounds may dissolve slowly as evidenced by the low percentage of cerium found in the tissues [19].

Metabolism:
As an element, cerium is neither created nor destroyed within the body. The particular cerium compound may be altered as a result various chemical reactions within the body, particularly dissolution. Exposure to cerium has been shown to change hepatic levels of some cytchrome (CYP) P_{450} isoenzymes in a species and strain sensitive manner for mice.

Elimination:
Elimination of orally administered cerium has been shown to be age dependent in animals, with suckling animals absorbing cerium into GI tissues.

Toxic Effect on humans:
Depending on the site of the disease, specific materials should be used in order to reduce toxicity. Liposomes are stable and effective vehicles for drug delivery, gene therapy and vaccines, and can be easily modified by other appropriate ligands, resulting in attractive formulations for targeted drug delivery. An epidemiological study reports a higher incidence of endocardial fibrosis among population consuming tubers grown in high cerium soil in India. A case control study found an association between increased toenail cerium concentration and the risk of first myocardial infarction. Numerous case reports have been published describing cases of workers who developed adverse lung effects, such as interstitial lung disease or pneumoconiosis, associated with accumulation of cerium in the lungs after prolonged occupational exposure of cerium fumes or dust. The human cases of cerium exposure demonstrate the accumulation of cerium particles in lungs and lymphoreticular system, with pulmonary function varying from normal to severe restriction and interstitial fibrosis in one case and granulomas in another.

Interstitial fibrosis accompanied by vascular thickening, reactive alveolar macrophages in the airspace, moderate chronic interstitial inflammation, deposits of grayish pigment, was observed in a 68 year old man who was employed as an optical lens grinder for 35 years. Data from a sub chronic toxicity test in Sprague –Dawley rats identified an increased incidence of alveolar epithelial hyperplasia as the critical effect for non cancer effects. Histologic examination revealed dose related epithelial and lymphoid hyperplasia and pigment accumulation in the lungs, lymph nodes and larynx. A population of immobilized, activated macrophages may serve to induce significant cell damage by effectively increasing the concentration of inflammatory cytokines and fibrogenic growth factors within the pulmonary epithelium.

In the lung, deposited particles are removed quite rapidly by macrophages. Mucociliary and macrophage clearance can only be circumvented by particles that are able to cross the mucus layer and reach the sol layer below the gel layer. Lai et al. (2005) reported the permeation of 200 nm PEGylated (with polyethylene glycol) particles across mucus. Thus, PE Gylation seems to be a promising approach to bypass the bronchial clearance of pharmaceutical particles. Tang et al. 2009 demonstrated that a biodegradable copolymer of poly (sebacic acid) and poly (ethylene glycol) rapidly penetrated the mucus barrier in the lungs of patients with cystic fibrosis, possibly by the efficient partitioning of polyethylene glycol (PEG).

Genotoxicity:
No information was located regarding genotoxic effects of cerium compounds in humans and only three studies were identified with pertinent information in mammals and in lower organisms. The available information is insufficient to ascertain the genotoxicity of cerium. Genotoxicity affects of others particles like titanium oxide, colbalt, gold, copper, zinc, aluminium are available [7, 21, 28, 33, 34, 50, 52]. Various genotoxic changes occurring in different parts of the body [26, 29, 32]. Concerning gene therapy, new concepts for the transfer of DNA into the nucleus are of special interest [27]. Various polycationic compounds (polymers, lipids, inorganic nanoparticles) have been used as non-viral transfection agents. DNA compacted with polycations accesses the nucleus of cells more efficiently than non-compacted DNA or lipid-DNA complexes.

However, complexes consisting of only polycation and DNA tend to aggregate in tissue fluids. Addition of PEG to complexes was shown to influence particle characteristics including structure

and stabilization, preventing aggregation. Unfortunately, high DNA complexation efficacy is often associated with toxicity. Consequently, nanoparticles formulated from biodegradables polymers are being extensively studied as a non-viral alternative to polycationic polymers. Poly (lactide-co-glycolide-acid) (PLGA), biodegradable polyester, has been recognized for its ability to deliver DNA.

However, complexation and delivery of nucleotides by PLGA nanoparticles are limited by their negative charge. As previously stated, positive surface charge seems to be essential for an effective binding of the negatively charged DNA to the carrier. In contrast, Harush-Frenkel et al. 2010 showed increased local and systemic toxic effects with cationic nanoparticle based PEG-PLA (polylactide). Conversely, anionic nanoparticles of similar size were much better tolerated and did not present a systemic toxicity effect, although a moderate change was noted in platelet count, with no clinical significance. Overall, these observations suggest that anionic PEG-PLA nanoparticles are useful pulmonary drug carriers. Conversely, several groups of nanomaterials (i.e. carbon nanotubes, carbon black, fullerenes, silica, metals or metal oxides) can induce inflammation and/or fibrosis in the lung. Polyamidoamine (PAMAM), a group of materials that appear to be very promising as nanocarriers for drug delivery, can induce autophagic cell death.

Objective of research:

The present research is to evaluate the toxicity of Cerium nanomaterials in rats by repeated daily oral treatment for 28 days by monitoring the effects of animal behavior, body weight gain and feed intake and also to assess the hematological like RBC, WBC, WBC differential count, Platelet count, Hemoglobin etc. in blood and various biochemical parameters like calcium, chlorine, glucose, creatinine in serum and targeted biochemical parameters like Lactate dehydrogenase (LDH), Protein estimation and alkaline phosphatase (ALKP) in serum, liver and kidney of rats.

MATERIALS AND METHODS:

Materials used in the study were Cerium oxide <25nm (CeO_2-NM), Chromium oxide Bulk (CeO-Bulk), Tris HC1, Quinidine sulphate, DTNB and other chemicals. The study was conducted at Indian Institute of Chemical Technology(IICT),tarnaka for the period of 9months(Jan 2013-Sep 2013. Repeated oral dose toxicity study was carried out in the adult albino male and female Wistar rats (Rattus norvegicus) of 6-8 weeks old weighing 100-150 gm for repeated dose toxicity study [45] (OECD guidelines 420 adopted in 2001). The rats were obtained from National Institute of Nutrition (NIN), Hyderabad, and maintained in controlled condition in the Indian Institute of Chemical Technology (IICT) animal house, for a week prior to the experiment.

Methods applied for work:

The animals were divided randomly into seven groups (I, II, III, IV) each consisting of 3 rats. Animals of group Ito III were orally treated with Cerium Oxide nanomaterial <25nm (CeO_2-NM) and group IV to VI with Cerium oxide bulk (CeO_2-Bulk) in the doses of 30, 300 & 600 mg/kg body weight representing Low, Medium and High doses respectively and group IV was given equal volume of distilled water and served as control for 28 days. The maximum one-milliliter water was used for administration of test material at one time.

The treatment was done between 11-11.30 AM every day, the doses for repeated dose toxicity study was adjusted on weekly basis according to body weight of the animals. The control and treated animals were maintained in the animal house at 22°C and relative humidity around 30-70%. The animal house was maintained 12 hrs dark and 12 hrs light cycle and the animals were given standard laboratory feed and water *ad libitum*. Body weights, feed intake were recorded daily.

On the termination of the experimental period (after 28 days) rats were sacrificed by cervical dislocation and blood, brain liver and kidney tissues were collected from the control and treated rats. Biochemical parameters like Calcium, Chloride, Creatinine, Glucose, Lipid profile, Alkaline phosphate (ALP), protein profile in serum, liver and kidney were estimated following standard procedures in control and treated rats.

Various parameters were estimated using different method:

Estimation of Haematological Parameters in Blood: Haemoglobin (Hb) by Cyanmethemoglobin (CMG) method., Haematocrit (HCT) by using microhematocrit method, Clotting time was determined using capillary tube method. Red Blood Cell (RBC) Count by using Neubauer Chamber/ Hemocytometer., Platelet Count by Neubauer Chamber/ Hemocytometer, White Blood Cell (WBC) Count by using Neubauer Chamber/ Hemocytometer, WBC differential Count. Determination of Red Blood Cell indices: The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are absolute values and calculated from hemoglobin, hematocrit and RBC count as described by Brown.

Estimation of Biochemical Parameters in Serum: Calcium (Ca^{+2}) by Cresolphthalein Complexone Method, Glucose by Glucose Oxidase (GOD) / Peroxidase (POD) Method, Chloride (Cl^{-}) by Colorimetric Method, Creatinine by Picrate Method, Alkaline Phosphatase by p-Nitrophenyl Phosphate (PNPP) Method, Lactate Dehydrogense (LDH).

Estimation of Total Protein in Serum:
Protein by Lowry method, Albumin by Bromocresol green (BCG) Method, Globulin, A/G Ratio.

Estimation of Lipid Profile in Serum: Cholesterol by Enzymatic Method**,** Triglycerides by Enzymatic Colorimetric Method, High Density Lipoprotein-Cholesterol by Phosphotungstate Method**,** Low Density Lipoprotein (LDL) – Cholesterol, Alkaline Phosphatase (ALKP). Quality control was carried out and also Linearity.

RESULTS AND DISCUSSION:

Nanomaterials due to their different properties are known for their utility in various areas especially in the field of life sciences. They are being widely used in pharmaceuticals, diagnosis and treatment to various health problems. But significantly not much work has been carried out in the area of assessing their toxicity. The repeated oral toxicity study was conducted with CeO_2-NM andCeO_2-Bulk against male and female albino Wistar rats with special reference to Hematological parameters like RBC, WBC, WBC differential count, Platelet count, Hemoglobin etc. in blood and various biochemical parameters like calcium, chlorine, glucose, creatinine in serum and targeted biochemical parameters like Lactate dehydrogenase (LDH), Protein estimation and alkaline phosphatase (ALKP) in serum, liver and kidney of rats. The 28 days repeated dose oral toxicity showed CeO_2-NM and CeO_2-Bulk did not affect the body weight gain and feed intake of the treated rats. Further, CeO_2-NM significantly inhibited some of the hematological parameters like platelets count, granulocytes, % hematocrit in both male and female rats. Similarly, Glucose, Albumin, Triglycerides, LDL in serum and Protein, ALP in serum, kidney and liver were significantly inhibited in both male and female treated rats in dose dependent order. CeO_2-NM significantly inhibited Protein, ALP in Medium and High doses in both male and female treated rats.

However, CeO_2-Bulk could not induce any significant alterations in these parameters and the changes were near to control in both male and female treated rats. Further, a maximum inhibition of 62 percent in male and 60 percent in female rats were recorded at high dose of CeO_2-NM (**Tables 1-6** i.e in hematological parameters). In Calcium and chlorine i.e.estimated in serum CeO_2-NM inhibited in dose dependent manner but it's not significant where as in glucose and creatinine estimation CeO_2-NM significantly inhibited at High dose in both male and female treated rats. CeO_2-Bulk could not induce any significant alterations in the above mentioned parameters and the changes were near to control in both male and female treated rats. Maximum inhibition of 22 and 20 percent were recorded at high dose of male and female rats treated with CeO_2-NM respectively.

Albumin was significantly inhibited at high dosetested with CeO_2-NM in serum of male and female treated rats further the changes were dose dependent. The alterations observed with CeO_2-Bulkwere insignificant in both male and female rats. Maximum inhibition of 32 and 24 percent were recorded at high dose in male and female rats treated with CeO_2-NM respectively. Cholesterol

and HDL did not show any significant inhibition at all the three doses, but showed inhibition in dose dependent manner where as in triglycerides and LDL there is significant inhibition at high dose of both males and females, there is maximum inhibition of 36 percent and of 25 percent in males and females respectively.

However, CeO_2-Bulk significantly inhibited at medium and high doses in female treated rats. Protein estimation in serum, liver and kidney shows significant activation at high doses in both male and female rats treated with CeO_2-NM in all the three extracts and the activation is in dose dependent manner. However, CeO_2-Bulk insignificantly activated at all doses in female treated rats. Maximum activation of 85 and 98 percent were recorded in male and female rats treated with CeO_2-NM respectively (Tables 7-9). LDH estimation in serum and kidney shows activation in dose dependent manner in both male and female rats treated with CeO_2-NM in all the three extracts and the activation is not significant. However, CeO_2-Bulk insignificantly activated at all doses in both female treated rats in dose dependent manner. Maximum activation of 15 and 17 percent were recorded in male and female rats treated with CeO_2-NM respectively. LDH estimated in kidney has shown insignificant inhibition in both males and female rats treated with CeO_2-NM in a dose dependent manner. Maximum inhibition of 15 and 17 percent were recorded in male and female rats treated with CeO_2-NM respectively.

In case of ALP that is estimated in serum, it showed significant activation at medium and high doses in male rats treated with CeO_2-NM, but it showed insignificant activation in female rats. The activation in male and female rats treated with CeO_2-NM is in dose dependent manner. But whereas the ALP estimated in kidney and liver extracts showed significant activation in a dose dependent manner in both males and female rats treated with CeO_2-NM. However, CeO_2-Bulk significantly activated at medium and high doses in case of ALP estimated in liver extract. Maximum activation of 166 and 150 percent were recorded in male and female rats treated with CeO_2-NM respectively (**Tables 10-12**).

TABLE 1: SHOWING THE HEMATOLOGICAL PARAMETERS OF MALE ALBINO WISTAR RATS TREATED WITH CERIUM OXIDE NANO PARTICLES BY REPEATED DOSING FOR 28DAYS

S.No	Parameters	Control	Low dose (30mg/kg)	Medium dose(300mg/kg)	High dose(60mg/kg)
1.	Haemoglobin (g/dL)	16.00 ± 0.32	16.20 ± 1.20 (+2)	17.02 ± 1.00 (+6)	17.52 ± 0.05 (+10)
2.	Haematocrit (%)	35.02 ± 0.07	36.6 ± 0.01 (+4)	41.5 ± 0.11 (+18)	49.5 ± 1.33* (+41)
3.	RBC (x 106 / cmm)	6.55 ± 0.02	6.85 ± 0.48 (+5)	7.05 ± 0.52 (+8)	7.55 ± 0.51 (+16)
4.	Platelets (x105 / cmm)	5.40 ± 0.05	5.05 ± 0.02 (-6)	4.95 ± 0.15 (-8)	2.04 ± 0.14* (-62)
5.	Maximum corpuscular volume (MCV µm3)	53.1 ± 0.01	54.01 ± 0.03 (+2)	59.00 ± 0.01 (+11)	66.1 ± 0.23* (+24)
6.	Mean corpuscular haemoglobin (MCH ρg/ml)	24.3 ± 0.13	24.7 ± 0.01 (+4)	25.4 ±0.12 (+6)	26.92 ±0.12 (+12)
7.	Mean corpuscular haemoglobin concentration (MCHC g/dL)	45.55 ± 0.21	45.8 ± 1.03 (+2)	46.7 ± 1.25 (+4)	48.02 ±1.52 (+7)
8.	Red cell distribution width (RDW %)	13.64 ±0.14	13.71 ± 0.41 (+5)	14.71 ± 0.01 (+8)	14.91 ±0.15 (+9)
9.	Mean platelet volume (MPV µm3)	6.70 ±0.02	6.90 ±0.05 (+3)	8.12 ± 0.04* (+21)	8.01 ± 0.02* (+20)
10.	PCT (% haeomatocrit)	0.073 ±0.02	0.081 ± 0.01 (+16)	0.091 ± 0.04* (+30)	0.097 ± 0.01* (+38)
11.	PDW (% Plate distribution width)	4.3 ± 0.03	5.51 ±0.01* (+28)	8.71 ± 0.02* (+102)	9.89 ± 0.01* (+130)

Data represent mean± SD of three biological replicates from each group.
*P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 2: SHOWING THE WBC DIFFERENTIAL COUNT IN MALE ALBINO WISTAR RATS TREATED WITH CERIUM OXIDE NANO PARTICLE BY REPEATED DOSING FOR 28DAYS

Sno.	Parameters	Control	Low dose (30mg/kg)	Medium dose (300mg/kg)	High dose (600mg/kg)
1.	WBC ($x10^3$/cmm)	5.00± 0.13	5.01± 0.23 (+0.2)	9.01± 0.56* (+80)	11.50± 1.05* (+130)
2.	Lymphocytes ($x10^3$)	4.00± 0.51	4.12± 0.35 (+3)	7.23± 0.17* (+81)	9.06± 0.34* (+127)
3.	Monocytes (%)	10.10± 0.03	11.70± 0.01 (+17)	12.41± 0.02* (+24)	12.50 ± 0.08* (+25)
4.	Monocytes ($x10^3$)	0.10± 0.50	0.51± 0.33* (+410)	1.00± 0.84* (+900)	1.01± 0.87* (+900)
5.	Lymphocytes (%)	82.10± 1.63	81.95± 1.19 (-0.06)	81.62± 0.54 (-0.5)	71.51± 0.69 (-3.03)
6.	Granulocytes (%)	7.81± 2.87	7.62± 2.01 (-5)	6.80± 1.12 (-15)	6.00± 2.45* (-25)
7.	Granulocytes ($x10^3$)	0.1± 1.71	0.51± 0.40* (+410)	1.00± 0.26* (+900)	1.06± 0.03* (+906)
8.	Neutrophils (%)	14.00 ± 0.02	15.00 ± 0.58 (+7)	15.25 ± 0.58 (+8)	15.32 ± 1.53 (+9)

Data represent mean± SD of three biological replicates from each group.
*P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 3: SHOWING THE HEMATOLOGICAL PARAMETERS OF FEMALE ALBINO WISTAR RATS TREATED WITH CERIUM OXIDE NANO PARTICLES BY REPEATED DOSING FOR 28DAYS

S.No	Parameters	Control	Low dose (30mg/kg)	Medium dose(300mg/kg)	High dose(60mg/kg)
1.	Haemoglobin (g/dL)	13.45 ± 1.52	13.95 ± 1.20 (+4)	15.16 ± 1.20 (+13)	15.86 ± 2.99 (+18)
2.	Haematocrit (%)	35.6 ± 0.57	36.6 ± 0.66 (+3)	41.4 ± 0.19 (+15)	42.1 ± 1.33 (+17)
3.	RBC (x 106 / cmm)	6.09 ± 0.27	6.70 ± 0.56 (+12)	6.89 ± 0.04 (+15)	7.55 ± 0.55* (+26)
4.	Platelets (x105 / cmm)	6.70 ± 0.05	4.54 ± 0.22* (-32)	4.07 ± 0.06* (-42)	3.67 ± 0.07* (-47)
5.	Maximum corpuscular volume (MCV µm3)	58.5 ± 0.02	59.5 ± 0.03 (+1)	61.3 ± 0.05 (+4)	66.1 ± 0.12 (+12)
6.	Mean corpuscular haemoglobin (MCH ρg/ml)	25.6 ± 0.06	24.73 ± 0.02 (-5)	22.6 ±0.13 (-13)	20.09 ±0.02 (-19)
7.	Mean corpuscular haemoglobin concentration (MCHC g/dL)	44.05 ± 1.25	39.3 ± 0.23 (-11)	36.6 ± 0.05 (-17)	35.9 ±0.02 (-18)
8.	Red cell distribution width (RDW %)	15.15 ±0.44	15.55 ± 0.41 (+4)	16.40 ± 0.31 (+9)	18.56 ±0.45* (+24)
9.	Mean platelet volume (MPV µm3)	7.05 ±0.01	7.35 ±0.05 (+5)	7.6 ± 0.03 (+9)	7.69 ± 0.01 (+10)
10.	PCT (% haeomatocrit)	0.47 ±0.12	0.40 ± 0.02* (-20)	0.38 ± 0.05* (-24)	0.36 ± 0.06* (-28)
11.	PDW (% Plate distribution width)	9.7 ± 0.03	5.06 ±0.02* (-49)	4.60 ± 0.01* (-54)	4.05 ± 0.01* (-60)

Data represent mean± SD of three biological replicates from each group.
*P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 4: SHOWING THE WBC DIFFERENTIAL COUNT IN FEMALE ALBINO WISTAR RATS TREATED WITH CERIUM OXIDE NANO PARTICLES BY REPEATED DOSING FOR 28 DAYS

S.no.	Parameters	Control	Low dose (30mg/kg)	Medium dose (300mg/kg)	High dose (600mg/kg)
1.	WBC ($x10^3$/cmm)	13.15 ± 0.35	13.45 ± 0.09 (+3)	14.7 ± 0.13 (+13)	16.83 ± 0.17* (+29)
2.	Lymphocytes ($x10^3$)	10.01 ± 0.02	10.30 ± 0.03 (+3)	10.78 ± 0.01 (+8)	12.96 ± 0.01* (+30)
3.	Monocytes (%)	8.1 ± 0.12	8.3 ± 0.01 (+4)	8.91 ± 0.06 (+11)	9.14 ± 0.04* (+14)
4.	Monocytes ($x10^3$)	1.01 ± 0.02	1.35 ± 0.02* (+35)	1.36 ± 0.05* (+36)	1.43 ± 0.06* (+43)
5.	Lymphocytes (%)	75.85 ± 0.01	75.10 ± 0.12 (-2)	73.01 ± 0.05 (-4)	72.75 ± 0.08 (-4)
6.	Granulocytes (%)	16.5 ± 0.02	16.95 ± 0.01 (+6)	18.01 ± 0.05 (+13)	18.9 ± 0.01 (+18)
7.	Granulocytes ($x10^3$)	2.15 ± 0.52	2.3 ± 0.56 (+15)	2.67 ± 0.15* (+33)	2.83 ± 0.01* (+41)
8.	Neutrophils (%)	17.01 ± 1.02	16.32 ± 0.58 (-4)	15.25 ± 0.58 (-10)	14.67 ± 1.53 (-14)

Data represent mean± SD of three biological replicates from each group.
*P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 5: SHOWING THE HEMATOLOGICAL PARAMETERS OF FEMALE ALBINO WISTAR RATS TREATED WITH CERIUM-BULK BY REPEATED DOSING FOR 28DAYS

S.No	Parameters	Control	Low dose (30mg/kg)	Medium dose(300mg/kg)	High dose(60mg/kg)
1.	Haemoglobin (g/dL)	13.91 ± 1.12	13.95 ± 1.20 (+0.3)	16.16 ± 0.39 (+15)	16.38 ± 0.36 (+17)
2.	Haematocrit (%)	33.10 ± 0.57	33.6 ± 0.43 (+2)	36.1 ± 0.32 (+9)	39.8 ± 0.33 (+19)
3.	RBC (x 106 / cmm)	6.09 ± 0.27	6.21 ± 0.03 (+4)	7.09 ± 0.11 (+12)	7.12 ± 0.13 (+19)
4.	Platelets (x105 / cmm)	6.70 ± 0.05	6.79 ± 0.15 (-2)	6.60 ± 0.15 (-2)	5.98 ± 0.43 (-11)
5.	Maximum corpuscular volume (MCV µm3)	58.5 ± 0.02	56.10 ± 0.24 (-3)	55.10 ± 0.10 (-5)	53.50 ± 0.10 (-9)
6.	Mean corpuscular haemoglobin (MCH ρg/ml)	22.4 ± 0.16	21.60 ± 0.02 (-2)	20.95 ±0.13 (-5)	20.60 ±0.09 (-6)
7.	Mean corpuscular haemoglobin concentration (MCHC g/dL)	44.95 ± 1.25	44.00 ± 0.06 (-2)	42.00 ± 0.15 (-7)	41.90 ±0.15 (-9)
8.	Red cell distribution width (RDW %)	15.15 ±0.44	17.30 ± 0.41 (+15)	17.40 ± 0.31 (+16)	17.51 ±0.45 (+17)
9.	Mean platelet volume (MPV µm3)	7.05 ±0.01	7.21 ±0.05 (+3)	7.30 ± 0.03 (+4)	8.10 ± 0.01 (+15)
10.	PCT (% haeomatocrit)	0.47 ±0.12	0.48 ± 0.02 (+2)	0.49 ± 0.05 (+4)	0.52 ± 0.06 (+10)
11.	PDW (% Plate distribution width)	9.7 ± 0.03	9.01 ±0.02 (-8)	8.63 ± 0.01 (-11)	8.21 ± 0.01 (-15)

Data represent mean± SD of three biological replicates from each group. *P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 6: SHOWING THE WBC DIFFERENTIAL COUNT IN FEMALE ALBINO WISTAR RATS TREATED WITH CERIUM-BULK BY REPEATED DOSING FOR 28 DAYS

S.no.	Parameters	Control	Low dose (30mg/kg)	Medium dose (300mg/kg)	High dose (600mg/kg)
1.	WBC ($x10^3$/cmm)	16.15 ± 0.35	16.71± 0.04(+4)	17.7 ± 0.12(+11)	18.83 ± 0.17(+18)
2.	Lymphocytes ($x10^3$)	10.01 ± 0.02	10.51± 0.03(+5)	10.70 ± 0.01(+7)	11.96 ± 0.01(+19)
3.	Monocytes (%)	8.1 ± 0.12	7.77± 0.01(-3)	7.41± 0.06(-7)	6.91± 0.04(-14)
4.	Monocytes ($x10^3$)	1.01 ± 0.02	1.01± 0.02(0)	1.20± 0.05(+12)	1.89± 0.06(+19)
5.	Lymphocytes (%)	75.85 ± 0.01	75.10 ± 0.12(-2)	74.01 ± 0.05(-3)	72.75 ± 0.08(-4)
6.	Granulocytes (%)	16.5 ± 0.02	16.95 ± 0.01(+5)	18.01 ± 0.05(+12)	18.9 ± 0.01(+18)
7.	Granulocytes ($x10^3$)	2.20± 0.52	2.3 ± 0.56(+12)	2.40± 0.15(+12)	2.46 ± 0.01(+15)
8.	Neutrophils (%)	17.91 ± 1.02	17.32 ± 0.58(-4)	16.25 ± 0.58(-10)	15.67 ± 1.53(-11)

Data represent mean± SD of three biological replicates from each group. *P < 0.05 significantly different from control. Values in parenthesis indicates the percentage increase (+) or decrease (-).

TABLE 7: ESTIMATION OF PROTEIN IN SERUM OF RATS TREATED WITH CERIUM-NANO ANDCERIUM-BULK BY REPEATED DOSING FOR 28 DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	6.50	6.20	6.10	6.26 ± 0.21	-
Low	6.50	6.40	6.30	6.40 ± 0.10	7 (+)
Medium	7.20	7.10	7.30	7.20± 0.10*	20 (+)
High	7.50	7.42	7.36	7.43 ± 0.07*	23 (+)
Female Rats Treated With NM					
Control	7.75	7.78	7.62	7.71 ± 0.09	-
Low	7.75	7.73	7.69	7.72 ± 0.03	10 (+)
Medium	9.75	8.75	9.52	9.34± 0.13	16 (+)
High	10.00	10.30	10.12	10.14 ±0.15*	26 (+)
Female Rats Treated With Bulk Material					
Control	7.75	7.78	7.62	7.71 ± 0.09	-
Bulk low	7.65	7.76	7.72	7.72± 0.08	0.1 (+)
Bulk medium	7.75	7.69	7.78	7.74 ± 0.05	0.3 (+)
Bulk high	8.50	8.52	8.49	8.50 ± 0.01	10 (+)

Data represent mean± SD of three biological replicates from each group. [a]**(µg)**
*P < 0.05 significantly different from control

TABLE 8: ESTIMATION OF PROTEIN IN KIDNEY EXTRACT OF RATS TREATED WITH NANO AND BULK CERIUM OXIDE BY REPEATED DOSING FOR 28 DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	195	196	197	196± 1.00	-
Low	195	200	198	198± 1.52	1 (+)
Medium	225	226	225	225± 0.31	14 (+)
High	300	308	310	306 ± 5.83*	56 (+)
Female Rats Treated With NM					
Control	195	196	197	196± 1.00	-
Low	231	199	200	209± 1.52	7 (+)
Medium	255	251	257	254± 3.05*	29 (+)
High	365	363	366	364± 1.53*	85 (+)
Female Rats Treated With Bulk Material					
Control	195	196	197	196± 1.00	-
Low	203	217	221	214 ± 1.02	9 (+)
Medium	210	223	216	216± 2.08	10 (+)
High	225	230	228	227 ± 2.51	16 (+)

Data represent mean± SD of three biological replicates from each group.
[a](µg)
*P < 0.05 significantly different from control.

TABLE 9: ESTIMATION OF PROTEIN IN LIVER EXTRACT OF RATS TREATED WITH CERIUM-NANO ANDCERIUM-BULK BY REPEATED DOSING FOR 28 DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	145	147	147	147± 1.53	-
Low	175	176	176	176 ± 1.00	19 (+)
Medium	205	203	207	205± 2.00*	39 (+)
High	290	291	293	291 ± 1.53*	98 (+)
Female Rats Treated With NM					
Control	145	147	146	148± 1.53	-
Low	160	165	162	162± 1.53	9 (+)
Medium	195	198	196	196± 1.50*	32 (+)
High	265	266	268	266± 1.52*	79 (+)
Female Rats Treated With Bulk Material					
Control	145	147	146	148± 1.53	-
Low	155	154	159	156 ± 1.53	5 (+)
Medium	165	173	162	166± 0.58	12 (+)
High	175	164	184	175 ± 1.53	18 (+)

Data represent mean± SD of three biological replicates from each group.
[a]**(mg)**
*P < 0.05 significantly different from control.

TABLE 10: ESTIMATION OF ALP IN SERUM OF RATS TREATED WITH CERIUM-NANO AND CERIUM-BULK BY REPEATED DOSING FOR 28 DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	0.077	0.075	0.079	0.08± 0.04	-
Low	0.072	0.078	0.073	0.08± 0.03	0
Medium	0.069	0.070	0.073	0.07± 0.02	13 (-)
High	0.067	0.069	0.068	0.07± 0.01	13 (-)
Female Rats Treated With NM					
Control	0.091	0.093	0.098	0.09± 0.04	-
Low	0.090	0.091	0.099	0.09± 0.01	0
Medium	0.065	0.063	0.068	0.07± 0.03*	22 (-)
High	0.051	0.053	0.056	0.05± 0.03*	44 (-)
Female Rats Treated With Bulk Material					
Control	0.091	0.093	0.098	0.09± 0.04	-
Low	0.088	0.089	0.096	0.09± 0.03	0 (-)
Medium	0.089	0.090	0.086	0.08± 0.02	2 (-)
High	0.086	0.083	0.084	0.08± 0.01	2 (-)

Data represent mean± SD of three biological replicates from each group.
[a] µ g/hr/ml
*P < 0.05 significantly different from control.

TABLE 11: ESTIMATION OF ALP IN KIDNEY EXTRACT OF RATS TREATED WITH CERIUM-NANO AND CERIUM-BULK BY REPEATED DOSING FOR 28DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	0.192	0.193	0.198	0.20 ± 0.01	-
Low	0.221	0.223	0.226	0.22 ± 0.01	10 (+)
Medium	0.264	0.264	0.260	0.27 ± 0.01*	35 (+)
High	0.270	0.278	0.269	0.28± 0.02*	40 (+)
Female Rats Treated With NM					
Control	0.192	0.193	0.198	0.20 ± 0.01	-
Low	0.270	0.278	0.278	0.28 ± 0.01*	40 (+)
Medium	0.353	0.356	0.360	0.36 ± 0.02*	80 (+)
High	0.379	0.378	0.380	0.37 ± 0.01*	85 (+)
Female Rats Treated With Bulk Material					
Control	0.192	0.193	0.198	0.20 ± 0.01	-
Low	0.202	0.201	0.208	0.21± 0.01	5 (+)
Medium	0.220	0.211	0.218	0.22± 0.02	10 (+)
High	0.237	0.241	0.240	0.24± 0.01	12 (+)

Data represent mean± SD of three biological replicates from each group.
[a]**mg protein**
*P < 0.05 significantly different from control.

TABLE 12: ESTIMATION OF ALP IN LIVER EXTRACT OF RATS TREATED WITH CERIUM-NANO ANDCERIUM-BULK BY REPEATED DOSING FOR 28 DAYS

Groups	1	2	3	Mean±SD	% Activation/ Inhibition
Male Rats Treated With NM					
Control	0.003	0.004	0.003	0.01 ± 0.02	-
Low	0.009	0.010	0.019	0.013± 0.01*	30 (+)
Medium	0.013	0.018	0.024	0.018 ± 0.02*	80 (+)
High	0.023	0.026	0.025	0.025± 0.03*	150 (+)
Female Rats Treated With NM					
Control	0.003	0.004	0.003	0.003± 0.02	-
Low	0.003	0.002	0.003	0.003± 0.01	00
Medium	0.010	0.003	0.006	0.006± 0.01*	111 (+)
High	0.009	0.008	0.007	0.008± 0.02*	166 (+)
Female Rats Treated With Bulk Material					
Control	0.003	0.004	0.003	0.003± 0.02	-
Low	0.004	0.003	0.001	0.003± 0.01	00 (+)
Medium	0.005	0.004	0.005	0.004 ± 0.01*	55 (+)
High	0.005	0.006	0.004	0.005± 0.02*	66 (+)

Data represent mean± SD of three biological replicates from each group.
[a]**(mg protein)**
*P < 0.05 significantly different from control

DISCUSSION: These results indicated that CeO_2-NM able to produce more potent toxicity in comparison to its counterpart CeO_2-Bulk. Further, these results suggested that as particle size decreases, this cerium nano material showed increased toxicity, even though the same material is relatively less effective in bulk form. The results of the present investigation revealed that the treatment of repeated doses of Cerium oxide in female albino Wistar rats have shown significant changes in the Haematological parameters. Blood of Cerium oxide treated rats was evaluated for hematological toxicity. Haemogram was estimated and results were shown haematopoietic slightly increased the haemoglobin, blood cell count, and haematocrit values slightly increased as compared to control but these increases were not significant. Clotting time, Platelet count, WBC count, WBC differential count evaluating Eosinophils, Lymphocyte, Monocytes, Neutrophils counts in all the treated groups were similar as compared to control. Similarly, as in the present study reported the significant changes in food consumption, body weight, haematological, biochemical and histopathological parameters of rats treated with three sub-acute doses of Cerium oxide.

Similarly, the results of the treatment of repeated doses of Cerium oxide in albino Wistar rats have shown significant changes in the Clinical Biochemical parameters. Serum Biochemical parameters were evaluated for the diagnosis of various diseases. Biochemical parameters are the good indicators for biological functions. Creatinine and Chloride are the indicators of renal function. The Alkaline Phosphatase (ALKP) is used to detect liver diseases or bone disorders.

The concentrations of Calcium, ALKP, and Protein slightly increased as compared to control but these increases were not significant. Similarly, as in present study, signs of toxicity, mortality, changes in tissue weight, pathology and serum and blood parameters were reported in rat treated for 90 days with 30, 300 and 600 mg/kg doses of Cerium oxide. Lipid profile is a group of tests that are often ordered together to determine risk of coronary heart diseases. The concentration of Cholesterol, HDL, LDL, Triglycerides were slightly varying from the control values and these changes were significant.

CONCLUSION: Our results have shown that CeO_2-Bulk did not induce any adverse effects in the target biochemical parameters in the treated rats and the changes observed were near to controls and mostly insignificant indicating that the counter part of nanoparticles i.e. bulk material is less potent than the nanoparticles in causing toxicity in the exposed animals. These results suggested that as particle size decreases, this cerium nanoparticle showed increased toxicity, even though the same material is relatively inert in bulk form. These

studies highlight the need for further experimentation to fully elucidate the mechanisms responsible for the behavior of nanomaterials specifically to develop a comprehensive evaluation, of the human health implications, from exposure to nanoscale materials. More studies are needed to characterize the toxicity of a broader range of commercial nanoscale materials as a function of chemical composition, shape, surface characteristics, and method of production.

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