Subchronic Oral Toxicity of Copper Nanoparticles

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FINAL APPROVAL

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DECLARATION

I declared that this work, apart from help, is my own effort. No part of this thesis is presented before. And there is no plagiarism in this thesis work.

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DEDICATION

I dedicate this thesis to Allah, who is the real source of Help and power, who gave me strength to accomplish my work, and Iacknowledged this thesis to my parents who have been my strength, and encouraged me a lot throughout my educational career, who are real source of inspiration for me. Without their help and encouragement I would never be able to complete my work.

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Table of content

Table of Contents

Dedications.....i

Declaration of Originality.....ii

Table of Contents.....viiii

List of Graphs.....ix

List of Tables.....x

List of Figures.....xii

Abstract.....xiii

List of Abbreviations and Symbols.....xv

Acknowledgements.....xvi

1 **Introduction**.....1

1.1 Nanotechnology.....1

1.2 Nanomaterials.....2

1.3 Toxicology.....3

1.3.1 *In vivo* toxicity assessment.....4

1.3.2 Oral LD50.....5

1.4 Nanotoxicology.....5

1.4.1 Nanotoxicity in humans.....6

1.4.2 Variables that influence Nanotoxicity.....6

1.4.2.1 Dose-Dependent Toxicity.....6

1.4.2.2 Size-Dependent Toxicity.....6

1.4.2.3 Surface-Area-Dependent Toxicity.....7

1.4.2.4 Crystalline-Structure-Dependent Toxicity.....7
1.4.2.5 Surface Coating Dependent Toxicity.....7
1.5 Potential routes of exposure for nanomaterial.....8
1.5.1 Inhalation.....8
1.5.2 Ingestion.....8
1.5.3 Dermal.....9
1.5.4 Parenteral.....9
1.6 Copper Nanoparticles.....9
1.6.1 Bioactivities of Copper-nanoparticles.....10
1.6.2 Application of Copper Nanoparticles.....11
1.6.3 Copper nanoparticles exposure.....12
1.6.4 Toxicity of Copper nanoparticles.....12
1.7 Research Objectives.....15
2 **Materials and Methods**.....17
2.1 Nanoparticles 17
2.2 Characterization of Nanoparticles.....17
2.2.1 Dynamic Light Scattering (DLS).....17
2.2.2 Transmission Electron Microscopy (TEM) of Nanoparticles.....18
2.3 Nanoparticles and animals.....18
2.3.1 Accommodation and Husbandry.....18
2.3.2 Acclimatization period.....19
2.4 Sub-chronic Repeated Oral Toxicity 19
2.4.1 Observations for Clinical Signs of Toxicity.....20
2.4.2 Sample Collection.....20
2.5 Biochemical Analysis of Blood.....20
2.6 Heamtological Analysis of Blood.....20

2.7 Necropsy Finding and Organ weight measurrement.....21

2.8 Histopathological Examination of Organs.....21

2.9 Statistical Analysis.....21

3 **Results**.....22

3.1 Characterization of copper Cu nanoparticles.....22

3.2 Clinical Observations.....23

3.3 Effect of Cu Nanoparticles on food intake, water consumption and Body weight.....24

3.4 Necropsy and average organ weight.....25

3.4.1 Necropsy findings.....25

3.4.2 Organ Weight.....25

3.5 Serum Biochemical and heatological parameters.....27

3.5.1 Analysis of serum biochemical parameters.....27

3.5.2 Analysis of serum hematological parameters.....31

3.6 Histopathological Examination.....33

4 **Discussion**.....36

4.1 Conclusions.....39

4.2 Future recommendation.....40

5.**References**.....41

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List of Figures

1. Timeline for beginning of industrial prototyping and nanotechnology Commercialization: Four generations of nanoproducts (Renn and Roco, 2006).....3
2. Sub-chronic repeated oral toxicity in albino rats.....19
3. TEM image showing the shape and size of the copper Cu nanoparticles (magnification is x 60K).....22
4. Size distributions of copper Cu nanoparticles in deionized H_2O.....23
5. Necropsy and Necropsied animal.....26
6. Vital organs from necropsied animals.....26
7. Rats Kidney Histopathology dosed with copper Cu nanoparticles.....34
8. Rats liver Histopathology dosed with copper Cu nanoparticles.....34
9. Rats Kidney Histopathology dosed with copper Cu nanoparticles.....35

List of Tables

1. Weekly average body weights for copper Cu nanoparticles.....24
2. Average organ weight of rats treated with copper Cu nanoparticles.....27
3. Serum biochemical parameters of control and copper Cu nanoparticlese exposed rats.....31
4. Blood hematology parameters of control and copper Cu nanoparticles exposed rats.....32

5.References.....40

Table of Graphs

1. Weekly average body weight for Cu nanoparticles.....25
2. Blood Urea Nitrogen level in rats treated with different doses of Cu NPs.....28
3. Serum Creatinie level in rats treated with different doses of Cu NPs.....28
4. ALT (SGPT) level in rats treated with different doses of Cu NPs.....29
5. ALP level in rats treated with different doses of Cu NPs 29
6. AST level in rats treated with different doses of Cu NPs.....30
7. Serum Uric Acid level in rats treated with different doses of Cu NPs.....31
8. Different Hematological parameters level in rats treated with different doses of Cu NPs.....33

List of Abbreviations and Symbols

RBC	Red Blood Cells
PCV	Packed Cell Volume
ROS	Reactive Oxygen Species
MCV	Mean Corpuscular Hemoglobin Concentration
MCHC	Mass Cell Volume
ALT	Alanine aminotransferase
ALP	Alkaline Phosphatase
LD50	Lethal Dose
LC50	Lethal Concentration
BUN	Blood urea nitrogen
OECD	Organisation for Economic Co-operation and Development
Hb	Haemoglobin
WBC	White Blood Cells
EDTA	Ethylene Diaminetetraacetic Acid
DMSO	Dimethyl Sulfoxide

ABSTRACT

Nanotechnology is among the quickly developing areas of science and innovation with the expanding progress being made in the subjects of medicine, cosmetics engineering and electronics. Metallic nanoparticles have extensive medical, consumer and industrial applications due to their unique characteristics such as high surface-to volume ratio, broad optical and electronic properties, ease of synthesis, facile surface chemistry and functionalization. Exposure of these particles to humans and other biological systems has aroused global concerns regarding their fate in biological systems resulting in a demand for their toxicity assessment.

This study was designed to evaluate the *in vivo* sub-chronic oral toxicity of copper (Cu) nanoparticles. The rats were treated orally with different doses (75, 125, 250 and 500 mg/kg) of Cu nanoparticles for 90 days and observed weekly for signs of toxicity such as body weight, food and water consumption, loss of appetite, diarrhea and vomiting, etc. The animals were sacrificed after 90 days exposure to copper nanoparticles, the blood was collected for biochemical and hematological analysis. The organs were removed and observed visually for signs of abnormality (necropsy finding) and organ weights were also determined. The organs were preserved in formalin solution for histopathological examination.

All animals from 250, and 500 mg/kg dose group showed obvious symptoms of poisoning such as loss of appetite, diarrhea and vomiting, etc. as compared to control. In addition, rats in 250, and 500 mg/kg Cu nanoparticles treated dose group showed passive behavior, tremor, arching of back, paralysis, increase heart beat and depressed respiration.

There were no significant differences in body weight was observed in 75, 125 mg/kg dose group as compared to control, but following exposure to 250, and 500 mg/kg dosages of Cu nanoparticles, the body weight of the rats was found significantly reduced.

No significant organ-weight changes were observed in rats treated with different doses (75, 125, 250 and 500 mg/kg) of Cu nanoparticles as compared to control after 90 days. However, increased in the average weight of the liver and decreased in the average weight of kidney were found in rats from the high-dose (500 mg/kg) group.

There were no statistically significant changes observed in rat blood glucose, total protein, serum albumin, serum globulin, serum triglyceride and cholesterol in all dose groups. However, aspartate aminotransferase (AST/SGOT), alkaline phosphatase (ALP), alanine aminotransferase (ALT/SGPT), blood urea, serum creatinine and uric acid levels were significantly increased in

rats from the 250 mg/kg and 500mg/kg Cu nanoparticles treated dose group compared with those from the control group.

Hematological data showed that there was significant decrease in hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in the 250 and/or 500 mg/kg groups compared with the control. The white blood cells (WBCs) count decreased significantly along with percent increase in lymphocytes, monocytes eosinophils, basophils following exposure to 250 and/or 500 mg/kg of Cu nanoparticles as compared to control group.

No significant changes were observed in75mg/kg,125mg/kg and 250 mg/kg dose group during histopathological examination of different organs. Moderate changes were found in photomicrograph of kidney, liver and spleen of the rats treated with high dose (500 mg/kg) of Cu nanoparticles.

The finding of this thesis will advance the knowledge about the toxicological effects and safety of copper nanoparticles in view of their tremendous applications in various fields of life.

1. Introduction and Literature Review

1. 1 Nanotechnology

The origin of the term ''nanotechnology'' is connected with the Greek word ''nano,'' which means 'dwarf.' Growth of the nanotechnology industry in the late times and nanomaterials are being produce at a quick rate for use in innumerable industries (Warheit, 2008). Nanotechnology is "an understanding and control of matter at dimensions between 1 and 100 nanometers (nanometer is 1 x 10-9 m or one millionth of a millimeter), where exceptional phenomena empowers its novel applications" by the creation and manipulation of materials at the nanometer (nm) scale (Sun H *et al.,* 2009).The union of nanotechnology with different fields of sciences including physics, chemistry, and biology has brought the idea of combination of nanoparticles from their separate metals (Ingle, A. P. *et al.,* 2014).Nanotechnology also deals with materials and devices on the size of one or couple of nanometers. Materials at this scale display properties that contrast physically, artificially, and naturally from their bigger partners. As indicated by the U.S. National Science Foundation and National Nanotechnology Initiative "nanotechnology is the capacity to comprehend, control, and control matter at the level of individual atoms and molecules, and additionally at the supra sub-atomic level including groups of particles (in the scope of around 0.1 to 100 nm) to make materials, devices and frameworks with in a general sense new properties and capacities on account of their little structure" (Roco, 2007).

Nanotechnology is not another field. We have utilized nanomaterials for truly quite a while, e.g. gold nanoparticles in the Roman Lycurgus cup (fourth century A.D.), the iron oxide nanoparticles in Maya blue paint (Jose-Yacaman *et al.,* 1996) (roughly 700 A.D.) and Michael Faraday's colloidal gold arrangements, initially reported in 1857 (Farady, 1857). In particular light conditions, these gold nanoparticles suspensions could create diverse different colored solutions green, violet, blue and ruby. These particles are still steady and are in plain view in the Royal Institution in Great Britain. Be that as it may, the distinction today is that we have started

to see how to control the optical and electrical properties of matter, late advances in union of nanomaterials and characterization tools.

1.2 Nanomaterials

Nanomaterials are generally at the 1–100 nm scale and have an inconceivable source of utilizations, for example, in medication, hardware and vitality generation. Beautifiers, sunscreens, coatings, batteries, fuel added substances, paints, colors, tires and concrete are the illustrations of purchaser items that in light of nanotechnology. Nanomaterials may likewise use for exceptional therapeutic purposes, for example, to create novel medication conveyance frameworks, to improve the execution of medicinal gadgets, or to deliver analytic imaging materials (Aydn, Sipahi, & Charehsaz, 2012)

1.2.1 Properties of nanomaterials

Special properties of nanomaterials are attributed to the quantum effects and large surface to volume ratio in contrast to their bulk counterpart. The level of reactions may increase with the large specific surface area of particles, which are kinetically or thermodynamically unfavorable (Jefferson, 2000). Gold nanomaterial, which is a very stable and relaiable material, becomes highly reactive when particle size is small enough (Haruta, 2003). Only very less kinds of manufactured nanoparticles are presently in industrial production such as titanium dioxide (TiO2), carbon black, zinc oxide (ZnO), iron oxide (Fe3O4, Fe2O3), etc. and others are expected to appear in the future (Borm and Kreyling 2004). Nanomaterials may have distant shapes or structures like spheres, tubes, needle-like, platelets, etc. Metallic nanoparticles have fascinated researcher for over a century and are presently vigorously used in biomedical sciences and engineering. Today these materials can be blended and changed with different substance useful gatherings which permit them to be conjugated with antibodies, ligands, and medications of hobby and consequently opening an extensive variety of potential applications in biotechnology, attractive partition, and preconcentration of target analytes, focused on medication conveyance, and vehicles for gene and drug delivery and more importantly diagnostic imaging Iron oxide, aluminum oxide, copper, titanium oxide ,silver and so on are a few cases of metallic nanoparticles. (Mody *et al.*, 2010)

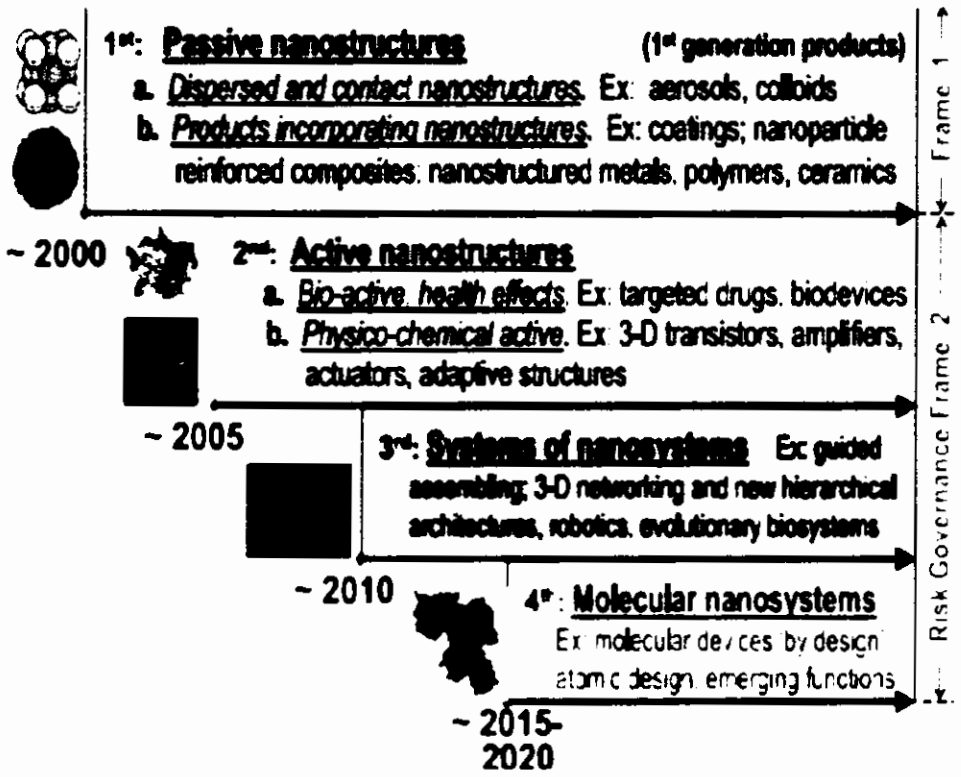


Figure 1 Timeline for beginning of industrial prototyping and nanotechnology Commercialization: Four generations of nanoproducts (Renn and Roco, 2006).

1.3 Toxicology

The Society of Toxicology characterizes toxicology as "the investigation of the unfriendly physicochemical impacts of chemical, physical and biological agents on living beings and ecosystem, involving the hindrance and amelioration of such an adverse impact". Examples of such agents include cyanide (chemical) radiation (physical) and the snake venom (biological). Albeit all substances are toxins, it is the correct dosage that separates the toxic substance and cure. Fundamental perceptions on the harmfulness of chemicals were made by Paracelsus in the mid sixteenth century (Gallo, 2008). Toxicological reviews are imperative for the assurance of people and the earth from the deadly impacts of toxicants and the advancement of more specific toxicants, for example, anticancer and other clinical medications and pesticides.

Toxicity may be acute or chronic, and there are number of biological factors that may influence an individual's sensitivity for adverse health effects from exposure to toxic substances. These factors are age, gender, genetics, diet, physiological or the health condition of the organism. Exposure of humans and other organisms to toxicants may be intentional ingestion, occupational, environmental, deliberate (suicidal or homicidal) poisoning and accidental. The kind and severity of toxic impacts of a particular compound may vary with the way of entry into the body, whether through the alimentary canal, the inhalation, or the skin. Experimental means of administration for toxicity assessment such as injection (intravenous, intraperitoneal, intramuscular, or subcutaneous) may also present significantly inconsistent (tenfold variation) results. Following exposure, compounds are metabolized through various plausible ways of metabolism, may be either detoxifying or activating with numerous potential toxic endpoints (Hodgson, 2004).

Today in most of the developed countries the laws and regulations have approved to control the marketing of industrial chemicals, drugs, food additives, pesticide, etc. Such regulations often set down a precise rule of toxicity testing to generate information that facilitates government regulators to decide whether the benefits of a particular substance are more important than its risks to human health and the environment.

1.3.1 *In vivo* toxicity assessment

Laboratory animals are used for the safety assessment of drugs, food additives, pesticides, and industrial chemicals. *In vivo* studies regulate the toxicity from exposure range in duration from short-term or extend over a longer period of time. These are generally performed under the rules pronounced by Food and Drug Administration (FDA) and other regulatory agencies. Acute (short-term) toxicity testing is generally performed in various rodent species to define the shape of the dose-response curve and lethal dose range of a peculiar substance. Results of these studies are very helpful to set doses for subchronic and chronic experiments. Subchronic studies are generally conducted in both sexes of rodent and non-rodent species. These are carried out using multiple doses for a period of three to six months.

However, chronic toxicity tests are regulated in multiple species for a period that approaches the life span of the animal. These types of experiments are mostly complicated and time consuming that needs a multiple intermediate clinical evaluation, including daily observations of animals for signs of toxicity, frequent observation of food consumption and body weight. At the end of a

study all animals are dissected to detect alterations in the tissue morphologies and complete blood chemistries (Paget and Barnes, 1964). Sub -chronic and chronic toxicity studies provide preclinical information that is very helpful for assessing safety and risk of drugs, chemicals, food additives, etc.

1.3.2 Oral LD50

LD50 (lethal dose) or LC50 (lethal concentration) is often used to measure the acute toxicity of chemicals. The dose of test substance that produces death in half of the tested animals is stated as LD50 and the LC50 of that substance and generally measured in milligram per unit weight of test animal (mg/kg) (Turner, 1965). A high LD50 or LC50 means that a substance is less toxic because large quantities are required to cause death. A low LD50 or LC50 means that a substance is highly toxic, just a little quantity of it causes death in 50 percent of the tested animals. LD50 and LC50 measure acute effects as a result no information is available about chronic health effects of chemicals (Barile, 2010; Ghosh, 1984). Following are the three categories of substances based on the value of LD50 or LC50, extremely toxic (50 mg/kg or less), moderately toxic (50 – 500 mg/kg), slightly toxic (500 – 5000 mg/kg) or relatively harmless (5000 mg/kg or more).

1.4 Nanotoxicology

Nanotoxicology deals with the quantitative assessment of the severity and frequency of nanotoxic effects in relation to the organisms (Oberdorster *et al.*, 2005). Another control of nanotoxicology has developed to examine the potential unfavorable impacts of nanoparticles. In the course of recent years, the quantity of progressing nanotoxicology studies to explore the organic pathways taken by nanoparticles and actuated poisonous impacts have expanded considerably. In any case, next to no is thought about the basic poisonous quality systems in charge of the harmful activities of nanoparticles. In vivo considers uncovered that ultrafine particles have reliably affected mild, yet important pulmonary inflammatory responses and extrapulmonary impacts in organs far off from the lungs (Oberdorster *et al.*, 2005; Elder *et al.*, 2006). Animal toxicity studies have uncovered that pulmonary exposure to nanoparticles produces a higher unfriendly inflammatory response than bigger particles of indistinguishable

piece at comparable mass focuses (Warheit, 2004). The toxicity of nanoparticles is essentially connected with their one of a kind physicochemical property. These exceptional nanoscale properties are liable to influence the chemistry and physics as well as their conduct in organic frameworks (Seaton, 2006; Drobne, 2007).

14.1 Nanotoxicity in humans

The connection of nanoparticles with people and the earth is not a late occasion. It is evaluated that the normal individual expends 10^{12} submicron-sized particles every day in a typical eating regimen as a consequence of sustenance added substances comprising fundamentally of titanium dioxide (TiO_2) and aluminosilicates (Lomer *et al.*, 2002). Incidental nanoparticles are additionally found in such basic sources as wood smoke, and vehicles and heater deplete (Barregard *et al.*, 2006). Levels of incidental nanoparticles in the outdoor environment near heavy traffic areas can range from 5000 to 3,000,000 particles/cm3 (Oberdorster *et al.*, 2005).Potential courses of nanoparticle introduction incorporate inward breath, dermal, and on account of biomedical applications, parenteral. Harmfulness coming about because of nanoparticle introduction could happen at the different entryways of passage, for example, the lungs and skin.

1.4.2 Variables that influence Nanotoxicity

1.4.2.1 Dose-Dependent Toxicity

Generally, negative health impacts of nanomaterials try not seen to be corresponding with molecule mass measurements. In fact, randomly, a high convergence of nanomaterials may advance molecule collection and could subsequently diminish dangerous reactions contrasted with lower groupings of the same particles (Buzea *et al.*, 2007).

1.4.2.2 Size-Dependent Toxicity

Gold nanoclusters (1.4 nm) were appeared to be lethal to cells attributable to their particular association with real scores of DNA, while littler or bigger gold particles did not carry on along these lines (Pan *et al.*, 2007). Moreover, quantum spots were accounted for to limit to

various cell compartments in connection to their size. Others have proposed that silica nanoparticles of 40–80 nm in measurement can enter the cell core and limit to particular subnuclear areas in the nucleoplasm yet don't localize with nucleoli.

1.4.2.3 Surface-Area-Dependent Toxicity

The relative division of surface particles to mass molecules is drastically diverse in nano-sized versus micro-sized particles. Subsequently, while less than 1% of molecules of a micro-particle involve surface positions, more than 10 % of the atoms in a 10-nm molecule reside on its surface; this in this manner adds to changes in surface physical and chemical properties as materials are decrease in size (Jones and Grainger, 2009). For example, taking after inward breath introduction of rats to 20-nm or 250-nm titanium dioxide particles, the half-times for alveolar freedom of polystyrene test particles were corresponding to the titanium dioxide molecule surface zone per million macrophages.

1.4.2.4 Crystalline-Structure-Dependent Toxicity

Titania exists in a diversity of cryastal structures, the most effectively examined of which are its rutile, anatase, and brookite frames (Fadeel and Bennett, 2010). Of note, the cytotoxic properties of titanium dioxide nanoparticles seem to associate with their stage arrangement. Thus, in a far reaching investigation of titanium dioxide nanoparticles (3-10 nm), exhibited that anatase titanium dioxide was 100 times more dangerous than a proportional specimen of rutile titanium dioxide. The era of receptive oxygen species under UV light related well with the watched natural reactions.

1.4.2.5 Surface-Coating-Dependent Toxicity

Surfaces of nanomaterials make contact with cells, and a detailed understanding of surface composition is therefore outstanding to understanding the interactions of nanomaterials with biological systems (Jones and Grainger, 2009). Surface contamination may result from adventitious air- or water-borne contaminants or arise from synthetic modifications of the nanomaterial. Adsorption of the ubiquitous bacterial endotoxin, lipopolysaccharide, is one of the

most common problems for all biomaterials, and could also contribute to the cellular responses evoked by nanoparticles, in particular immunological responses.

1.5 Potential routes of exposure for nanomaterial

1.5.1 Inhalation

The most common route of exposure to any aerosol particle in the workplace, including a NP, is through inhalation. In this route, the deposition of NPs in the respiratory tract is determined by the aerodynamic diameter of a stand-alone particle, or NP agglomerate, in which many weakly attached discrete NPs form a particle larger than 100 nm (Jaques and Kim, 2000). Deposition increases with exercise due to an increase in breathing rate and a change from nasalto- mouth breathing (Daigle *et al.,* 2003). Deposition also increases among persons with existing lung diseases or conditions (Brown *et al.,* 2002). Based on animal studies, discrete NPs may enter the bloodstream from the lungs and translocate to other organs (Oberdorster *et al.,* 2002).

1.5.2 Ingestion

Ingestion may likewise accompany inhalation exposure, since particles that are cleared from the respiratory tract by means of the mucociliary lift might be gulped (ICRP, 1994). The capacity of mucosal layers and the gastrointestinal tract to ingest and, in few cases, encourage systemic dispersion of NPs, for example, liposomes, proteins and infections (e.g., rotavirus, hepatitis E) is surely understood. Moreover, it has been demonstrated that inorganic particles, for example, 500 nanometer (nm) titanium dioxide (Jani *et al.,* 1994) and nanoscale gold (Hillyer, 2001), can possibly cross the digestive tract coating and translocate to systemic organs such the liver, spleen, lung and peritoneal tissues. There is likewise confirming that littler particles can be exchanged more promptly than their bigger partners over the intestinal divider (Behrens *et al.,* 2002). These regular procedures are without further excitement of search for potential applications in creating nanoscale vehicles for oral medication and supplements conveyance: poly (DL-lactide-coglycolide) - covered particles (Brayden and Baird, 2001), liposomes (Hussain *et al.,* 2001), unsaturated fat polymer particles (Mathiowitz *et al.,* 1997) and virosomes (Rae *et al.*, 2005).

1.5.3 Dermal

Dermal introduction can be another course for nanomaterials to pick up section into the human body. Entrance of in place skin can happen through various pathways, including sweat pipes, startup consumption by means of between or intracellular modes and hair follicles. The capacity of nanoscale particles to cross the skin's external layer of startup consumption remains a subject of serious study and open deliberation. A few studies demonstrate that particles as substantial as 1000 nm can enter human skin after flexing in vitro (Tinkle, 2003), while different studies demonstrate that nanoscale titanium dioxide re mained on the furthest layer of unperturbed human skin in vivo following six hours with none recognized in the more profound stratum corneum, epidermis or dermis (Schulz *et al.*, 2002).

1.5.4 Parenteral

Nanomaterials can be likewise brought into human bodies by means of the parenteral course either by chance through slices and other harm to in place skin, or deliberately for medication conveyance, restorative imaging, or different applications. Built nanomaterials for medication applications can convey notably enhanced qualities: (1) upgraded dissolvability particularly for hydrophobic substances; (2) expanded solidness through coatings fit for keeping away from the resistant framework; (3) enhanced specificity through multifunctional capacities and dynamic and detached focusing on; and (4) the improved capacity to infiltrate particular obstructions like the blood-mind boundary (Lockman *et al.*, 2003). Engineered nanomaterials that enter the body parenterally can collaborate with plasma proteins in minor courses, for example, covalently clinging to proteins without changing the capacity of the protein, and non-unimportantly, by cooperating with the body's frameworks like communications normal for certain characteristic nanoscale particles, for example, proteins and infections.

1.6 Copper nanoparticles

Nano-sized copper particles are one of the fabricated nanoparticles that are presently industrially produced and accessible. Now days, nano-copper particles are used as the additive in lubricants, polymers/plastics, metallic coating and inks, and so on. Because of superb retouching impacts of nano-copper particles, they are included into oil as an added substance to viably

decrease contact and wear, or to repair a worn surface (Liu *et al.*, 2004). Nano-copper particles are homogeneously stored on the surface of graphite to enhance the charge–discharge property essentially, for example, coulombic effectiveness, cycle attributes, and high rate execution as an anode material for lithium ion batteries (Guo *et al.*, 2002).The copper-fluoropolymer nano-composite is utilized as bioactive coatings that are equipped for hindering the development of target microorganisms, for example *Saccharomyces cerevisiae*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria* (Cioffi *et al.*, 2005).

Accordingly, nano-copper particles, like any of different nanomaterials, are liable to enter nature and human body by means of various ways, for example, such as effluent, spillage during shipping and handling, consumer products and disposal, and so on. In human body, copper is kept up in homeostasis (Jesse and Mary, 2004). In the event that the admission of copper surpasses the scope of the human resistance, it would bring about toxic impacts, for example, hemolysis, jaundice and even death.

1.6.1 Bioactivities of Copper-nanoparticles

Antimicrobial activity of copper nanoparticles showed the adequacy of copper nanoparticles across different pathogenic organisms. In addition to the control of development of yeasts and molds, copper nanoparticles have likewise seen to be viable against gram-positive and gram-negative microbes (Schrand *et al.*2010; Theron *et al.* 2008).

Das *et al.* (2010) concentrated on the antibacterial action of copper nanoparticles against three microbes to be specific Staphylococcus aureus, Bacillus subtilis, and Escherichia coli by the Kirby–Bauer dispersion strategy. They found that copper nanoparticles were compelling development inhibitors against these microscopic organisms. Copper nanoparticles likewise exhibited the size-dependent antibacterial action (Duran *et al.*, 2010; Prabhu *et al.*, 2010).Copper nanoparticles have been researched for use in biotechnological applications that may battle these infections. Scientists have attempted to join copper nanoparticles with a polymer material to make a composite equipped for discharging metal species in a controlled way keeping in mind the end goal to repress the development of growths and other pathogenic microorganisms (Cioffi *et al., 2005*). Antiviral movement of copper nanoparticles, which affirms that copper Cu nanoparticles additionally have promising antiviral action.

Fujimori *et al.* (2012) researched the antiviral action of nanosized copper (I) iodide particles having a normal size of 160 nm against a flu An infection of swine beginning (pandemic [H1N1] 2009) utilizing plaque titration test. They claimed that copper nanoparticles can be beneficial for protection against viral attacks and may have new applications for the development of filters, face masks, protective clothing, and kitchen cloths. Anyaogu *et al.* (2008) reported antialgal movement of functionalized copper nanoparticles against strains of green growth, cyanobacteria, and diatoms .According to (Jose *et al.*,2011) copper nanoparticles corrupt DNA in a single oxygen-mediated fashion even without any outer specialists like hydrogen peroxide or ascorbate. This makes copper nanoparticles as a magnificent possibility for targeted therapy. Antibacterial action of copper nanoparticles has been abundantly considered when contrasted with its movement against growths. Among the distinctive types of parasites, Saccharomyces cerevisiae is said to be a model living being for concentrating on the antifungal movement of nanomaterials (Longano *et al.*, 2012).

1.6.2 Applications of Copper Nanoparticles

Copper Cu nanoparticles have a number of industrial applications and are accessible commercially. Its late utilization incorporates facial splash, hostile to oxidants, anode materials for lithium-particle batteries, polymers, metallic coatings, plastics, bimetallic catalyst, fillers in electrically conductive polymer composites, antibacterial specialists and inkjet printing nanocopper inks (Barrabes *et al.*, 2006; Guo *et al.*, 2002; Lee *et al.*, 2008b; Liu *et al.*, 2004; Ren *et al.*, 2009; Yang *et al.*, 2006). Cu nanoparticles have a more prominent patching impact as an oil added substance productively diminished wear, contact and repaired worn surfaces

(Tarasov *et al.*, 2002). Zerovalent Cu nanoparticles are utilized as an added substance as a part of animals and poultry sustain furthermore utilized as a part of osteoporosis treatment drugs (Lei *et al.*, 2008). The introduction to designed nanomaterial has expanded subsequent to the last couple of years. Subsequently numerous inquiries are raised with respect to the toxicological and ecological effect of nanomaterials.

1.6.3 Copper nanoparticles exposure

Occupational exposure of copper dusts or fumes is harmful to human health, including an expanded danger of malignancy among copper smelter specialists (Magaye *et al.*, 2012). In the early studies on the genotoxicity and cancer-causing nature of water solvent copper mixes, for example, copper sulfate, they were seen to be genotoxic, with qualities including the actuation of chromosomal distortions and micronuclei in White Leghorn chick bone marrow cells (Bhunya *et al.*, 1996) and chromosomal abnormalities in Swiss mice (Agarwal *et al.*, 1990). However, the information on the genotoxicity and cancer-causing nature of water insoluble copper particles are rare.

1.6.4 Toxicity of Copper nanoparticles

Different studies demonstrated that copper nanoparticles can bring about various parameters of harmful impacts to organic frameworks. Copper nanoparticles demonstrated a size and focus subordinate harmfulness to dorsal root ganglion neurons of rats (Prabhu *et al.*, 2010). Lei *et al.* (2008) guaranteed that copper nanoparticles could bring about scattered spot hepatocytic rot and across the board renal proximal tubule putrefaction in the rodent. Chen *et al.* (2006) demonstrated that no one but copper nanoparticles can affect toxicological impacts and extreme neurotic wounds to the kidney, liver and spleen of mice when contrasted and copper at micrometer size. It is as of now realized that the harmfulness brought about by miniaturized scale copper is lower than the poisonous quality of copper nanoparticles and the lethality created by copper particles in copper nanoparticles media and the danger of copper oxide NPs can't be just clarified by Copper particles discharged into the cell medium (Chen *et al.* 2006; Karlsson *et al.* 2008).

Cytotoxic impacts and responsive oxygen species (ROS) levels were corresponded with the physico-synthetic properties of the copper nanoparticles and the cell sorts. Liver being the basic organ for copper storage, homeostasis and discharge of a few animal categories and an acquired issue of copper digestion system can bring about Wilson's sickness in people (Tao and Gitlin, 2003).

Like others nano-sized particles, Cu nanoparticles are also manufactured at industrial level and available commercially. Hence Cu nanoparticles enter the environment and human body through different paths such as effluent, spillage during shipping and handling, consumer products and

disposal, etc. similar to any of other nanomaterials. Size-depended toxicity of Cu nanoparticles was reported in a study conducted on mice and the oral LD50 found 413 mg/kg body weight (Chen *et al.*, 2006).

Meng *et al.* (2007) examined the in vitro and in vivo toxicological impacts of nano-copper and watched that these nanoparticles may not bargain the mice straightforwardly, be that as it may, they lead to the aggregation of unreasonable alkalescent substance and overwhelming metal particles (copper particles) finished the metabolic alkalosis and copper particle over-burden.

The antibacterial impact of Cu nanoparticles was examined by (Yoon *et al.*, 2007) against Bacillus subtilis (B. subtilis) and E. coli. Among them B. subtilis demonstrated most prominent vulnerability when contrasted with E. coli regarding percent debasement.

Sharma (2007) studied the toxicity of copper nanoparticles by administrating them through the route of Intravenous, intraperitoneal or intracerebral. Copper nanoparticles induced brain dysfunction in normal animals and aggravating the brain pathology caused by whole-body hyperthermia.

Zhangh *et al.* (2012) evaluate the systemic toxicity and potential influence of intranasally instilled copper nanoparticles (23.5 nm) at three different doses on the neurotransmitter secretion. Copper nanoparticle-exposed mice exhibit pathological lesions at different degrees in certain tissues and also effect on the neurotransmitter levels in the brain (Zhangh *et al.*, 2012).

Toxicity assessment of copper nanoparticles (10-15 nm) in rat indicated that all concentration of copper nanoparticles induces toxicity and histo-pathological changes in liver and lung tissues (Doudi and Setorki, 2014).

A few investigations performed with rats and mice indicated genuine destructive impacts of NPs on mind. In the rodent, a work to Cu-NPs (40 and 60 nm distance across) incited the multiplication of endothelial cells of mind vessels when they were regulated to low fixations (around 1.5 μg/mL). Higher focuses (around 50 μg/mL) impelled an expansion of prostaglandine E2. Extracellular levels of TNFα and ILβ were essentially high, and the lethality at long last influenced the blood-cerebrum boundary (Trickler *et al.*, 2012).

Prabhu *et al.* (2010) concentrated on the impact of copper nanoparticles on dorsal root ganglion (DRG) of rodent. In their study, they presented these neurons to copper nanoparticles with expanding focuses (10–100 μM) and sizes (40, 60, and 80 nm) for 24 h. Light microscopy, histochemical recoloring for copper, lactate dehydrogenase examine for cell demise, and MTS

showed CuO nanoparticles had a great potential regarding cytotoxicity and DNA damage (Karlsson *et al.*, 2008).

Pregnant and non-pregnant mice (C57Bl/6 J) were exposed to Cu NPs or laboratory air in the whole-body chamber for 4 hrs/day. Bronchoalveolar lavage (BAL) fluid was analyzed for total and differential cells. Cytokine/chemokine concentrations were determined in the BAL fluid and the plasma of dams/non-pregnant mice and pups. Result showed profound pulmonary inflammation in dams and strong immunomodulatory effects in offspring (Adamcakova-Dodd *et al.*, 2015).

Pettibone *et al.* (2008) investigated the pulmonary inflammatory response of mice following whole-body inhalation exposure to well-characterized copper and iron nanoparticles in acute and subacute studies. Although no significant pathology was found following acute exposure, following sub-acute exposure, both iron- and copper-exposed mice showed increased inflammation compared to sentinels. Furthermore, copper nanoparticle-exposed mice had significantly higher inflammatory response immediately and three weeks post exposure (Pettibone *et al.*, 2008).

1.7 Research objectives

Nanotoxicology has widespread implications in addressing the occupational and environmental toxicity concerns of nanomaterials. Though as the lists of known toxic effects of nanomaterials and nanoparticles continue to grow, there is still a huge gap in our knowledge about the toxicity of nanomaterials. The objective of this proposed work is to investigate the toxicological profile of engineered copper nanoparticles *in vivo*. Starting from clinical observations and going through hematology, biochemical profile and histopathological examinations of different organs.

The key objectives of this study are

- To study the signs of abnormality and toxicity weekly during the 90 days of oral treatment with Cu nanoparticles.
- To analyze the hematological parameters in the blood samples such as: white blood cell count, differential counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils),

red blood cell count, hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin, mean cell hemoglobin, mean cell hemoglobin concentration, and platelet count.

- To examine the changes in blood biochemistry such as: aspartate aminotransferase (AST), alanine transaminase-(ALT), alkaline phosphatase (ALP), serum total protein, urea and creatinine
- To investigate the histopathological changes in different organs include stomach, liver, spleen, pancreas and kidney.

2. Materials and Methods

2.1 Nanoparticles

Copper Cu nanoparticles were purchased from Plasma Chem, Germany. Cu nanoparticles were obtained as a nanopowder suspension in ethanol with average particle size of 40 nm. The stock solutions (10 mg/ml) of nanoparticles were prepared in sterilized double distilled water.

2.2 Characterization of Nanoparticles

The hydrodynamic diameter, size and morphology of nanoparticles were determined by using dynamic light scattering and transmission electron microscopy.

2.2.1 Dynamic Light Scattering (DLS)

The DLS for characterization of the size of the nanoparticles in solution was performed on a Malvern Instruments Zetasizer Nano-ZS instrument. DLS analyzes the velocity distribution of particle movement by measuring dynamic fluctuations of light scattering intensity caused by the brownian motion of the particle. This technique yields a hydrodynamic radius or diameter to be calculated via the Stokes-Einstein equation from the aforementioned measurements. It yields an overall measurement of the particle perpendicular to the light source at that instant. For the particle sizing in solution (DLS), Malvern Zetasizer Nano-ZS uses the Dispersion Technology Software (V4.20) for data collection and analysis of particle size. Nanoparticles, already in a suspension forms (Fe_2O_3, Cu and Ag) were diluted from bulk concentrations to 1 mg/ml working stock concentrations and sonicated for 30 second at 35 W. Nanoparticles' stock solutions (1 mg/ml) were then used to make 25–50 µg/ml solutions in water added) for size measurement trials (Murdock et al., 2008).

2.2.2 Transmission Electron Microscopy (TEM) of Nanoparticles

TEM characterization was performed to obtain nanoparticle size and morphology on a JEOL JEM 1010 tungsten-tip instrument at an accelerating voltage of 100 KV. Nanoparticles were examined after making suspension in water and subsequent deposition onto carbon-coated TEM grids. Information on mean size and standard deviation was calculated from measuring over 100 nanoparticles in random fields of view in addition to images that show the general morphology of the nanoparticles (Murdock *et al.*, 2008).

2.3 Nanoparticles and animals

Male BALB/c rats were purchased from National Institute of Health, Islamabad. They were housed in conventional animal rooms with 2-3 animals per cage at National Institute for Biotechnology and Genetic Engineering (NIBGE) and identify by marking with different colors. Water and food available ad libitum throughout the acclimation and experimental period. Each treatment group was consisting of five 6-7-week old male rats. Four different doses 75, 125, 250 and 500 mg/kg of copper were used in this study along with control.

2.3.1 Accommodation and Husbandry

Laboratory veterinarian checked all the animals' health condition on their arrival in the laboratory. Three animals were housed per cage in stainless steel cages in room that were designed to maintain proper environmental conditions like temperature, humidity, air exchange and photo cycle. Room temperature and relative humidity were maintained within a range of 22± 3°C and 40-70 % respectively. 12 hours light and dark cycle was maintained in animal house room. Room was properly air exchanged and water was in supply. Animals were given standard laboratory diet in pellet form and filtered water was provided by adlibitum throughout the period of the study.

Figure 2 Sub-chronic repeated oral toxicity in albino rats

2.3.2 Acclimatization Period

Animals were observed for 1 week prior to the treatment. Only healthy animals, free from any clinical symptoms were use in the test. Test animals were randomly assigned to each dose groups and marked for individual identification.

2.3.3 Test Samples Dose Preparation

Solution/suspension of the nanoparticles was obtained with vigorous mixing and sonication from the stock solution 10 mg/ml.

2.4 Sub-chronic Repeated Oral Toxicity

A 90-days repeated-dose oral toxicity test was conducted as per Organization for Economic Co-operation and Development Test Guideline TG420 and 423 with modifications for dosage, biochemical parameters, and histopathological evaluation. Doses were administered orally through gavage. Five animal groups were formed with five animals in each group. Four groups were treated with different dosage of copper nanoparticles. One group was treated with normal saline as a control. Four different doses 75, 125, 250 and 500 mg/kg of copper Cu nanoparticles were used and selected by preliminary dose response experiments.

2.4.1 Observations for Clinical Signs of Toxicity

All animals were observed for mortality, wellness parameter during the whole experimental period. Animals were observed at least two times on the day of treatment, with special attention given during the first 4 hours and daily for 90 days. Body weight, food and water intake were measured weekly. All 25 rats were observed daily for signs of abnormality and toxicity during the 90- days of treatment.

2.4.2 Sample Collection

Day after last treatment (day 91) the treated animals along with control were anesthetized with isoflurane. Blood samples were collected from an abdominal artery. After collection of blood samples, the animals were sacrificed under deep anesthesia with isoflurane. Different internal organs were examined for Necropsy findings and organ weight measurement. All the organs were preserved in 10% phosphate-buffered formalin solution for pathologic examination.

2.5 Biochemical Analysis of Blood

Serum biochemical analysis was carried out using an auto analyzer (Hitachi, Japan). The serum level of total protein, total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) was determined according to Reitman and Frankel (Reitman and Frankel, 957). Blood urea nitrogen (BUN) uric acid, total cholesterol, triglycerides, blood glucose and creatinine (Cr) were observed using Henry's method (Henry et al., 1974).

2.6 Hematological Analysis of Blood

Hematologic analyses were performed by using an automatic hematologic analyzer. Parameters measured in the blood samples were: white blood cell count, differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), red blood cell count, hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin, mean cell hemoglobin, and mean cell hemoglobin concentration.

2.7 Necropsy Findings and Organ Weight Measurement

After collection of blood samples, the animals were sacrificed under deep anesthesia with isoflurane. After exsanguination, the external surface, all orifices, the cranial cavity, and the thoracic and abdominal cavities and their contents were be visually observed for any signs of gross abnormality. Absolute organ weights were measured for each rat. For paired organs, the sum of those organs was used. After collection, the organs preserved in 10% phosphate-buffered formalin solution in preparation for histopathological examination. Organs collected will include stomach, small intestine, large intestine, liver, spleen, pancreas and kidneys.

2.8 Histopathological Examination of Organs

After 24 h the tissues were removed from formalin solution for histopathological examination and were embedded in paraffin block. The paraffin embedded tissues were cut into 5 µm thick sections. The sections were placed on glass slide and stained with hematoxylin and eosin (H&E). The stained (H&E) slides were examined for histopathological changes using light microscope (Labomed, USA) and the photographs were also taken.

2.9 Statistical Analysis

Statistical analysis of all the data was performed using statistical software Minitab version 16 (Minitab Inc, USA). The comparison between treatment groups and control group was done using one-way ANOVA followed by the Tukey's test. $P < 0.05$ was considered statistically significant.

3 .Results

3.1 Characterization of copper Cu nanoparticles

Transmission electron microscope (TEM) and dynamic light scattering were used for characterization purpose. The size of copper Cu nanoparticles used in this study was observed with a TEM and were the same as described by the supplier. The size of Cu nanoparticle was 40 24 + 2 nm (Figure 3). However, aggregated nanoparticles were observed after analyzing data using DLS. The size of copper Cu nanoparticles in deionized H_2O was in the range of 28-68 nm (Figure 4). Physico-chemical interactions between the nanomaterials could be the possible reason for this aggregation of nanoparticles in suspension. Hence, before using constant re-suspension was performed to create a homogeneous solution.

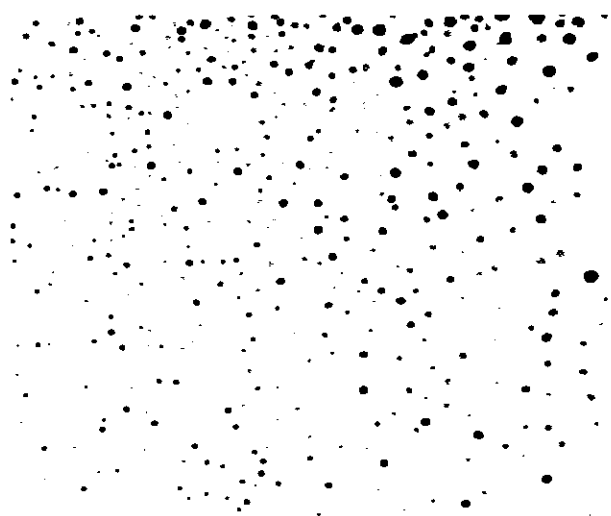


Figure 3 TEM image showing the shape and size of the copper Cu nanoparticles (magnification is x 60K).

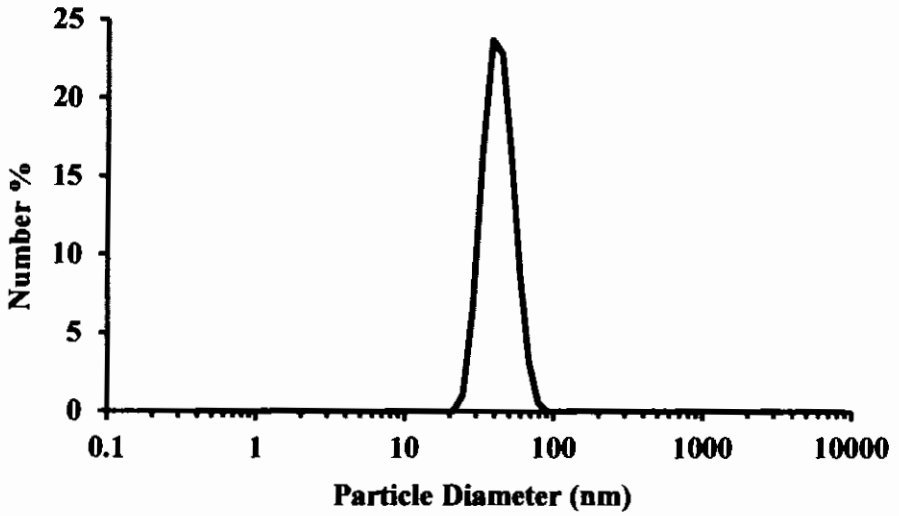


Figure 4 Size distributions of copper Cu nanoparticles in deionized H_2O.

3.2 Clinical observation

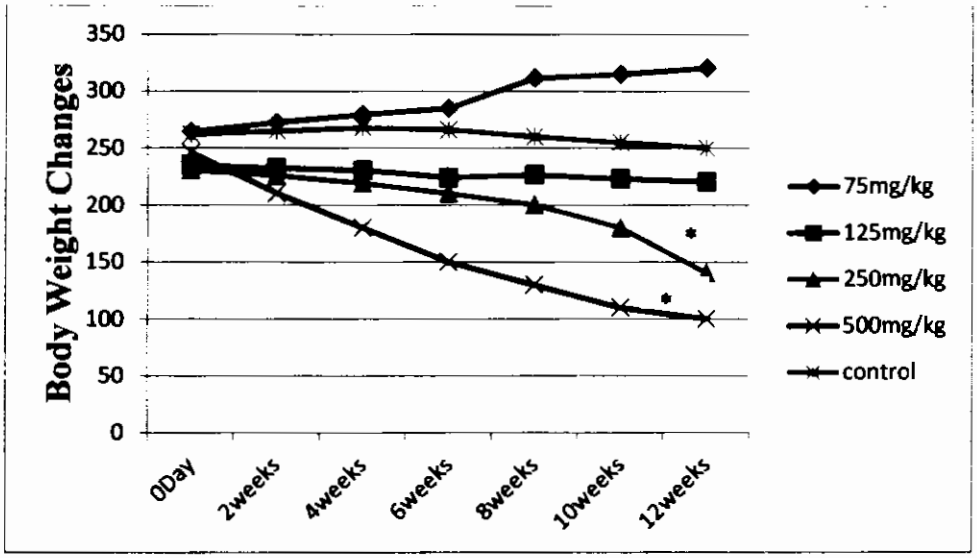
Rats treated orally with different concentration (75, 125, 250, and 500 mg/kg) of copper Cu nanoparticles were observed daily for 90 consecutive days. Rats dosed with copper Cu nanoparticles at 75, 125 mg/kg exhibited no clinical signs of toxicity and no significant decrease in body weight as compared to control. However, all animals treated with copper Cu nanoparticles at 250, and 500 mg/kg showed obvious symptoms of poisoning such as loss of appetite, diarrhea and vomiting, etc. as compared to control. In addition, some rats in 250, and 500 mg/kg copper Cu nanoparticles treated dose group showed passive behavior, tremor, arching of back, paralysis, increase heart beat and depressed respiration. During the period of experiment, percentage mortality in a group exposed with copper Cu nanoparticles orally with 500 mg/kg bw/day dose was 20% (1 rat) while there was 0% death or mortality in a group treated with 75,125 and 250 mg/kg bw/day of copper Cu nanoparticles in comparison with control group.

3.3 Effect of Cu nanoparticles on food intake, water consumption, and bodyweight

In the present study, the effects of different doses of copper Cu nanoparticles were compared to control group. There were no sigificant differences in body weight in 75, 125 mg/kg dose group as compared to control , but following exposure to 250, and 500 mg/kg dosages of copper Cu nanoparticles, the body weight of the rats was found to have been significantly ($P < 0.05$) reduced (Table **1**) and Graph **1**. For food intake, statistically significant decrease ($P<0.05$) were observed in the 250, and 500 mg/kg dose group at weeks 6, 9 and 12 as compared with the control groups. Water consumption significantly increased in all groups at various time points. This was most recognizable in the 500 mg/kg group compared with the controls and occurred at weeks 1, 2, 3, 4, and 12. Significantly increased water consumption was also seen in the 250 mg/kg group at weeks 4 and 8 ($P < 0.05$), and in the 125 mg/kg group at week 8 and 12 ($P<0.05$).

Table 1 weekly average body weights for copper Cu nanoparticles

Group	Dose mg/kg	Weekly Average Body Weights						
		0Day	2week	4week	6week	8week	10 week	12week
G1	75mg/kg	264.5±61.1	269.5±56.8	273.5±58.8	279±59.2	283±62.6	287±67.2	291.5±70.6
G2	125mg/kg	265.1±70.2	264.2±76.5	266 ±73.5	264±47.2	268 ±70.4	271±47.2	273.5±65.7
G3	250mg/kg	240.6±65.3	239.8±61.7	235.1±59.8	230±47.2	220.1±45.2	215±40.2	210.5±50.5
G4	500mg/kg	256±47.2	250.2±45.2	245±40.0	242±39.2	240±35.0	235±37.2	230±30
G5	Control	262±62.2	264±64.5	268±67.2	273±65.2	276±60.2	280±57.2	285±47.2



Graph 1: Weekly average body weight for copper Cu nanoparticles. A significant decrease (p >0.05) in body weight of rats high dosed group as compare to control group is marked with symbol "*"

3.4 Necropsy and average organ weight

3.4.1 Necropsy findings

Pale yellowish discoloration on the posterior surface of the left lateral lobe of the liver was observed in two rats from the 500 mg/kg dose group. A light yellow discoloration of the left lateral lobe of the liver (about 1 mm diameter), and a light yellow-colored cyst with adjacent fat near the right kidney were also observed in another 3 rats in the 500 mg/kg dose group. No observable necropsy changes were observed in other organs of rats in all dose groups.

3.4.2 Organ weight

No significant organ-weight changes were observed in rats treated with different doses (75, 125, 250 and 500 mg/kg) of copper Cu nanoparticles as compared to control after 90 days except for an increase ($P < 0.05$) in the average weight of the liver for the high- dose (500 mg/kg) group rats as compared to control and for decreases ($P < 0.05$) in the average weight of kidney for the 500 mg/kg dose group.

Figure 5 Necropsy and Necropsied animal

Figure 6 vital organs from necropsied animals
A) Brain B) Testes C) Kidneys D) Spleen E) Heart F) Liver G) Lungs

Table 2 Average organ weight of rats treated with copper Cu nanoparticles

Groups	Dose	Average Organ Weight						
		Heart	Lungs	Liver	Spleen	Kidney	Brain	Testis
G1	75mg/kg	0.95±0.13	2.9±0.19	2.86±1.47	0.61±0.14	1.95±0.42	1.75±0.13	0.70±0.18
G2	125mg/kg	0.93±0.20	2.10±0.41	2.90 ±1.9	0.6 ±0.07	1.94±0.37	1.78±0.05	0.68±0.20
G3	250mg/kg	0.93±0.16	2.9±0.31	2.88±0.94	0.6±0.07	1.93±0.27	1.77±0.09	0.68±0.13
G4	500mg/kg	0.94±0.46	2.8±0.45	3.65±1.3*	0.6±0.04	1.35±0.3*	1.76±0.19	0.69±0.17
Control	D.W	0.95±0.50	2.10±0.47	2.7±1.50	0.59±0.18	1.95±0.45	1.78±0.20	0.71±0.19

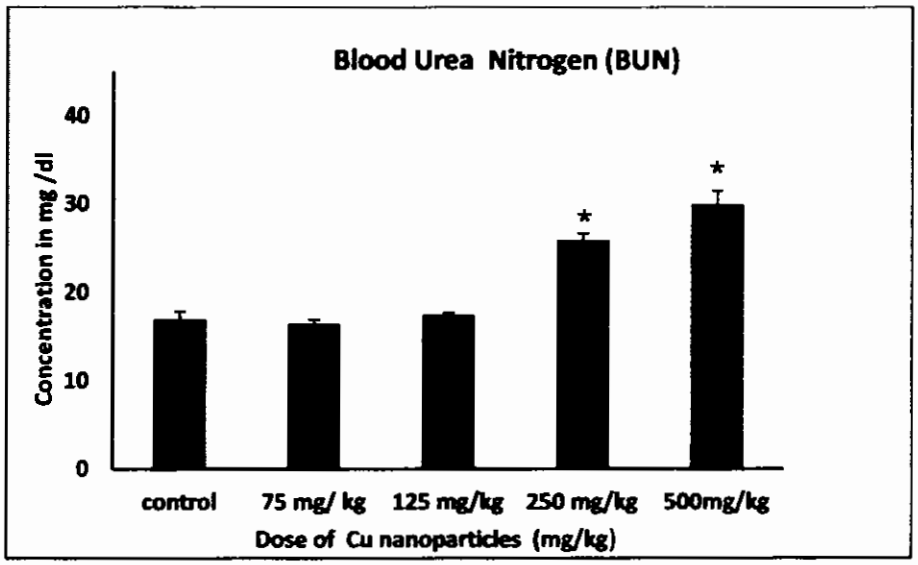
- **A significant decrease and increase ($p > 0.05$) in organ weight of rats high dosed group as compare to control group is marked with symbol "*"**

3.5 Serum biochemical parameters and hematological observations

Rats treated with copper Cu nanoparticles were studied further. Serum biochemical parameters and hematological observations were examined.

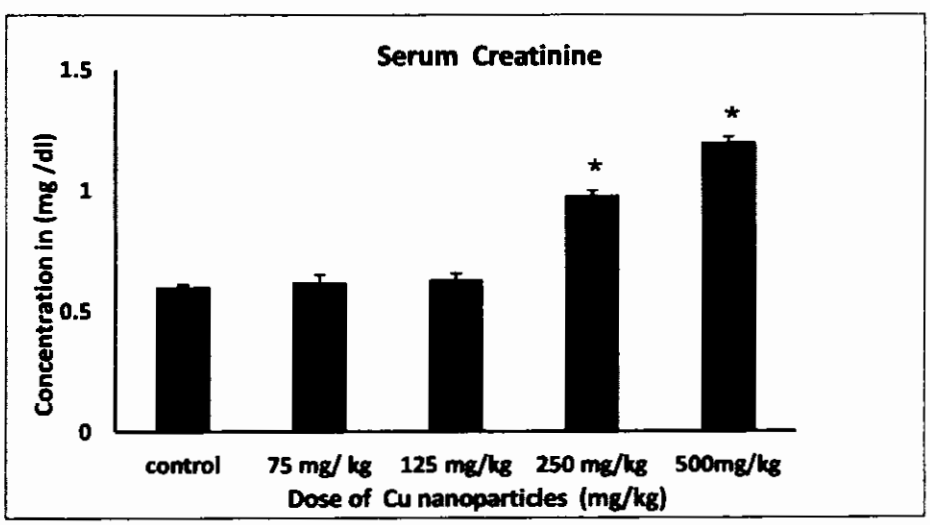
3.5.1 Analysis of serum biochemical parameters

There were no statistically significant changes in in rat blood glucose, total protein, serum albumin, serum globulin, serum triglyceride and cholesterol data related to administration of copper Cu nanoparticles in all dose groups as shown in (Table 3). However, aspartate aminotransferase (AST/SGOT), alkaline phosphatase (ALP), alanine aminotransferase (ALT/SGPT), blood urea, serum creatinine and uric acid levels were significantly increased in rats from the 250 mg/kg and 500mg/kg Cu nanoparticles treated dose group compared with those from the control group.



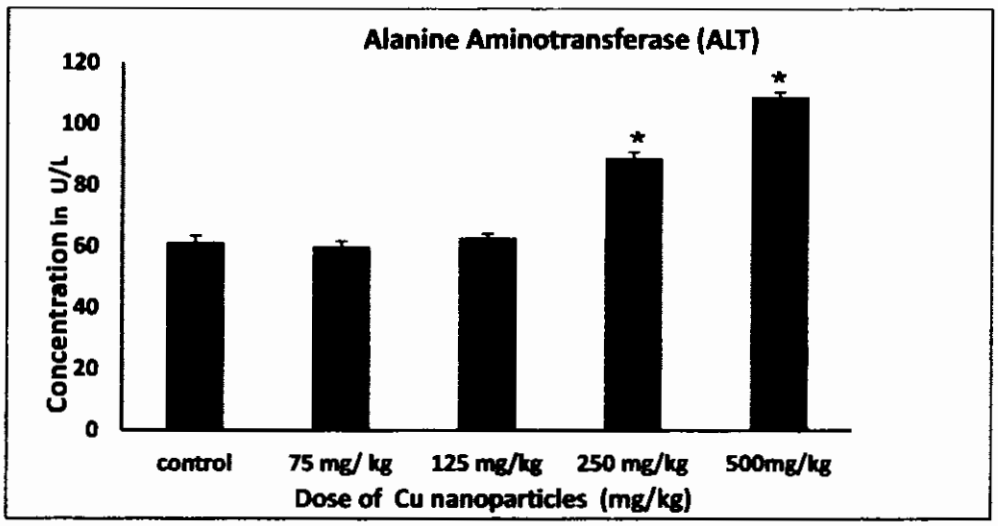
Graph 2: Blood Urea Nitrogen level in rats treated with different doses of Cu NPs

A significant increase (p >0.05) in Blood Urea level Nitrogenof rats high dosed group as compare to control group is marked with symbol "*"



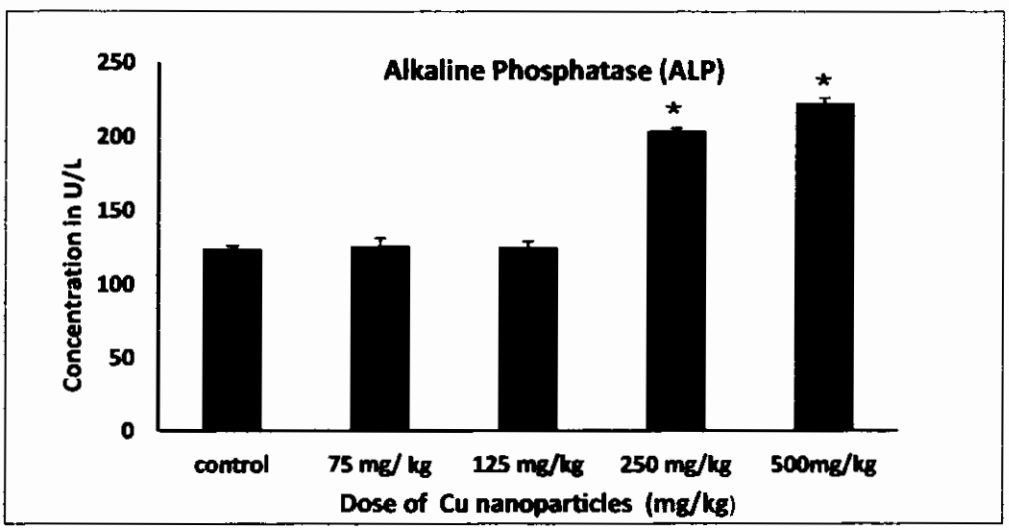
Graph 3: Serum Creatinie level in rats treated with different doses of Cu NPs

A significant increase (p >0.05) in serum createnine level of rats high dosed group as compare to control group is marked with symbol "*"



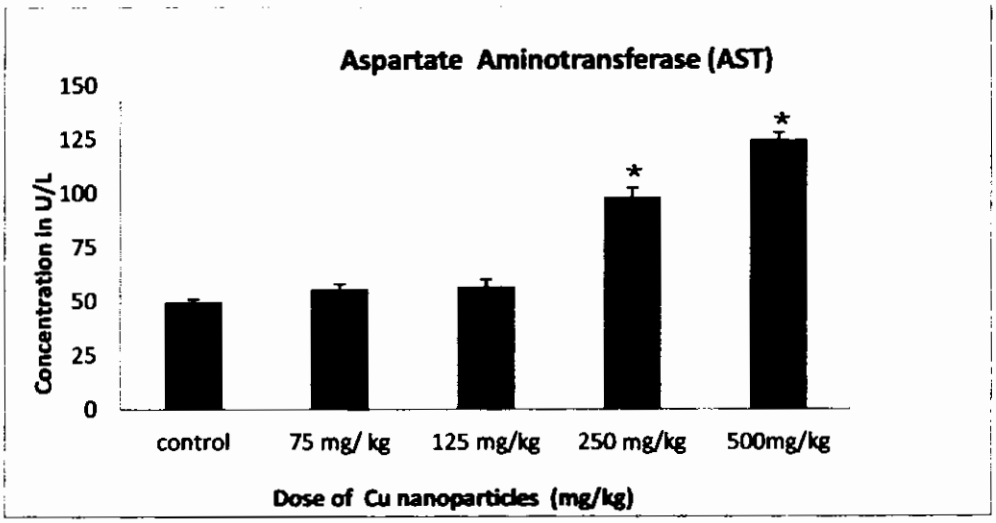
Graph 4: ALT level in rats treated with different doses of Cu NPs

A significant increase (p >0.05) in ALT level of rats high dosed group as compare to control group is marked with symbol "*"



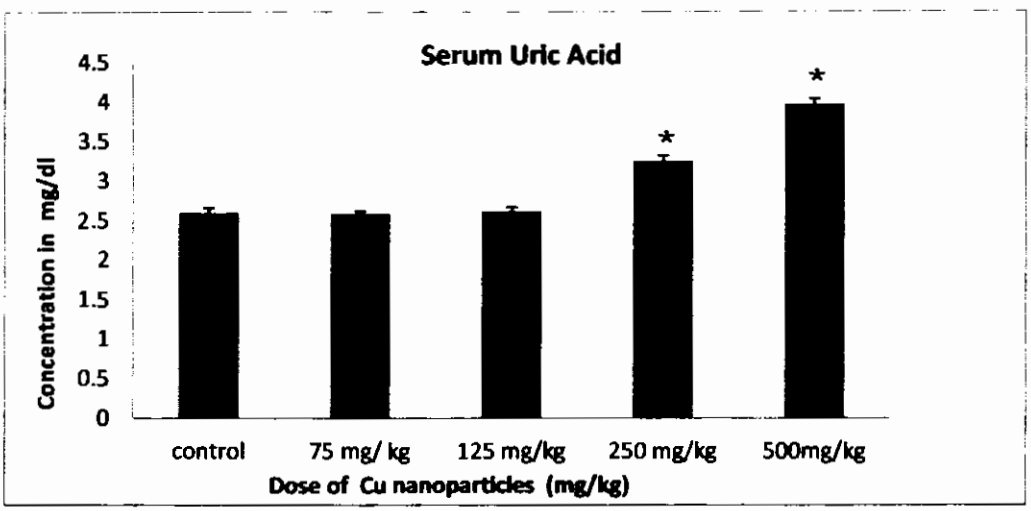
Graph 5: ALP level in rats treated with different doses of Cu NPs.

A significant increase (p >0.05) in ALP level of rats high dosed group as compare to control group is marked with symbol "*"



Graph 6: AST level in rats treated with different doses of Cu NPs

A significant increase (p >0.05) in AST level of rats high dosed group as compare to control group is marked with symbol "*"



Graph 7: Serum Uric Acid level in rats treated with different doses of Cu NPs

A significant increase (p >0.05) in Serum Uric Acid level of rats high dosed group as compare to control group is marked with symbol "*"

Table 3 Serum biochemical parameters of control and copper Cu nanoparticlese exposed rats

Serum Biochemistry Parameter	Treatment Groups (after 12 weeks)				
	G1 Dose=75mg/kg	G2 Dose=125mg/kg	G3 Dose=250mg/kg	G4 Dose=500mg/kg	Control
Blood Glucose mg/dl	123.2±10.3	121.7±15.8	116.25±12.7	115.6±6.01	106±6
Blood Urea mg/dl	16.5±7.08	17.5±9.86	24±2.5	30±0.47	17±4
Serum Creatinie mg/dl	0.62±0.05	0.63±0.24	0.98±0.05	1.2 ±0.04	0.6±0
Bilirubin Total mg/dl	0.49±0.05	0.52±0.04	0.50±0.08	0.51 ±0	0.5±0
ALT(SGPT)	60±19.5	63±5.9	89±9.5	109±7.3	61.5±19.5
Alkaline Pbosphate U/L	126±13.3	124 ±12.2	204±5.3	223.6±13.4	124±14.1
AST U/L	55.8±5.3	57 ±2.2	98.2±4.3	125±3.4	50±4.1
Serum Uric Acid mg/dl	2.59±0.2	2.62 ±0.25	3.25±0.22	3.97±0.141	2.6±0.1
Serum Cholesterol mg/dl	75.2±3.63	72±18	73.7±5.21	74 ±11.4	71.5±3.5
Serum Triglycerides mg/dl	101.2±87.3	101.5±111.5	105.2±32.9	103.6±101.1	106.5±5.5
HDL Cholesterol mg/dl	75.5±5.93	75.0±3.90	74.5±3.90	76.3±8.21	76.5±2.5
Total Protein G/dl	5.89±0.19	6.02±0.63	6.12±0.62	6.20±0.74	6.11±0.35
Serum Albumin G/dl	4.3±0.36	4.4±0.52	4.55±0.67	3.86±0.18	4.4±0.7
Serum Globulin G/dl	4.07±0.32	4.0±0.12	4.01±0.21	4.03±0.57	4.05±0.35

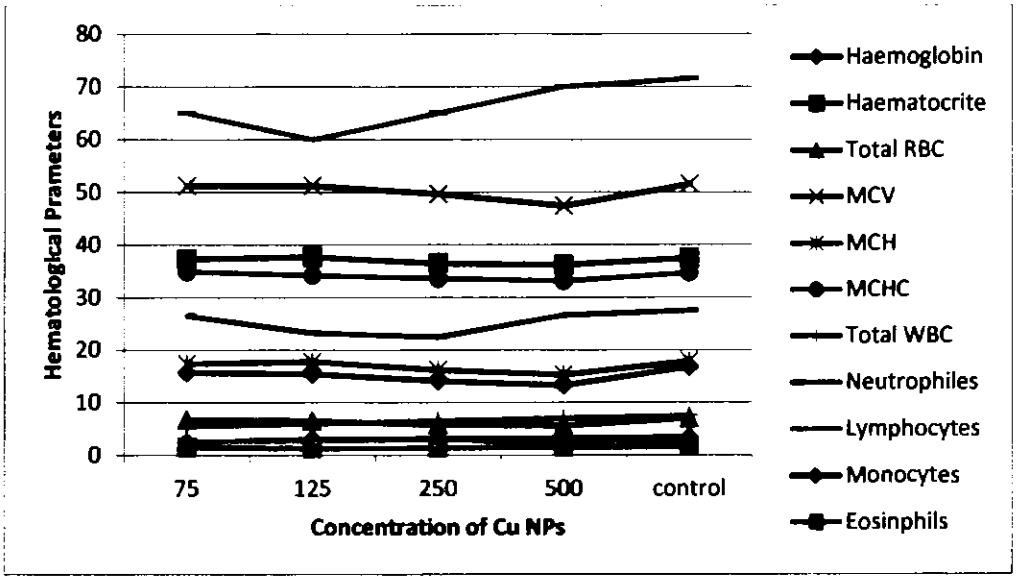
3.5.2 Analysis of Blood Hematology parameters

Effect of copper Cu nanoparticles on blood hematology was determined using various parameters as shown in (Table 4). Hematological data showed that there were significant ($P<0.05$) decrease in hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in the 250 and/or 500 mg/kg groups compared with the controls. The WBCs count increased significantly ($P<0.05$) along with

lymphocytes, monocytes eosinophils, basophils following exposure to 250 and/or 500 mg/kg of copper nanoparticles as compared to control group.

Table 4 Blood hematology parameters of control and copper Cu nanoparticles exposed rats

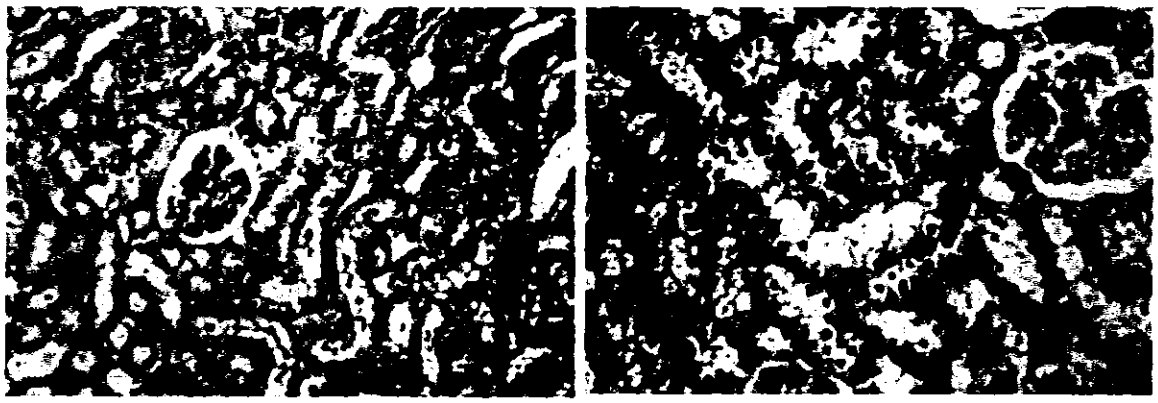
Hematological Parameter	Treatment Groups (after 12 weeks)				
	G1 Dose=75mg/ kg	G2 Dose=125mg /kg	G3 Dose=250mg/ kg	G4 Dose=500mg /kg	Control
Haemoglobin g/dl	15.7±1.13	15.5±0.64	14.1±0.81	13.3±0.26	16.8±0.7
Haematocrite %	37.3±2.8	37.7±1.98	36.5±16.5	36.2±4.82	37.5±1.35
Total RBC, $10^6/\mu L$	6.73±0.45	6.53±0.24	5.74±0.18	5.56 ±0.13	7±0.24
MCV, fL	51.2±0.08	51.2±1.76	49.6±11.3	47.4 ±1.69	51.5±0.1
MCH, pg	17.4±0.18	17.8±0.66	16.2±0.61	15.3±0.79	17.9±0.15
MCHC, g/dL	34.9±0.40	34.2±0.77	33.6±1.95	33.1±0.32	34.7±0.25
Total WBC, $10^3/\mu L$	5.5±0.26	6±1.95	6.5±0.62	7±1.72	7.5±2.15
Neutrophiles %	26.5±7.69	23.27±2.86	22.5±3.77	26.6±3.09	27.5±2.5
Lymphocytes %	65±0.8	60±4.49	65±4.55	70±2.86	71.5±3.5
Monocytes %	2.9± 0.59	3±0.86	3.1±0.96	3.21±0.65	3.44±0.98
Eosinophils %	1.5±0.5	1.3±1.11	1.4±0.82	1.5 ±0.94	1.6 ±1
Basophils %	2.70±0.46	2.71±0.57	2.99±0.96	2.12±0.76	2.73±0.86



Graph 8: Different Hematological parameters level in rats treated with different doses of Cu NPs.

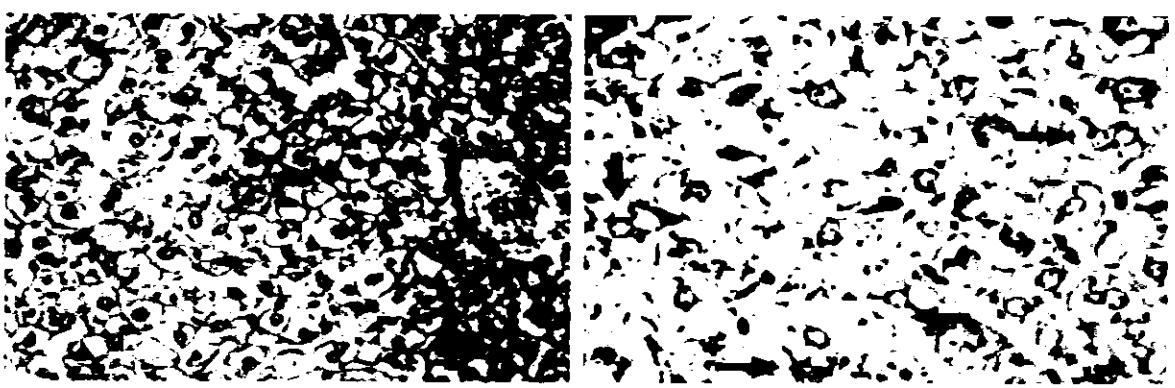
3.6 Histopathological evaluation

Histopathological evaluation of liver, spleen and kidney was performed for animals treated with dose of 500mg/kg copper Cu nanoparticles orally for 90 days. The histological photomicrograph of rat's kidney from control group **(Figures 8 (A))** shows no abnormalities whereas in the rats kidney with high dose (500 mg/kg/) of copper Cu nanoparticles, the swelling of proximal tubule was observed **(Figure 8 (B))** .Moreover, the histological photomicrograph of rat's liver from control group **(Figures 9 (C))** shows normal hepatocytes. The histological photomicrograph of rat's liver from copper Cu nanoparticles treated group shows moderate degree of vacuolation in high (500mg/kg) dose group **(Figures 9(D))** which indicated the fatty degeneration in liver cells. The histological photomicrograph of rat's spleen from control group shows no abnormalities **(Figure 10 (E))** whereas spleen of rats treated with copper Cu nanoparticles shows moderate decrease in cellularity in white pulp and increase in multinucleated giant cells **(Figure 10(F)).**



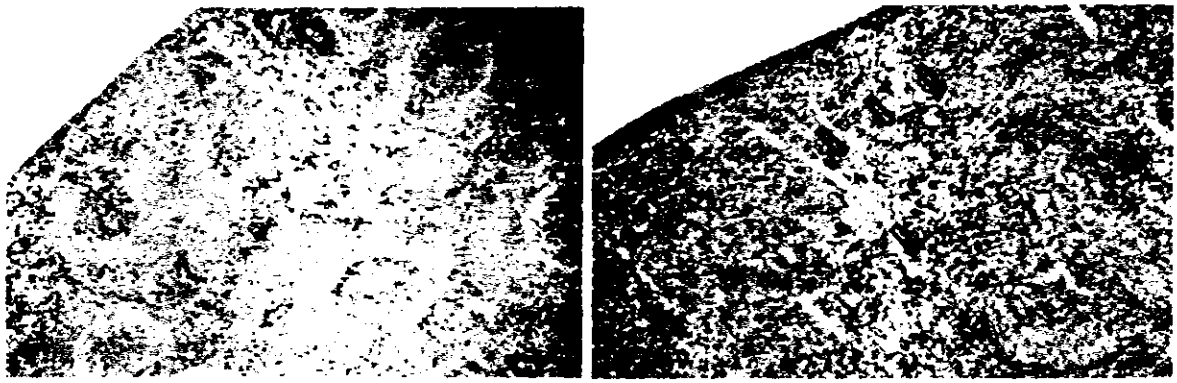
(A) (B)

Figure 8 Rats Kidney Histopathology dosed with copper Cu nanoparticles. (A) Photomicrograph of the Kidney from control rat with no abnormalities (B) Photomicrograph of the Kidney with high dose (500 mg/kg/) copper Cu nanoparticles orally treated rats, the swelling proximal tubule was observed. (Hematoxylin and eosin staining, 400 x).



(C) (D)

Figure 9 Rats Liver Histopathology dosed with copper Cu nanoparticles. (C) Photomicrograph of the liver from control rat, showing central vein (CV) and normal hepatocytes, (Hematoxylin and eosin staining, 400 x).(D) Photomicrograph of the liver from copper Cu nanoparticles treated mice (500 mg/ kg),showing moderate degree of vacuolation in the cytoplasm of hepatocytes (Hematoxylinand eosin staining, 400 x). Black arrows point to vacuolation.



(E) (F)

Figure 10 Rats Spleen Histopathology dosed with copper Cu nanoparticles (E) Photomicrograph of the spleen from control rat, showing no abnormalities (Hematoxylin and eosin staining, 400 x). (F) Spleen of rats treated with copper Cu nanoparticles shows moderate decrease in cellularity in white pulp and increase in multinucleated giant cells (megakaryocytes; open arrows)

4. Discussion

Nanotechnology is just going to affect almost every field of human life, since nanomaterials possess unique chemical, physical and mechanical properties. Nevertheless, the same properties that make these particles exhilarating for technology also make them alarming public health concerns because their toxicities are unknown and unexplored. Many studies has been designed at acute and sub-acute levels for the study of toxicity of nanoparticles, however a lot of unknowns remain about their effects and the mechanism after exposure at subchronic and chronic levels.

The present study describes a 90-day repeated dose, subchronic oral toxicity study designed to investigate the systemic toxicity of copper Cu nanoparticles in rats. During the test period only one rat in the 500 mg/kg group were found dead. In the current study clinical signs of toxicity such as loss of appetite, diarrhea and vomiting, etc along with passive behavior including tremor, arching of back, paralysis, increase heart beat and depressed respiration were observed in rats treated with high doses (250, and 500 mg/kg) of copper Cu nanoparticles to tested in this study. In another study similar signs of toxicity, including anorexia, diarrhea, lethargy, and body weight loss, were observed in rats treated with copper Cu nanoparticles (Lei *et al.,* 2008) these indicators of toxicity were identical to the effects of excessive Cu compound treatment (Semple *et al.,* 1960) (Winge and Mehra 1990). To observed potential of toxic compound, body weight of animal is sensitive indicator about the toxicity of that compound (Bailey *et al.,* 2004). The body weight and the amount of food consumption in the high dose group of copper Cu nanoparticles decreased significantly during the test period. Similar toxic manifestations has been reported by Chen *et al.* (2006) when they have administrated nano and micro copper (Cu) via oral gavage to mice for a comparison of their toxicities (Chen *et al.,* 2006). These notable changes in body weight and food consumption were regarded as nanoparticle related changes at high dose level and were likely due to the decreased intake of food poor digestion of food or enhanced degradation of proteins and lipids because of the toxicity of chemical treated rats(Mansour *et al.,* 2010).

Hematological data in this study showed significant decrease in hemoglobin, hematocrit, and mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin

concentration in the 250 and 500 mg/kg groups compared with the controls. The WBCs count increased significantly along with lymphocytes, monocytes eosinophils, basophils following exposure to 250 and 500 mg/kg of copper nanoparticles as compared to control group. This interpretation was well supported by other different studies where chronic copper Cu intoxication causes hemolytic anemia with diverse hematological changes, including decreased red blood cells, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin and white blood cells in rodents (Winge and Mehra 1990) (Chung *et al.*, 2009) (Al-Naimi *et al.*, 2013).Furthermore, the hematological changes in rats following exposure to copper Cu nanoparticles show a microcytic anemia that generally manifest iron deficiency. High copper Cu levels in blood have been shown to competitively inhibit iron absorption and utilization and to be correlated with decrease in serum iron levels (Hébert *et al.*, 1993) (Arredondo and Núñez 2005).In the differential white blood cells count, in tested high dose group decrease in lymphocytes indicated the adverse effects of copper Cu nanoparticles on the immune system. The increased percentages of neutrophils and monocytes might be related to the inflammatory response of the affected organs and the decreased lymphocytes. These results were thought to be related to the effect of copper Cu nanoparticles on red blood cells and immune organs (spleen and thymus).

Copper Cu nanoparticles treated rats showed a dose-related response in the rise of serum aspartate aminotransferase (AST), Alanine transaminase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE) as well as uric acid . These results related to the rat exposure to copper Cu nanoparticles proved that copper Cu nanoparticles cause significant damage to the liver and kidney at high dose level to be tested in this study. The distribution of these nanoparticles into liver, kidney, and spleen was due to dissociation of copper Cu from copper Cu nanoparticles which cause obvious functional and structural damage.

Previously It has been reported that, copper Cu nanoparticles caused liver and kidney damages with biochemical alterations, such as increased AST, ALT, total bilirubin (TBIL), BUN, and CRE (Lei *et al.*, 2008) (Manna *et al.*, 2012).

Our findings confirmed previous studies showing that subchronic oral exposure of copper Cu nanoparticles induces severe damage to the kidney and liver (Chen *et al.*, 2006) (Meng *et al.*, 2007) (Lei *et al.*, 2008) (Manna *et al.*, 2012).The major histopathological findings, including mononuclear cell infiltration, dilated sinusoid, degenerated or binucleated hepatocytes,

cytoplasmic vacuolation in hepatocytes, dilated tubules and degenerated tubular cells in the rats treated with Cu nanoparticles. Our results are supported by the findings of two different studies which showed that nano copper (Cu) and zinc oxide (ZnO) induce sternly toxicological effects and heavy injuries on kidney, liver, pancreas, bone and spleen of experimental mice (Chen *et al.*, 2006; Wang *et al.*, 2008).

Impairment of cellular and humoral immune responses has been resulted due to excessive copper Cu intake as described in another study. Excessive copper Cu intake results in impairment of both cellular and humoral immune responses (Pocino *et al.*, 1991). Recently, Cu (II) chloride was found toxic for splenocytes and thymocytes because it causes apoptosis in these cells, especially cell death has been observed in $CD4^+$ T cells (Mitra *et al.*, 2012) (Mitra *et al.*, 2013).

The changes in organ weight included decreased kidney weight and increased liver weight in rat treated with tested high dose of copper Cu nanoparticles. These toxicologically significant findings were well supported by correlated biochemical, hematological, and histopathological changes. Chattopadhyay and Biswas 2005) studied subchronic exposure of rats with copper chloride at 2 mg/kg/day displayed toxic effects on reproductive organs in term of development of these organs. Similarly in another toxicity study test substance related stress has been found a major cause of decrease in the weights of reproductive organs (Everds *et al.*, 2013).The routes of exposure to copper Cu nanoparticles are inhalation, ingestion, injection or physical contact. Oral administration of copper Cu nanoparticles resulted in dissociation of Cu ions from copper Cu nanoparticles in gastric pH conditions and it is due to high solubility of copper Cu nanoparticles in the acidic environment. Likewise, higher copper Cu levels in blood and tissues in rats treated with copper Cu nanoparticles showed distribution of absorbed Cu ions through circulation and accumulated in various tissues, which can be a toxic reservoir. Consistent with the above results, the toxicological study revealed that dissolution of copper Cu nanoparticles may have an important role in their toxicity (Chen *et al.*, 2006) (Meng *et al.*, 2007).

In general, few data exist on the toxicity of nanomaterials at subchronic and chronic level and, in particular, on copper Cu nanoparticles despite their tremendous applications in different field of life. Furthermore, there is a growing need for more *in vivo* studies to expand our knowledge on the possible toxic potential associated with copper Cu nanoparticle's exposure.

4.1 Conclusion

Recently, investigations towards copper Cu nanoparticles' environmental and health effects have become possible. It signifies that numbers of questions are likely to come up yet. Probably, exposure to metallic nanoparticles is a current problem of occupational and environmental hygiene. Following are the culmination points of the study;

- Copper Cu nanoparticles at high dose (250and 500 mg/kg) used in this study exhibited obvious symptoms of poisoning such as loss of appetite, diarrhea and vomiting, etc. as compared to control.

- Copper Cu nanoparticles at dose level of 250 mg/kg and 500 mg/kg showed significant decrease in body weight.

- The organ weight of selected organs was not affected at different dose level of copper Cu nanoparticles such as 75, 125 and 250 mg/kg. However, increases in liver and decreases in kidney weight were observed at high dose level 500 mg /kg of copper Cu nanoparticles.

- The changes in hematological parameters were observed in animals from the 250 mg/kg and 500 mg/kg of copper Cu nanoparticles treated dose group compared with those from the control group. No changes were observed in all other dose groups.

- The changes in blood biochemical parameters related to liver and kidney were found in rats from the 250 mg/kg and 500mg/kg of copper Cu nanoparticles treated dose group compared with those from the control group.

- Histopathological evaluation of animals treated with dose of 500 mg/kg of copper Cu nanoparticles showed moderate changes in liver, spleen and kidney. No changes were observed in all other dose groups.

  The results of this study indicated the toxicity of copper Cu nanoparticles at high dose levels to be tested in this study. These observations indicate that Cu nanoparticles must be used with caution in human related things like medicines, cosmetics etc.

4.2 Future directions

The experimental design used in the present dissertation can be used for the toxicological evaluations of other nanoparticles that have tremendous applications. The data obtained from these kinds of studies will give information to the manufacturers about the synthesis and safe use of these materials in different fields of life.

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